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Therapeutic approach for Myasthenia Gravis using conditioned mesenchymal stromal cells

Alexandra Clarissa Bayer Wildberger

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Sorbonne Université

Ecole Doctorale 515 « Complexité du Vivant »

PhD Thesis

Therapeutic approach for Myasthenia Gravis using conditioned mesenchymal stromal cells

Presented by

Alexandra Clarissa BAYER WILDBERGER

On the 29th of September, 2022

JURY

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Abbreviations

- Ab: Antibody(ies)
- Ach: Acetylcholine
- AChE: Acetylcholine Esterase
- AchE-I: Acetylcholine Esterase Inhibitor(s)
- AChR: AcetylCholine Receptor(s)
- AD-MSC: Adipose Derived MSC(s)
- Ag: Antigen(s)
- AID: AutoImmune Disease(s)
- AIRE: Autoimmune Regulator
- AMP: Adenosine Monophosphate
- APC: Antigen-Presenting Cell(s)
- AT: Adipose Tissue
- BAFF: B cell Activation Factor
- bFGF: basic Fibroblast Growth Factor
- BM: Bone Marrow
- CAR: Chimeric Antigen Receptor
- CBA: Cell-Based Assay
- CCL-: C-C motif Chemokine Ligand
- CD: Clusters of Differentiation
- CD: Crohn's Disease
- CFA: Complete Freund's Adjuvant
- CG: Clinical Grade
- CIA: Collagen Induced Arthritis
- CMS: Congenital Myasthenic Syndromes
- CNS: Central Nervous System
- COX2: Prostaglandin-Endoperoxide Synthase 2
- CT: Clinical Trial(s)
- CTLA-4: Cytotoxic T cell Lymphocyte Associated Antigen-4
- CXCL-: C-X-C motif Chemokine Ligand
- CXCR-: C-X-X motif Chemokine Receptor
- DAF: Decay Accelerating Factor
- DC: Dendritic Cell(s) (c, cortical; m, medullar)
- EAE: Experimental Autoimmune Encephalitis
- EAMG: Experimental Autoimmune MG
- ECM: Extracellular Matrix
- EOMG: Early Onset MG
- EPP: EndPlate Potential
- ER (a, b): Estrogen Receptor(s) (a, b)
- ESC: Embryonic Stem Cell(s)
- EV: Extracellular Vesicle(s)
- FcRn: Fc Receptor Neonatal
- FBS: Fetal Bovine Serum
- FOXP3: Forkhead Box P3
- GF: Growth Factor(s)
- GMCSF: Granulocyte Macrophage Colony Stimulating Factor
- GMG: Generalized MG
- GVHD: Graft Versus Host Disease
- HGF: Hepatocyte Growth factor
- HIF: Hypoxia-Inducible Factor
- HLA: Human Leukocyte Antigen system
- HLA-DR: Human Leukocyte Antigen – DR isotype.
- HO-1: Heme Oxygenase-1
- HSCT: Hematopoietic Stem Cell Transplantation(s)
- ICAM-1: InterCellular Adhesion Molecule-1
- IDO: Indoleamine-2,3 Dioxygenase
- IFN: Interferon
- Ig: Immunoglobulin(s)
- IGF-1: Insulin-like Growth Factor 1
- IL-: Interleukin-
- IL1-RA: Interleukin 1 Receptor Antagonist
- ILCs: Innate Lymphoid Cell(s)
- iNOS: Inducible Nitrite Oxide Synthase
- IP: IntraPeritoneal
- IS: Immunosuppressant(s)
- ISCT: International Society for Cellular Therapy
- ISG: IFN-Stimulated Gene(s)
- IV: Intravenous
- IVIg: Intravenous Ig(s)
- JOMG: Juvenile Onset MG
- LEMS: Lambert-Eaton myasthenic Syndrome
- LIF: Leukemia Inhibitory Factor
- LOMG: Late Onset MG
- LPS: LipoPolySaccharide
- LRP4: LDL Receptor Related Protein 4
- mAb: monoclonal Antibody(ies)
- MAC: Membrane-Attack Complex
- MCP: Monocyte Chemoattractant Protein
- MG: Myasthenia Gravis
- MGFA: Myasthenia Gravis Foundation of America
- MHC: Major Histocompatibility Complex
- MIR: Main Immunogenic Region
- miRNA: microRNA(s)

- MLR: Mixed Lymphocyte Reaction
- MMF: Mycophenolate Mofetil
- MMP-1: Matrix MetalloProteinase-1
- MS: Multiple Sclerosis
- MSC: Mesenchymal Stromal Cell(s)
- MuSK: Muscle-Specific Kinase
- NET: Neutrophil Extracellular Trap(s)
- NK: Natural Killer cell(s)
- NKT: Natural Killer T cell(s)
- NMJ: NeuroMuscular Junction(s)
- NMO: Neuromyelitis Optica
- NO: Nitric Oxide
- OA: Osteoarthritis
- OMG: Ocular MG
- PAMP: Pathogen-Associated Molecular Pattern(s)
- PBMC: Peripheral Blood Mononuclear Cell(s)
- PBS: Phosphate-Buffered Saline
- PD-1/2: Programmed Death 1/2
- PD-L1, PD-L2: Programmed Cell Death Ligand 1/2
- PGE2: Prostaglandin E2
- PTGS2: Prostaglandin Synthase 2
- PL: Platelet Lysate
- PLEX: Plasma exchange
- RA: Rheumatoid Arthritis
- RG: Research Grade
- RIPA: RadioImmunoPrecipitation Assay
- ROS: Reactive Oxygen Species
- RyR: Ryanodine Receptor
- SCIg: Subcutaneous Ig(s)
- SDF: Stromal Cell-Derived Factor
- SEB: Staphylococcal Enterotoxin B
- SFEMG: Single Fiber EMG
- SLE: Systemic Lupus Erythematosus
- SNMG: SeroNegative MG
- SSc: Systemic Sclerosis
- SVF: Stromal Vascular Fraction(s)
- STAT: Signal Transducer and Activator of Transcription
- TAMG: Thymoma-Associated MG
- TCR: T Cell Receptor
- TEC: Thymic Epithelial Cell(s) (c, cortical; m, medullar)
- TGF: Transforming Growth Factor
- TIMP-1: Tissue Inhibitor of MetalloPeptidase 1
- TLR: Toll-Like Receptor(s)
- TNF: Tumor Necrosis Factor
- TRA: Tissue Restricted Antigen(s)
- Treg: Regulatory T cell(s)
- TSG-6: TNF-Stimulated Gene 6
- UC: Umbilical Cord (MSC)
- UCB: Umbilical Cord Blood
- VCAM-1: Vascular Cell Adhesion Molecule-1
- VEGF: Vascular Endothelial Growth Factor
- VGSC: Voltage Gated Sodium Channel

I. STATE OF THE ART

Manuscript general organization

The state of the art is divided in 3 main chapters. In the first one Myasthenia gravis is introduced and different aspects of this disorder such as, diagnosis, pathophysiology and therapeutic options are discussed. Special attention is given to AChR⁺ Myasthenia, which represents a consequent proportion of the patients and consequently is the main focus of the researches in the hosting laboratory. The second chapter presents mesenchymal stromal cells; here, characteristics, biological properties and therapeutic uses of these cells are exposed and detailed. As the use of these cells is projected in the context of autoimmunity, a special review of immunomodulatory capacities, mechanisms and impacts on the different effectors of the immune system are presented. Current available results of mesenchymal stromal cells based-therapy in different autoimmune disorders are also reviewed. As in the present work, cellular conditioning is studied as a licensing strategy for these cells, a dedicated subchapter overviews mesenchymal stromal cells most common priming strategies and their effects on cell's properties. A final chapter summarizes the few studies reporting the use of MSC in MG, either in preclinical or small clinical set-ups. The state of the art is followed by the problematic seized by this work and the main objectives. The main results of this doctoral study are presented in the form of an article, in final preparation, including Abstract, Materials and methods, Results and Discussion. Finally, the manuscript presents the conclusion and perspectives of this research work.

1. Myasthenia Gravis

1.1. Neuromuscular junction: the target of Myasthenia gravis

Myasthenia gravis (MG) is a rare chronic autoimmune disease (AID) caused by auto-antibodies targeting the neuromuscular junction (NMJ). It is primarily characterized by fluctuating episodes of muscle weakness and abnormal fatigue that follow exertion and improve after rest (Berrih-Aknin and Le Panse, 2014; Gilhus et al., 2019). The autoreactive antibodies (Ab) cause morphological and functional alterations of the post-synaptic membrane at the NMJ, resulting in neuromuscular transmission impairment and the consequent fatigable muscle weakness. Distinct clinical and immunopathological MG subtypes have been identified and they differ in primary immune targets, clinical presentation, and response to treatment.

In order to understand the pathological action of MG auto-Ab, it is important to first introduce the normal structure and function of the NMJ. It is divided into three main parts: the presynaptic part

(nerve terminal), the postsynaptic part (motor endplate), and an area between the nerve terminal and motor endplate (synaptic cleft), as schematized in **Figure 1**.

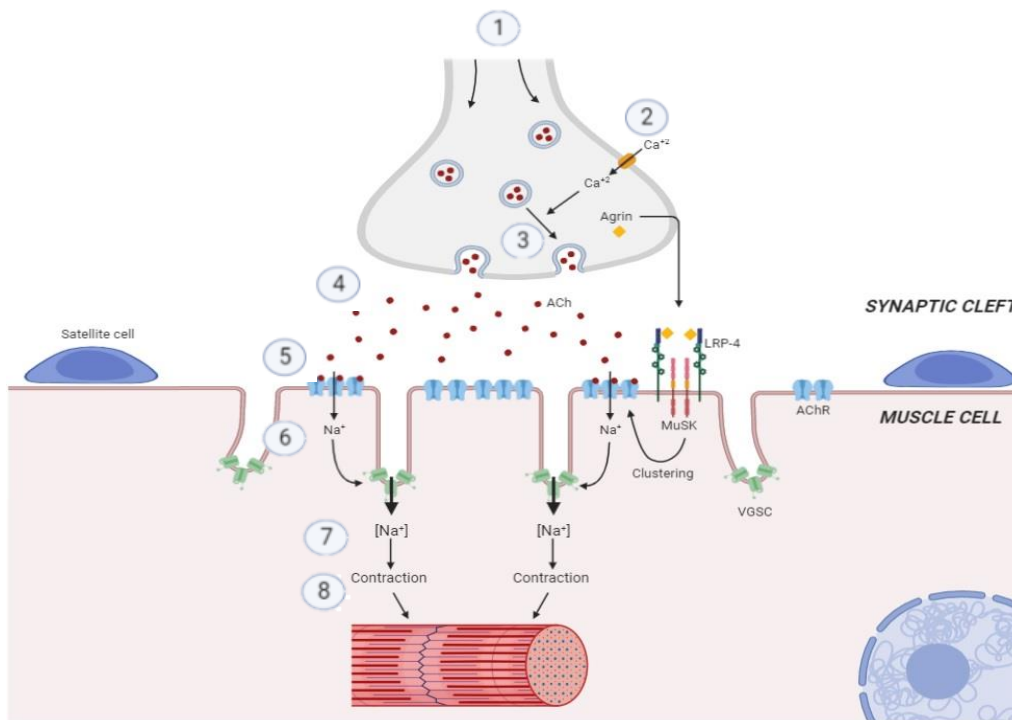


Figure 1. Simplified structure and function of the neuromuscular junction

Upon depolarization, an action potential travels down the axon (1), causing voltage-gated calcium channels to open, resulting in an influx of calcium ions into the nerve terminal (2). This causes the vesicles to migrate towards the nerve terminal membrane and fuse with the active zones (3), releasing acetylcholine (ACh) in the synaptic cleft (4). The released ACh subsequently binds to nicotinic ACh receptors (AChR) on the junctional folds of the motor endplate (5) and triggers the opening of ACh-gated ion channels allowing the influx of sodium ions into the muscle (6). The sodium influx changes the postsynaptic membrane potential and opens the voltage-gated sodium channels (VGSC) letting higher quantities of sodium get-in (7), this ultimately results in muscle contraction (8). (Vilquin et al., 2019)

Under physiological conditions, the post-synaptic membrane is characterized by deep junctional folds, with clusters of acetylcholine receptors (AChR) concentrated on the top, while voltage-gated sodium channels (VGSC) are concentrated in the depths. The process begins when the nervous system generates a signal, an impulse, called an action potential that triggers the following key coordinated steps:

1. The action potential travels down the neuron to the presynaptic axon terminal.
2. Voltage-dependent calcium channels open and Ca^{2+} ions flow from the extracellular fluid into the presynaptic neuron's cytosol.

3. Ca^{+2} influx causes acetylcholine (ACh) containing vesicles to dock and fuse to the presynaptic neuron's cell membrane.
4. The neurotransmitter ACh is released into the synaptic cleft.
5. Acetylcholine diffuses and binds to the nicotinic AChR at the motor end-plate.
6. AChR, which are ligand-gated cation channels, open when bound to ACh, allowing Na^{+} ions to flow into the muscle's cytosol. This induces a local depolarization of the membrane and forms the endplate potential (EPP). Then, AChR enter a short refractory period.
7. The EPP stimulates the opening of the VGSCs, and upon reaching the firing threshold, a further influx of Na^{+} ions ensues, and the action potential spreads along the muscle fiber.
8. EPP travel from the surface of the muscle cell along the membrane of T tubules that penetrate into the cytosol, it opens the stocks of intracellular Ca^{+2} that finally trigger the muscle contraction.

Other molecules such as agrin, MuSK (Muscle-Specific Kinase) and LRP4 (Lipoprotein Receptor-related Protein 4) are also present at the NMJ and play important roles. The development and maintenance of the NMJ are primarily dependent on the agrin-MuSK-LRP4 signaling system. LRP4 and MuSK are anchored in the post-synaptic membrane. Agrin, secreted by the nerve terminal, binds to LRP4, which then binds to the extracellular domain of MuSK, resulting in phosphorylation and activation of MuSK. Phosphorylated MuSK recruits then Dok-7, an adaptor protein that becomes phosphorylated and recruits additional signaling molecules essential for synapse formation and AChR clustering (Ohkawara et al., 2021). If AChR clustering is impaired, signal transmission at the NMJ is compromised.

Main immunological targets of MG autoreactive Ab include AChR (85% of cases, abbreviated AChR⁺ MG in this manuscript), followed by MuSK, detected in about 5% of⁺ MG patients and LRP4, dug out in a small proportion of MG patients without anti-AChR or anti-MuSK Ab (Gwathmey and Burns, 2015). Other Ab, directed against cortactin, a protein acting downstream from agrin/MuSK promoting AChR clustering (Gallardo et al., 2014) and against agrin (Gasperi et al., 2014) have also been described, but their presence is most often concomitant to one of the already cited auto-Ab.

In healthy patients, functional AChR are present in excess, which confer a safety factor to the NMJ. In AChR⁺ MG patients, there is widening and simplification of synaptic clefts and accumulation of debris in the synaptic zone. The degradation of the post-synaptic membrane results in reduction of the number of functional AChR, their availability and/or clustering, and in a reduction of the number of

the VGSCs channels. This leads to a significant reduction in the safety factor and consequently, in MG patients, the number of functional AChR contacted may be insufficient to drive the EPP to threshold, resulting in failure of neuromuscular transmission (Vilquin et al., 2019).

1.2. Historical aspects

The first recorded case of MG was most probably the Native-American Indian Chief Opechankanough in the early 17th century (Marsteller, 1988). Literature reported that, Chief Opechankanough, uncle of the famous Pocahontas, developed severe generalized muscle weakness that “wrecked his constitution”. Known for his vigor before the early Jamestown encounters, Chief Opechankanough later became so weak that his warriors had to carry him into battle, and his eyes became so droopy that his attendants had to prop them open for him to see. Although he could not walk during the last phase of his life, Chief Opechankanough was able to stand after a period of inactivity while being imprisoned in Jamestown, Virginia, for leading a deadly attack on English settlers. Chief Opechankanough died in 1644 and his clinical presentation is considered to be consistent with MG, including: the symptoms manifested in adulthood, severe weakness improved with rest, and the fact that other family members did not suffer similar problems (Nguyen-Cao et al., 2019).

The first written description of MG and its recognition as a distinct clinical entity was done by Thomas Willis in 1672 (Hughes, 2005). Since then, and until the end of 19th century, the disease was attributed different names: Erb’s disease, Erb-Goldflam Syndrome, both as a tribute to Erb and Goldflam who gave the most precise description of the disease. Later it was recognized as “*Myasthenia gravis pseudo-paralytica*”, as proposed by Friedrich Jolly in 1895 (Deymeer, 2021; Hughes, 2005; Keeseey, 1998). And finally, in 1899, the current known and used term “*Myasthenia gravis*” was accepted in the meeting of the Berlin Society of Psychiatry and Neurology (Keynes, 1961; Nguyen-Cao et al., 2019). The name, which is Latin and Greek in origin, means “grave, or serious, muscle weakness”.

MG was initially thought to be caused by a toxic or a microbial agent (Campbell and Bramwell, 1900; Goldflam, 1893; Keeseey and Aarli, 2007). In the 1930s, Mary Walker hypothesized the presence of a “curare-like” agent (a vegetal-derived alkaloid that causes muscle paralysis) in the blood of myasthenics, after showing that MG symptoms were temporary but successfully reduced with curare-antagonists: physostigmine and neostigmine (Walker, 1934, 1935). She later proposed that this chemical agent may be released by myasthenic muscles and that through blood-stream it may reach and affect other skeletal muscles, after demonstrating that fatigue of the forearm muscles in a MG patient could induce paralysis of the extra-ocular muscles (Walker, 1938). Several other studies examined the possibility that the hypothetical circulating curare-like factor originated in the

myasthenic thymus gland. Pathological abnormalities of the thymus gland, and especially tumors, had been associated with MG since the beginning of the 20th century (Keeseey, 2002)

The description of transient neonatal myasthenia in babies born from myasthenic mother, reinforced the hypothesis of a circulating agent in patients' blood and was thought capable to be transferred from mother to child (Levin, 1949; Wilson and Stoner, 1944).

It was not until late 1950s that paradigm shifted towards the consideration of immunological mechanisms in myasthenia. In 1958 D. W. Smithers, after studying the role of the thymus and its changes in autoimmunity, suggested that MG may be a result of autoimmune dysregulation (Smithers, 1959). One year later, John Simpson examined signs and symptoms of 440 MG cases and hypothesized that an auto-Ab against proteins situated at the motor end plate may be blocking nerve signaling in these patients (Simpson, 1960).

Further progresses in the elucidation of MG pathogenesis were possible thanks to the extraction of α -bungarotoxin from the venom of *Bungarus multicinctus* by Chang and Lee in 1963 (Chang and Lee, 1963). This protein, that has strong affinity to AChR and bounds irreversibly to it, allowed isolation of the receptor from electrical eel for the first time (Changeux et al., 1970). James Patrick and Jon Lindstrom administered electrical eel AChR to rabbits, aiming to produce Ab to purify this receptor. Upon repeated injections, immunized animals demonstrated striking clinical similarities to MG patients, including easy fatigability, flask paralysis, difficulties to breathe, and their clinical signs improve under treatment with the anticholinesterase inhibitor edrophonium (Patrick and Lindstrom, 1973). An experimental model of MG and of auto-immunity was discovered by serendipity.

In 1973, the number of AChR in human myasthenic muscles was reported to be reduced to 11–30% of that in normal human muscle (Fambrough et al., 1973) and years later, in consonance of what seen in the experimental autoimmune MG (EAMG) model, Lindstrom and colleagues demonstrated the presence of anti-AChR Ab in 87% of their MG patients cohort (Lindstrom et al., 1976). The link between AChR reduction and the presence of auto-Ab, and thus the pathogenicity of these ab, was evidenced by Tokya et al. who injected IgG from MG patients to mice and observed reduced amplitudes of miniature endplate potentials and reduced numbers of AChR at the NMJ, (Toyka et al., 1975). Then Pinching and colleagues reported the improvement of MG symptoms after plasma exchange (Pinching et al., 1976), and Engel et al. detected IgG and complement at the NMJ of MG patients (Engel et al., 1979)

Despite the advanced understanding of MG, there were still blanks to fill. Particularly, the pathogenic mechanisms acting in the 15% of patients with generalized MG who were seronegative for

AChR-Ab (SNMG) had not been elucidated by the time. It was suspected that SNMG patients had an AID since they responded to immunotherapy such as plasma exchange and immunosuppressants. In addition, the passive transfer of MG to mice when using the serum of SNMG patients; the development of transient neonatal myasthenia in infants of SNMG women, and the binding of IgG from SNMG patients to muscle, suggested such an autoimmune etiology (Rivner et al., 2018). In 2001, Hoch and colleagues showed that about 50% of SNMG patients have serum auto-Ab against MuSK (Hoch et al., 2001) and 10 years later Higuchi identified the novel antigen LRP4 as a supplementary target for auto-Ab in SNMG patients (Higuchi et al., 2011).

1.3. Epidemiology

MG occurs in both sexes, at all ages and in all races. A systematic review, including all population-based epidemiological studies of MG performed between 1950 and 2007, was published by Carr et al. and estimated an overall prevalence of 70,6 to 163,5 cases/per million person, with an estimated annual incidence of 8–10 cases/million person (Carr et al., 2010).

While the absolute estimates may vary, the incidence and prevalence of MG reported around the globe have been rising steadily and consistently over the past decades due to improvements in recognition, diagnosis, treatment, and an overall increase in life expectancy (Dresser et al., 2021; Punga et al., 2022). More recent studies show MG worldwide prevalence to be 124 cases/million person (Salari et al., 2021), with values ranging between 15 to 367 cases/million person depending on the geographic location. This represents 700.000 patients worldwide, 56.000–123.000 patients in Europe, 60.000 in the United States (Bubuioc et al., 2021) and 12.000 patients in France (recognized as affected by a long term condition by the Health system AMELI).

Geographical variation (that could point to the disease's etiology) could be hardly defined due to methodological biases of existing studies and scarce worldwide scale representative information. Limitations include: small study populations, different inclusion criteria and sources of data, disparate diagnosis criteria, underestimation of patients with milder disease, and most importantly, data unavailability in many countries such as those located in Latin-America (Bettini et al., 2017; Cea et al., 2018), Africa and Oceania (Bubuioc et al., 2021).

Ethnic differences have been described. Black women demonstrated a higher incidence of MG compared to white patients and black men (Heckmann et al., 2007). Black patients are more likely to develop treatment-resistant ocular MG than white patients, who show higher probabilities of developing more severe forms of generalized MG which often respond poorly to treatment. Oriental

subjects, on their turn, are more likely to develop juvenile ocular myasthenia with lower AChR titers and less likely to develop severe generalized disease (Lopez, 2022). (Nota Bene: the different forms of MG will be described below but they have to be named in this paragraph dedicated to epidemiology).

Regarding sex and age distribution of MG patients, MG can be considered as a disease of young women and older men. Incidence rates have a bimodal distribution pattern in women, with peaks around age 30 and 50 (Avidan et al., 2014a; Gilhus et al., 2019; Gwathmey and Burns, 2015). Women are more commonly affected before age 40, with a female to male ratio of 3:1 for early-onset MG. In the fifth decade of life, women and men are equally affected, while men have a higher proportion after age 50, with a male to female ratio of 3:2 (Dresser et al., 2021), **Figure 2**. The incidence increases steadily with age in men and the highest rates are reported between age 60 and 79.

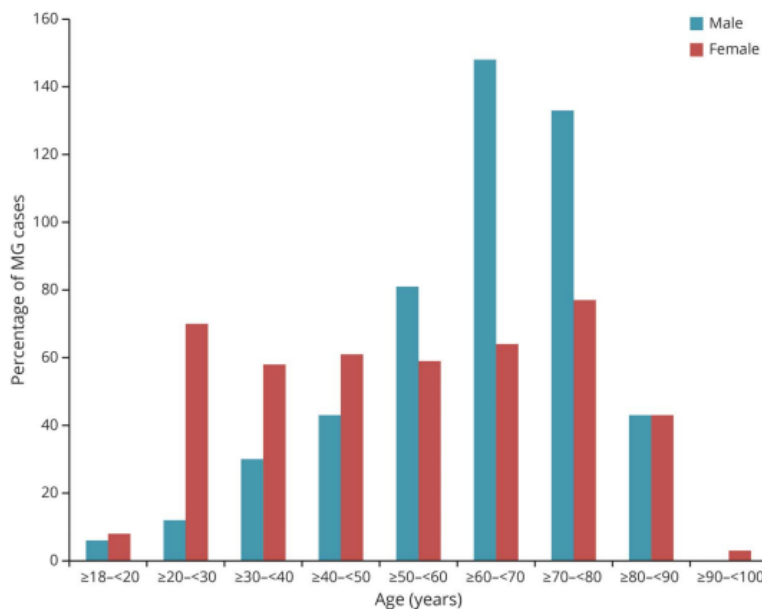


Figure 2. Distribution of the patients according to sex and age at diagnosis

Number and sex distribution of MG patients inscribed in the Spanish MG registry, per decades (Cortés-Vicente et al., 2020). Erratum: Y-axis should be labeled as “Number of MG cases” instead of “Percentage”.

As denoted here, genetic, hormonal and environmental/lifestyle factors contribute to a specific MG phenotype. MG may be therefore considered as an ensemble of symptoms with potential different clinical profiles rather than a single clinical entity.

1.4. Diagnosis

MG diagnosis might be difficult and easily missed because of the rarity of the condition and hence, unfamiliarity to physicians. Additionally, many of its symptoms can be nonspecific (and mimic other conditions) and they usually wax and wane perplexing the clinical presentation. Diagnosis is based on a series of clinical and paraclinical arguments as shown on **Figure 3**. Each diagnostic element is described in detail below.

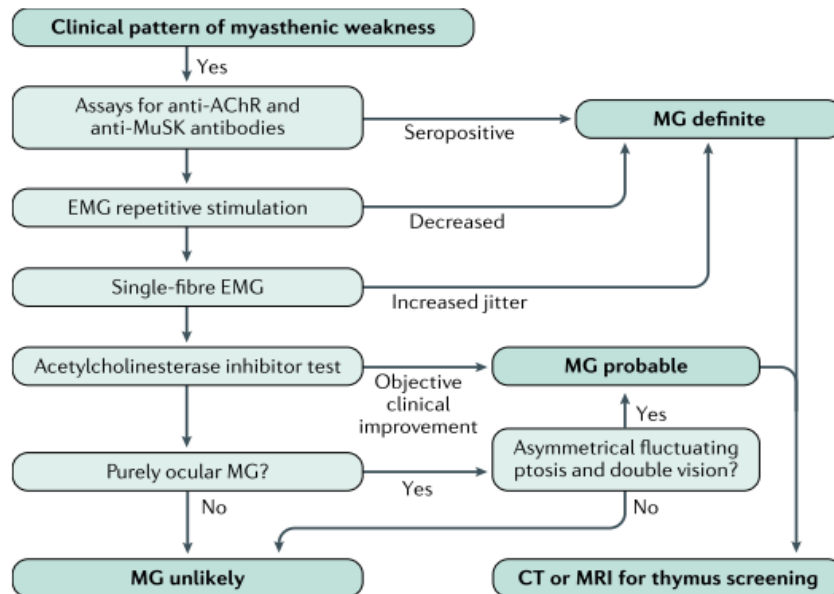


Figure 3. Simplified MG diagnosis algorithm

MG diagnosis starts with a clinical picture compatible with the disorder. The assessment of serological, neurophysiological and pharmacological tests helps to confirm diagnosis. (Gilhus et al., 2019)

1.4.1. Clinical Features

The core clinical feature of MG is fluctuating and fatigable weakness of muscle groups, varying rapidly in intensity over days or even hours during the daytime (with worsened symptoms in the evening), during menstruation or after exercise.

Other common MG symptoms may include (Berrih-Aknin et al., 2014; Ciafaloni, 2019; Gwathmey and Burns, 2015):

- Visual problems such as binocular diplopia (double vision) and frequently asymmetric ptosis (drooping eyelids), without pupillary abnormalities, bulbar disorders, weakness and fatigue of

members and cervical muscles. They eventually appear in most cases early in the disease course.

- In some patients, bulbar weakness resulting in flaccid dysarthria (pronunciation difficulty, nasal speech), dysphagia (swallowing difficulty), jaw closure weakness, and facial weakness (causing a mask-like appearance or typical “myasthenic snarl”) (Simpson, 1960) can be observed.
- Weakness of the neck or limbs. Limb weakness results in difficulty performing tasks that require the arms to be above the head, getting up from low seats or toilets, walking for prolonged distances, and climbing stairs.
- Combination of the following symptoms: ptosis + facial paresis + dysphonia + neck weakness.
- Purely muscular damage with no sensory or central nervous system alterations.

Some of these clinical features are represented in **Figure 4**.

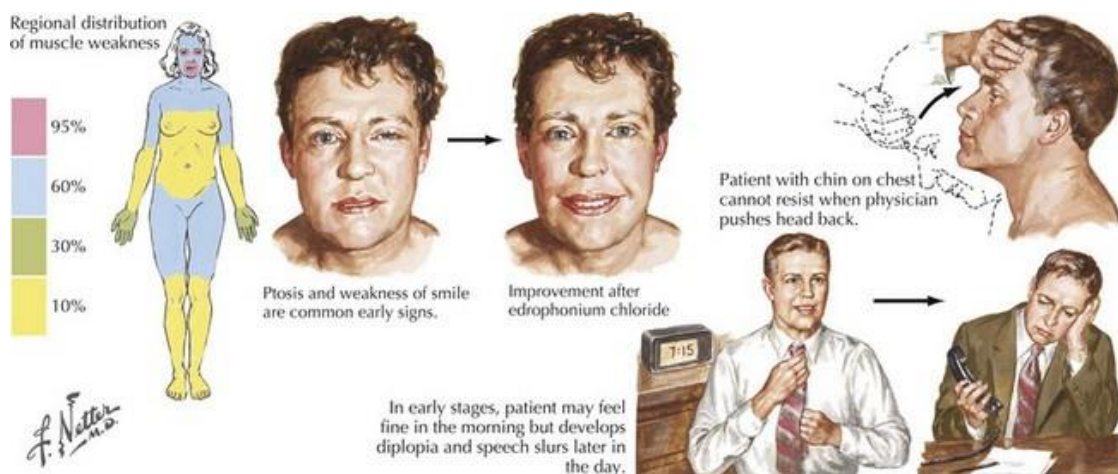


Figure 4. Clinical manifestations of MG in patients

MG almost always begins with ocular muscle weakness manifesting as ptosis, dysconjugate gaze, and eye closure weakness. The symptoms of MG often spread to involve “bulbar” muscles, causing fatigable and fluctuating dysarthria, chewing weakness, and dysphagia. Respiratory, neck, and limb muscles can also become weak. (Srinivasan et al., 2019)

Diagnostic tests are usually run to confirm the clinical diagnosis, and may include: immunological assays for auto-Ab detection, the edrophonium test, the ice pack test, and neurophysiological studies.

1.4.1.1. Serological tests

Serological Ab testing is the gold standard procedure for the diagnosis of MG, as auto-Ab presence is highly specific for MG. The first searched one is anti-AChR Ab, as they are present in 80 to 85% of MG patients. A positive Ab supports the diagnosis of MG, but the Ab titer does not correlate with the severity of the disease (Fichtner et al., 2020). If a negative test is obtained, next step includes testing for anti-MuSK Ab (positive in 50% of myasthenic patients with negative anti-AChR Ab) and anti-LRP4 Ab. Negative serological test for classical targets, do not exclude MG diagnosis (Gilhus and Verschuuren, 2015).

Developed and commercialized tests include: enzyme-linked immunosorbent assay (ELISA) and radioimmunoprecipitation assay (RIPA). While RIPA may be more sensitive for the detection of the auto-Ab in MG sera, the use of radioactive materials limits their use and favors ELISA application instead (Zisimopoulou et al., 2013). New generation tests, called cell-based assay or CBA, have been developed. For these assays, human embryonic kidney (HEK) cells, expressing the antigen of interest on the surface, are incubated with the patient's serum. Ab binding can be revealed by indirect immunofluorescence or flow cytometry. These assays were shown to be more sensible and are used to detect anti-AChR Ab of low affinity (Rodriguez Cruz et al., 2015), **Figure 5**.

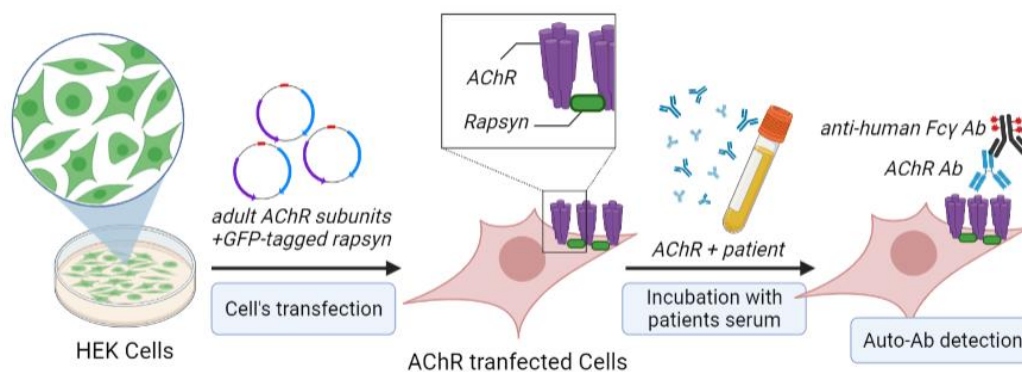


Figure 5. AChR cell-based assay principle

To perform the test, human embryonic kidney (HEK) cells are transfected with plasmids encoding the adult AChR subunits (2α , β , δ , and ϵ) and green fluorescent protein (GFP)-tagged rapsyn. Transfected cells are incubated with patient's serum, and if AChR auto-Ab are present they will bind to the clustered AChR. Binding is revealed by a conjugated anti-human Fc γ antibody. Signal emitted by the conjugated Ab can be detected by flow cytometry or immunofluorescence. Image created with Biorender.

1.4.1.2. Pharmacological tests

They are based on the clinical improvement of muscle weakness in myasthenic patients after intravenous (IV) administration of short-acting Ach esterase inhibitors.

The Ach is metabolized at the NMJ by Ach esterase (AChE) enzymes. Therefore, inhibition of the enzymes will increase the Ach concentration at the NMJ and hence improve the chance of interaction between the ACh and its receptors. Edrophonium (Tensilon) is a synthetic short-acting AChE competitive inhibitor that functions by forming non-covalent bonds at the allosteric site of acetylcholinesterase enzymes (Naji and Owens, 2021). If after slow IV administration of 2 and then 10 mg, the weakness briefly and temporarily improves, especially in the eye muscles, it may indicate MG (Berrih-Aknin et al., 2014; Pasnoor et al., 2018). Tensilon test was introduced in 1950s, but since 2018 it is no longer available in the United States due to its high rate of false-positive results (Naji and Owens, 2021).

An alternative to edrophonium is the slower, longer-acting neostigmine. The injection of 0.5 mg neostigmine subcutaneously or intra-muscularly is expected to produce improvements within 5–15 min post-injection and they may last hours (Rousseff, 2021). The test of oral cholinesterase such as pyridostigmine over a few weeks is quite justifiable to assess the functional status in daily life and over time. (Berrih-Aknin et al., 2014)

Even if pharmacological tests are useful, especially in ocular myasthenia, problems related to muscarinic side effects, drug availability, and some ambiguity in the choice of the endpoints have limited their use.

1.4.1.3. Radiological Studies

Chest X ray, computerized tomography scan, and magnetic resonance imaging might be recommended to evaluate potential presence of a thymoma, a tumor originating from the epithelial cells of the thymus.

1.4.1.4. Ice pack test

This debatable test uses the fact that cooling might improve neuromuscular transmission (Rousseff, 2021). It is mainly used by neuro-ophthalmologists to assess improvement in myasthenic patients presenting ptosis and diplopia, particularly when they are either old or medically unstable to do pharmacological test.

The predictive value of the test has not yet been established (Capone et al., 2008). This safe and straightforward bedside methodology is subjected to false-positive and false-negative results (Pasnoor et al., 2018). Another pitfall is its restricted application to only ocular-symptoms presenting patients and a possible placebo effect as with the Tensilon test.

1.4.1.5. Neurophysiological studies

These studies are commonly used to detect defects in neuromuscular transmission in patients with MG. Repetitive nerve stimulation (RNS) and single-fiber electromyography are the most commonly used tests.

In RNS, a small electrical impulse is applied to a nerve resulting in muscle contraction. In clinical practice, a train of 5 to 10 stimuli is delivered at a rate of 2 to 5 hertz. Muscles in patients with MG fatigue easily and don't bounce back from repeated stimulation as well as those of a healthy person, the compound muscle action potential (CMAP) declines in amplitude and area, resulting in the characteristic "decremental response" (Gwathmey and Burns, 2015), as shown in **Figure 6**.

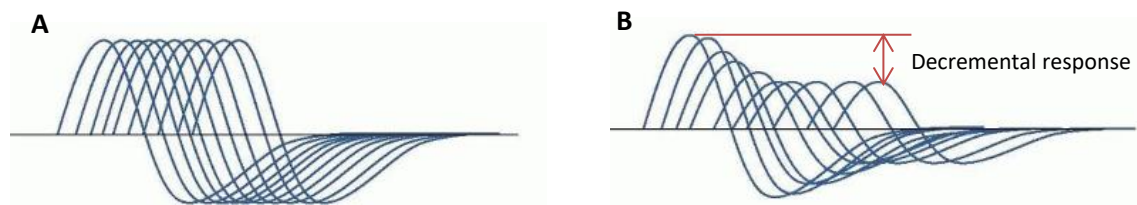


Figure 6. Representation of repetitive nerve stimulation of non-MG and MG patients

Slow (3 hertz) repetitive nerve stimulation (RNS) representation showing: (A) normal response and (B) decremental response in MG, where there is a decline in compound muscle action potential in response to nerve stimulation. (Ulane and Rowland, 2016)

A decrement is defined as the percentage of change between the amplitude or area of the fourth, fifth, or lowest potential compared with the first potential and is considered as abnormal when it exceeds 10% (Meriggioli and Sanders, 2004). This technique is helpful in seronegative cases or in situations where diagnosis is especially urgent.

If RNS studies are normal, single-fiber electromyography (SFEMG) may be informative. This technique is more sensible for neuromuscular transmission defects detection, but less specific than the classical EMG and more difficult to perform. A sterile needle electrode is inserted into a muscle which the patient gently contracts or activates. The variation in time interval between the two muscle fiber action potentials, known as "the jitter", is recorded for each identified pair. MG patients present increased jitter after multiple stimuli, instead of consistent latencies, as shown in **Figure 7**.

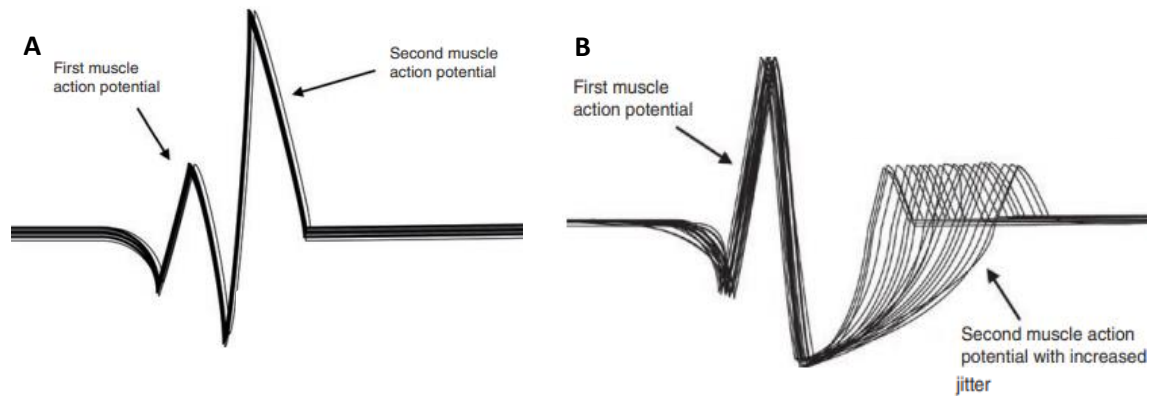


Figure 7. Normal and MG single-fiber electromyography representation

Representation of: (A) normal single-fiber electromyography (SFEMG), in which recording of multiple stimuli shows consistent latencies in the tested muscle fiber, and (B) SFEMG in a MG patient with characteristic increased jitter. (Sisti, 2019)

Increased jitter is the most sensitive measure of defective neuromuscular transmission. If NMJ transmission is significantly impaired, impulse blocking will be observed on SFEMG. Blocking constitutes a signature of clinical weakness (Berrih-Aknin et al., 2014; Gwathmey and Burns, 2015; Pasnoor et al., 2018).

1.4.2. Differential Diagnosis (LEMS and CMS)

Other clinical disorders can harbour similar signs and symptoms and may be mistakenly labeled as MG. Differential diagnosis includes other disorders of the NMJ such as, congenital myasthenic syndromes (CMS), Lambert Eaton myasthenic syndrome (LEMS), and botulism.

CMS is a rare heterogeneous group of genetic disorders affecting the neuromuscular junction, which are present before birth and inherited, mainly, in an autosomal recessive pattern. Mutations can target ion channels, enzymes, sensors and transporter proteins in presynaptic, synaptic and post synaptic function (Finsterer, 2019). In contrast with MG there is no autoimmune component, symptoms start at or shortly after birth rather than in adulthood and immunosuppressive therapy does not improve clinical symptoms in CMS, whereas it does in MG (Abicht et al., 2021).

LEMS is a rare autoimmune disorder in which IgG Ab are directed against the presynaptic voltage-gated calcium channels, the immune attack leads to a decreased Ach release in the synaptic cleft resulting in muscle weakness (Liu and Chakkalakal, 2018). In contrast with MG, LEMS starts with mild upper leg weakness, and oculobulbar symptoms appear lately. Muscle weakness is symmetrical and spreads caudal to cranially (in MG it is the reverse), and most LEMS patients have autonomic

dysfunction and reduced tendon reflexes (Okeafor and Awoyesuku, 2020; Titulaer et al., 2011), these features are summarized in **Figure 8**.

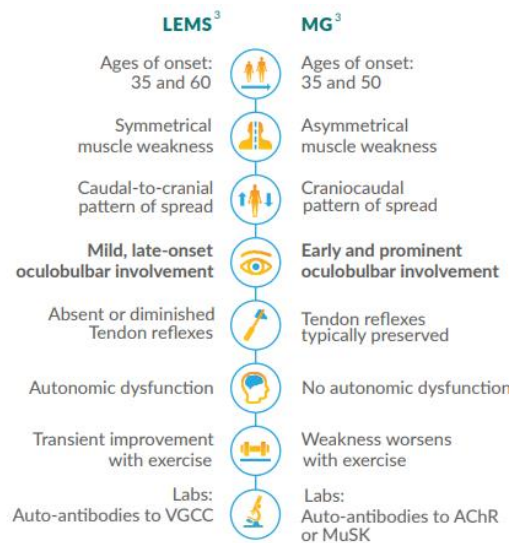


Figure 8. Differential diagnosis of Lambert-Eaton Myasthenic syndrome and Myasthenia Gravis

Representation of Key clinical features of differential diagnosis between Lambert Eaton myasthenic syndrome and myasthenia gravis. Obtained from <https://ce.mayo.edu>

Botulism is a rare and potentially fatal condition affecting the presynaptic neuromuscular junction and it is caused by the toxin produced by the anaerobic bacteria, *Clostridium botulinum*. Botulinum toxin induces paralysis by blocking presynaptic release of the Ach neurotransmitter at the NMJ (Rodríguez Cruz et al., 2020). When it occurs as a sporadic case it could be confused with MG because of the descending pattern of muscular weakness. However, botulism is a neuroparalytic process with flaccid paralysis while MG is fatigable weakness. Botulism may progress to quadriplegia and respiratory failure and there is an absence of sensory nerve damage (Okeafor and Awoyesuku, 2020).

In addition to previous described disorders, some neurological conditions in which nerve inflammation, demyelination, or lesions are present, may cause symptoms that mimic those of MG (Gwathmey and Burns, 2015; Juel and Massey, 2007).

Diagnosis of autoimmune MG has evolved from the simple confirmation of an acquired postsynaptic disorder, up to the sophisticated classification of disease subtypes along several axes: the type of autoantibody detected, the clinical status according to weakness distribution, the age of onset and the involvement of thymic pathology (Berrih-Aknin et al., 2014; Gilhus et al., 2019). Such classification proves to be very important in disease management, and is detailed in the next section.

1.5. Classification

Because clinical presentation, diagnosis, optimal treatment, and outcome vary among MG patients, subgrouping is necessary. The first classification of patients is done based on the clinical features and the severity of the disease, this classification helps health care professionals to tailor the treatments according to patient's needs. Patients are then classified according to auto-Ab status into: AChR⁺ MG, MuSK⁺ MG, LRP4⁺ MG and seronegative cases. Classical AChR⁺ MG cases constitute a heterogeneous group of patients that can be sub-classified according to other criteria. These main and minor categories are described below.

1.5.1. Clinical Classification

Historically, the Osserman Classification system was the most employed one. It separated patients with purely ocular involvement from those with generalized weakness, and further sub-classified patients with mild, moderate, or severe generalized weakness (Jaretzki et al., 2000). With time, modified versions have spread and standardization of systems was urgently needed. In 1997, an *ad hoc* committee of the American Myasthenia Gravis Foundation of America (MGFA) proposed the MGFA Clinical Classification (Jaretzki et al., 2000), these grading system divides MG into 5 main classes and several subclasses based on disease severity and the localization of the symptoms as shown in

Table 1.

Table 1. Myasthenia Gravis Foundation of America (MGFA) Clinical Classification

Class	Clinical symptoms
I	Any ocular weakness
II	Mild Weakness. May also have ocular muscle weakness of any severity
IIa	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
IIb	Predominantly affecting oropharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
III	Moderate weakness affecting other than ocular muscles. May also have ocular muscle weakness of any severity
IIIa	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
IIIb	Predominantly affecting oropharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
IV	Severe weakness affecting other than ocular muscles. May also have ocular muscle weakness of any severity
IVa	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
IVb	Predominantly affecting oropharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
V	Defined by intubation, with or without mechanical ventilation, except when employed during routine post-operative management

(Pham et al., 2021)

This classification should not be used to measure treatment outcomes (Jayam Trough et al., 2012). For MG therapy evaluation purposes, quantitative measure systems are needed. Different evaluation systems have been developed and are extensively reviewed by Barnett et al., (Barnett et al., 2018). Notwithstanding, The Task Force recommend Quantitative Myasthenia Gravis (QMG) Score to be used in all prospective therapeutic studies (Sanders et al., 2016). This scoring system is based on quantitative testing of sentinel muscle groups measuring endurance or fatigability (Barnett et al., 2018).

1.5.2. Forms with classical anti-AChR Ab

Nicotinic AChR of the muscle is the most common auto-antigen in MG and it is concentrated at the tips of the folds of the postsynaptic membrane. This cation-selective, ligand-gated, ion channel is formed by the assembly of five transmembrane subunits comprising two $\alpha 1$ plus one $\beta 1$, one δ , and one γ (fetal) or one ϵ (adult), as schematized on **Figure 9A** (Gilhus et al., 2019). Each AChR has two ligand binding pockets located at the interface between the principal α -subunits and the adjacent complementary γ -/ ϵ - or δ -subunit. When these sites are occupied by ACh, transitional conformational changes take place allowing cations passage (Na^+ , Ca^{2+} and K^+) across the membrane and triggering depolarization (Cetin et al., 2020). After opening, AChR move to a transient refractory phase in which ACh remain bonded to their receptor, but the channel does not allow cations passage. Finally, AChR regain their resting/closed conformation and are available for another cycle, **Figure 9B** (Auerbach, 2020).

Ab against AChR belong to IgG1/IgG3 subclass and appear to be polyclonal and heterogeneous. They can target the extracellular domains of all five subunits including the γ - or ϵ -subunit (Lazaridis and Tzartos, 2020), but most of them are directed against the main immunogenic region (MIR) located on the extracellular domain of the two α -subunits (Cetin et al., 2020). The Ab against AChR α subunit are more pathogenic than those against other subunits (Phillips and Vincent, 2016).

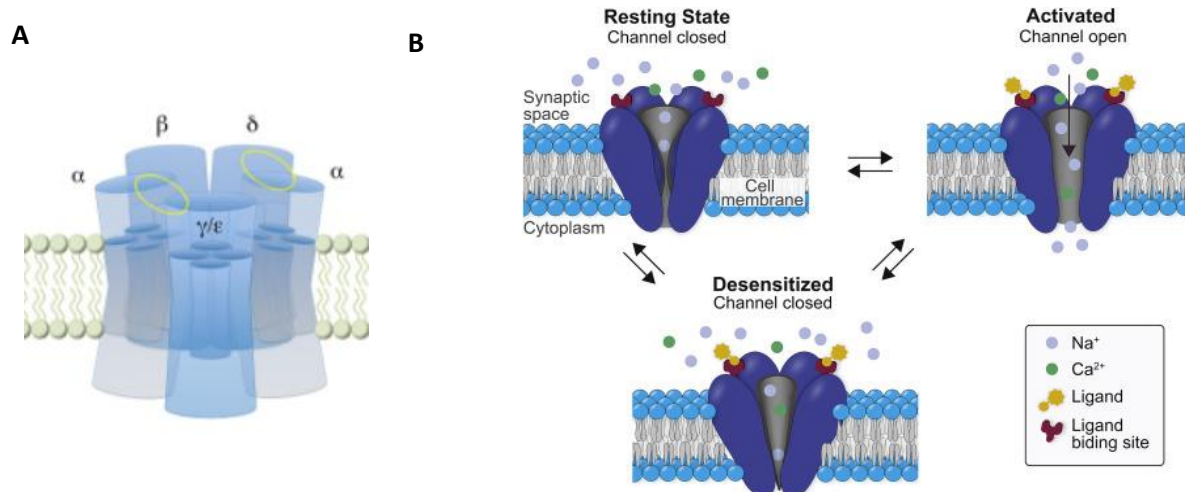


Figure 9. Didactic representation of nicotinic AChR structure and functional states

Simplified representation of: (A) the AChR structure, consisting of five subunits with the fetal specific γ -subunit or the adult specific ϵ -subunit. Each subunit has four helical transmembrane domains. The ACh binding sites are located between the $\alpha\gamma/\epsilon$ and $\alpha\delta$ subunits and shown with yellow ellipses. (B) AChR can adopt 3 main conformational states: In the resting state, the ACh binding sites are not occupied, and the pore is closed and non-conducting to cations. In the activated state, after ACh binding, the ion channel is open and permeable to small cations. In the desensitized state, the ACh binding sites are (usually) occupied, but the pore is closed and non-conducting. Adapted from (Auerbach, 2020; Gilhus et al., 2019).

The three mechanisms by which anti-AChR Ab disrupt neuromuscular transmission are through (1) cross-linking of the AChR leading to endocytosis of the AChR, (2) blockade of the ACh binding site, and (3) complement-mediated activation of membrane attack complex (MAC) and destruction of the postsynaptic membrane (Gwathmey and Burns, 2015). These mechanisms, represented in **Figure 10**, are responsible for severe AChR loss and postsynaptic membrane simplification.

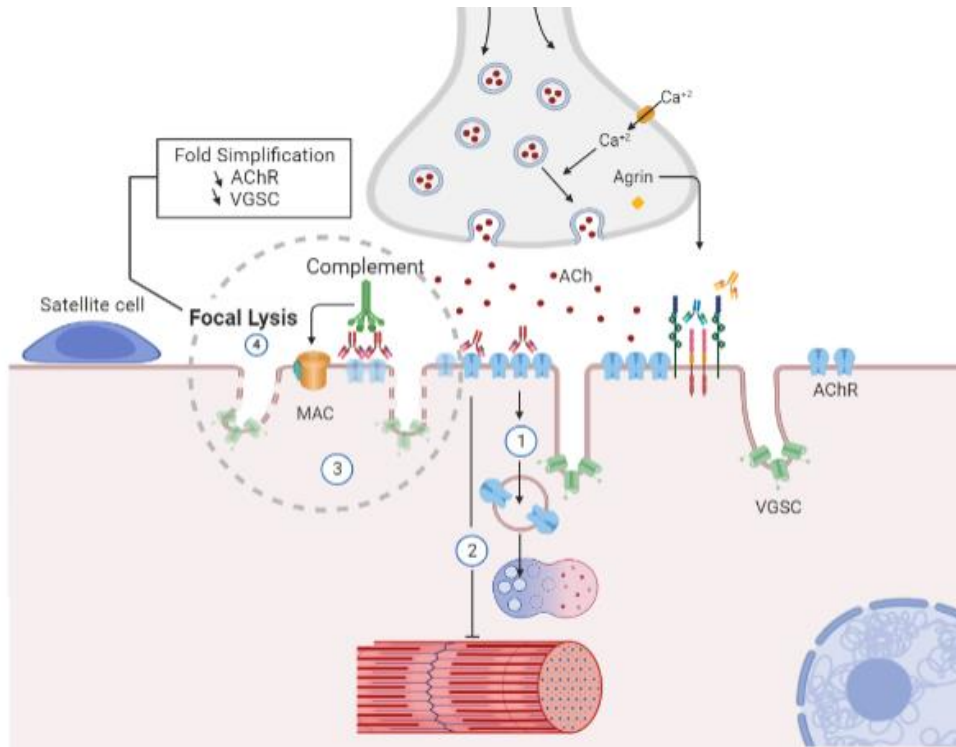


Figure 10. Effector mechanisms of anti-AChR Abs

Pathogenic Ab can impair neuromuscular transmission by: (1) cross-linking AChR molecules on the NMJ postsynaptic membrane, causing their endocytosis and their degradation (antigenic modulation), (2) binding to the ACh-binding sites of the AChR causing functional block of the AChR and (3) binding to the AChR and activating the complement cascade, resulting in the formation of membrane attack complex (MAC) and localized destruction of the postsynaptic NMJ membrane. This ultimately leads to reduced AChR and VGSC expression and to a simplified, altered morphology of the postsynaptic membrane of the NMJ of MG patients. Modified from (Vilquin et al., 2019)

AChR⁺ MG has long been recognized as a heterogeneous disease, with distinct clinical characteristics and different pathways of immune dysregulation. Sub-classification is possible according to different criteria, such as: age of onset, clinical presentation and thymic abnormalities. Principal subcategories are represented in **Figure 11** and detailed below.

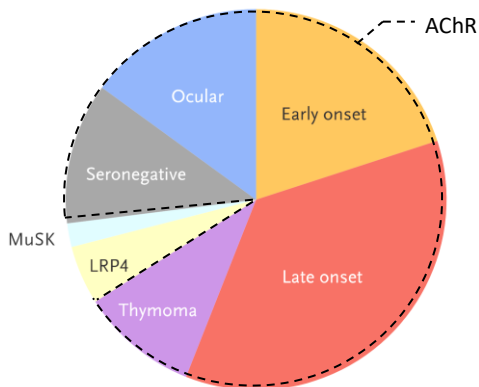


Figure 11. Subgroups of Myasthenia Gravis

Graph showing myasthenia gravis subgroups defined on the basis of clinical, antibody, and thymic features. Dotted parts correspond to subtypes of AChR⁺ MG cases. MuSK denotes muscle-specific kinase, and LRP4, LDL receptor-related protein 4. (Gilhus, 2016)

1.5.2.1. Juvenile-Onset (JOMG), Early Onset (EOMG) and Late Onset MG (LOMG)

In juvenile-onset myasthenia, symptoms appear at 18 years or younger. Among patients with juvenile-onset disease, childhood-onset disease (with symptoms appearing before puberty, or before age 12 years) is less frequent than adolescent-onset disease (in which symptoms appear between 12 to 18 years of age). JOMG is rare, with an annual incidence of 1,6 cases/million people and a prevalence of 3,6–13,8 cases/million people (Punga et al., 2022). Girls are more affected than boys and children are less likely to progress from ocular to generalized disease (Jayawant et al., 2013). JOMG share most of the clinical features and response to treatments of adult acquired MG (Ciafaloni, 2019).

In early-onset disease, symptoms begin between 19–50 years of age. EOMG has feminine predominance (ratio 3:1) (Berrih-Aknin et al., 2014; Gilhus et al., 2019), presents thymus hyperplasia, high AChR auto-Ab titers and absence of Ab to striated muscle (Limburg et al., 1983). The sex hormones may play a role in this form (Dragin et al., 2017a; Nancy and Berrih-Aknin, 2005).

LOMG (age >50 years) is typically more common in men and has been associated with the presence of thymoma and more severe forms of the disease (Punga et al., 2022). They usually present lower titers of anti-AChR Ab but with concomitant presence of anti-ryanodine and anti-titin Ab in up to 50% of patients (Berrih-Aknin et al., 2014; Limburg et al., 1983). These Ab that bind in a cross-striational pattern to skeletal muscle tissue sections mostly serve as biomarkers of disease severity (Dresser et al., 2021)

A very late-onset (VLOMG) subgroup of MG (onset at ≥ 65 years) has been recently proposed (Alkhawajah and Oger, 2015; Cortés-Vicente et al., 2020). VLOMG patients are frequently men, seropositive for anti-AChR Ab and not associated with thymoma (Cortés-Vicente et al., 2020; Punga et al., 2022). They present a higher frequency of life-threatening events such as myasthenic crises at disease onset but they achieve good outcomes with fewer immunosuppressants when diagnosed and treated properly (Barnett and Brill, 2020; Cutter et al., 2019).

These groups can be further subdivided, according to clinical weakness, into ocular MG or generalized MG.

1.5.2.2. Pure ocular form versus generalized form

When MG is clinically restricted to the extra-ocular muscles, it is referred as Ocular Myasthenia Gravis (OMG). Due to the high proportion of patients with initial ocular manifestations in the first year

after onset, the diagnosis of OMG is based on two years of purely ocular symptom (Berrih-Aknin et al., 2014; Hong et al., 2008).

Ocular symptoms are the first and sole manifestation in about 50% of patients (Wong et al., 2013). After a variable delay of usually several weeks to months, 50–80% progress to the generalized form of MG (GMG) presenting symptoms that involve any other voluntary muscles, typically bulbar or facial muscles, those of the limbs or trunk or even respiratory muscles, usually within 2 years (Hong et al., 2008; Zach et al., 2013).

OMG patients have lower anti-AChR Ab concentrations than patients with GMG (Limburg et al., 1983). Predictive factors for generalization include AChR seropositivity status (Hong et al., 2008; Kamarajah et al., 2018), thymoma and abnormal repetitive nerve stimulation test (Wong et al., 2013).

1.5.2.3. Thymoma-associated *versus* non-thymomatous MG

Different thymic abnormalities are described in anti-AChR⁺ MG patients. An important part of MG patients 60-70% (Gilhus, 2016), mainly females, present thymic follicular hyperplasia, characterized by B-cell infiltration leading to ectopic germinal center (GC) development (Berrih-Aknin and Le Panse, 2014); and 10–15% of MG patients develop a thymoma, predominantly after 50 years old without sex predominance (Lefevre et al., 2020). The physiopathological characteristics of these thymic abnormalities are better described in the section 1.7.1.2.

About 50% of patients with a thymoma develop anti-AChR Ab without clinical manifestations, and approximately 30% will develop MG, but this rate is highly variable depending on studies (Dresser et al., 2021). AChR⁺ women patients under 50 years old with B1 or B2 local invasive thymoma and ectopic GC in the adjacent thymic tissue are more prone to develop MG after thymectomy (Lefevre et al., 2020). Symptoms in thymoma-associated MG patients are usually more severe and more generalized, involving bulbar and respiratory muscles; needing more immunosuppressive treatments when compared to MG patients without thymoma.

1.5.2.4. Low affinity AChR⁺ MG

Some patients without detectable Ab titers against the soluble native form of AChR assessed by RIPA (Rodríguez Cruz et al., 2020) present clinical features and response to immunosuppressive treatments similar to AChR⁺ MG patients. For long-time they have been considered as SNMG patients until the development of CBA. This assay allowed the detection of low-affinity Abs that bind to AChR when clustered at high density, as observed at the NMJ, but not to the soluble form used in classical

methods (Evoli, 2017). A study showed that CBA was able to detect AChR Abs in 66% of a RIPA AChR and MuSK Ab negative MG cohort (Leite et al., 2008), later studies reported variable percentages (4-60%) (Devic et al., 2014; Masi et al., 2022; Park et al., 2018b; Zhao et al., 2015).

1.5.3. Forms without classical anti-AChR Ab

In this category are described characteristics of MG cases presenting Ab against 2 other important immunogenic targets: MuSK and LRP4. Ab against other extracellular or intracellular targets, such as agrin, Kv1.4 potassium channels, collagen Q, titin, the ryanodine receptor and cortactin have been found in some MG patients, but their pathological effects have not been proven. **Figure 12** shows antigenic targets of MG Ab.

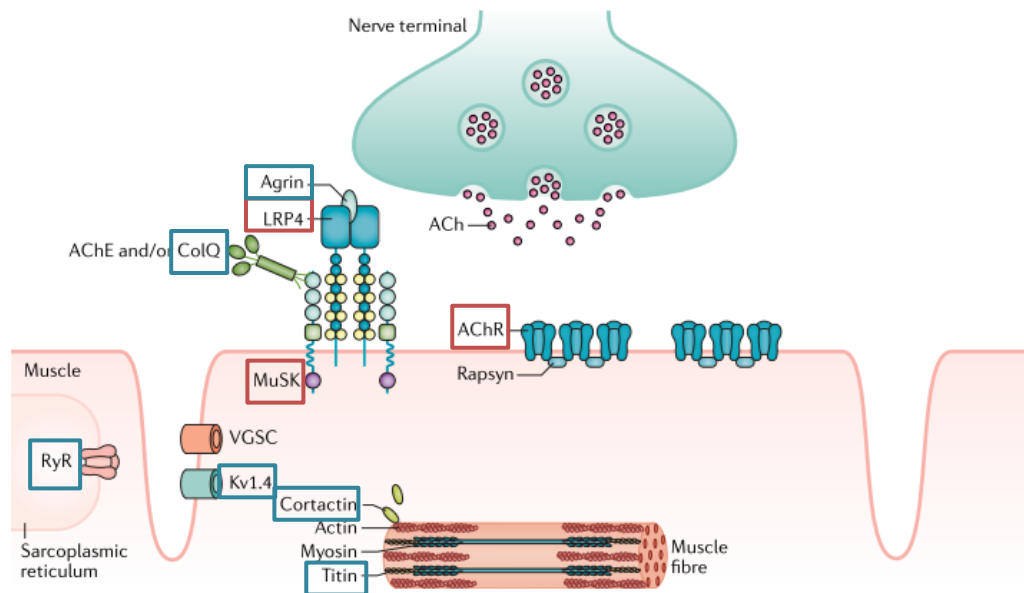


Figure 12. Representation of the antigenic targets of MG ant the neuromuscular junction

Targets of pathogenic MG antibodies are identified with red rectangles, while targets of non-pathogenic Ab are contoured in blue. (Gilhus et al., 2019)

1.5.3.1. MuSK Ab (MuSK⁺ MG)

MuSK is a single-pass transmembrane protein (**Figure 12**) constituted by three immunoglobulin-like domains (Ig1-3) and a Frizzled-like cysteine-rich domain on the extracellular region, this ectodomain is connected to the tyrosine kinase domain on the cytoplasmic region through a single transmembrane helix (Hubbard and Gnanasambandan, 2012). MuSK is responsible for AChR clustering at the NMJ and the maintenance of the postsynaptic membrane. It is activated through phosphorylation induced by the LRP4–agrin complex (Gilhus et al., 2019).

Ab against MuSK are found in approximately 5–10% of all MG patients and represent about 50% of patients with generalized MG who are seronegative for anti-AChR Abs. The disease has an early age of onset, with a peak of incidence by the end of the third decade, and it rarely occurs after age 70 (Rodolico et al., 2020). The clinical features of MuSK⁺ MG patients include female predominance, frequent bulbar involvement and rare ocular symptoms, rare thymic pathology, intolerance to AChE inhibitor treatment, and poor prognosis with frequent myasthenic crises (Park et al., 2018b).

MuSK⁺ MG is caused by auto-Ab production by short-lived plasmablasts (Stathopoulos et al., 2017). MuSK auto-Ab are mainly of the IgG4 subclass which can undergo Fab-arm exchange (Dresser et al., 2021), a process unique to this subclass and capital for their pathogenic capacity. In Fab arm exchange, IgG4 molecules can dissociate into two halves and recombine with other half IgG4 molecules resulting in bi-specific and functionally monovalent Ab (Fichtner et al., 2020), **Figure 13**.

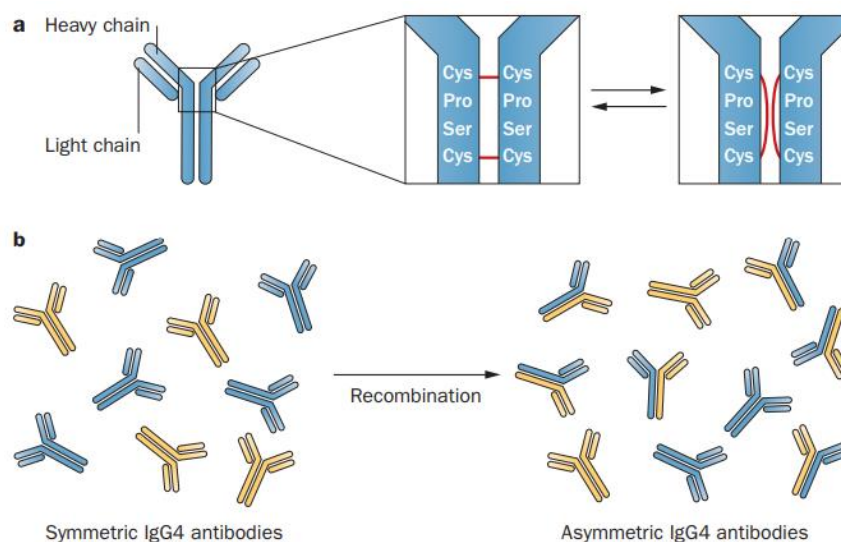


Figure 13. Fab arm exchange among IgG4 antibodies

The heavy chains of IgG4 antibodies switch between interchain and intrachain disulphide-bonded configurations. Intrachain disulphide-bonded IgG4 consists of non-covalently associated 'half-molecules' that can dissociate from one another and recombine with a different IgG4 half to produce asymmetric, bi-specific antibodies. (Cortazar and Stone, 2015)

Pathogenic anti-MuSK Ab disrupt the Agrin-Lrp4-MuSK-Dok-7 signaling pathway, interfere with AChR clustering and neuromuscular transmission, thereby causing MG disease (Takata et al., 2019). As other IgG4 Ab they are unable to fix and activate complement and are not strong triggers of cell-mediated cytotoxicity (Berrih-Aknin et al., 2014; Gwathmey and Burns, 2015). In contrast to AChR⁺ MG, MuSK⁺ MG presents a clear correlation between the disease severity and the Ab titer.

1.5.3.2. LRP4 Ab (LRP4⁺ MG)

LRP4 is a single-subunit transmembrane protein with a large extracellular domain that contains multiple low-density lipoprotein repeats. In adult skeletal muscle, LRP4 is concentrated at the NMJ, where it binds to agrin, which is normally secreted from the nerves. As previously mentioned, the LRP4–agrin complex triggers MuSK activation (Gilhus et al., 2019)

These Ab are present in approximately 2% of MG patients, they were discovered in a subgroup of patients who were double seronegative for anti-AChR and anti-MuSK (Berrih-Aknin et al., 2014) and their reported frequency varies widely (Evoli, 2017; Gwathmey and Burns, 2015). Many LRP4⁺ MG patients also have Ab against agrin (Dresser et al., 2021) and cases of concomitant existence of LRP4 and AChR and/or MuSK auto-Ab has been reported but remain rare.

Children and female patients have a higher LRP4⁺ MG prevalence, age at onset is variable, but patients tend to manifest it before age 50 (Dresser et al., 2021; Pevzner et al., 2012; Rivner et al., 2020). It often involves ocular and limb muscles. The clinical symptoms are usually mild, except in patients in which LRP4 auto-Ab co-occur with agrin auto-Ab. There are no thymic changes and in most cases, satisfactory responses to treatment are achieved (Evoli, 2017).

LRP4 Ab are predominantly IgG1 followed by IgG2 and IgG3 (Rivner et al., 2018). Active and passive immunization in mice have shown that these auto-Ab can induce MG-like weakness by disrupting Agrin-LRP4 binding and complement activation (Evoli, 2017). Of note, LRP4 Ab has also been reported in other neurological diseases, including amyotrophic lateral sclerosis and some cases of multiple sclerosis (MS), thus, further studies are required to validate its specificity in the diagnosis of MG. (Dresser et al., 2021; Rivner et al., 2017; Tzartos et al., 2014)

1.5.3.3. Other Ab

Agrin Ab (Agrin⁺ MG)

Agrin is an extracellular matrix (ECM) protein that binds to proteins in the muscle membrane, such as LRP4, dystroglycan and laminin, regulating the formation, maintenance and regeneration of the NMJ (Gilhus et al., 2019). Anti-agrin auto-Ab are detected in some patients with MG, often in association with other disease-specific Abs, mostly AChR Abs (Rivner et al., 2018). They inhibit MuSK phosphorylation and impair AChR aggregation. Mice immunized with neural agrin showed MG-like deficits, such as muscle weakness (Yan et al., 2018b). However, the pathogenic role of these Ab in human disease remains unclear (Evoli, 2017). Anti-agrin Ab have also been described in patients with amyotrophic lateral sclerosis (Rivner et al., 2017)

Collagen Q

Collagen Q (ColQ), is the collagen tail that concentrates and anchors AChE in the ECM of the NMJ. Ab against this molecules were reported in MG patients (Gilhus et al., 2019). A study searching for anti-ColQ Ab by CBA has shown that relative low titers of these Ab can be detected in the serum of 3% of patients with MG but also in a similar frequency in a healthy control cohort (Zoltowska Katarzyna et al., 2015). No pathogenic role of these Ab has been shown, but they may contribute to the varied clinical presentations in MG patients, and modify the response to AChE inhibitors (Gilhus et al., 2016).

Kv1.4.

Kv1.4 channels are concentrated in axonal membranes or near axon, and are also found in the cardiac ventricular endocardium. Anti-Kv1.4 Ab might cross-react with voltage-gated potassium channels in heart muscle in patients with MG (Gilhus et al., 2019). Ab against the α -subunit of the voltage-gated potassium channel Kv1.4 in skeletal muscle have been studied in two different MG cohorts: a Japanese one (Suzuki et al., 2005) and a Caucasian one (Romi et al., 2012) using immunoprecipitation assays. The rates of Ab occurrence was between 10 and 20% in both MG studied populations (Gilhus et al., 2019) but the clinical features were strikingly different. In the Japanese cohort, anti-Kv1.4 Ab were associated with severe MG subset, with bulbar involvement, myasthenic crisis, and concomitant myocarditis (Suzuki et al., 2005) while MG was mild in the European cohort (Romi et al., 2012). As for anti-agrin and anti-ColQ Abs, the role of anti-Kv1.4 Ab in the physiopathology of MG has not been demonstrated.

Intracellular antigens

Auto-Ab to intracellular striational antigens were also discovered in MG patients, among them: ryanodine receptor (RyR), titin, cortactin, myosin, tropomyosin, actin, filamin and others (Konecny and Herbst, 2019; Lazaridis and Tzartos, 2020). The RyR is the Ca^{2+} channel in sarcoplasmic reticulum and it participates in muscle contraction through release of calcium from the sarcolemma into the cytoplasm. Titin is a structural protein found in muscle that extends the entire length of the sarcomere and has an important role in muscle elasticity (Rivner et al., 2018). Due to their intracellular localization they are unlikely to play a pathogenic role in MG, nevertheless, anti- RyR and anti-titin Abs have diagnostic value as biomarkers for MG characteristics, such as severity, presence of thymoma and muscle damage (Gilhus et al., 2019).

Auto-Ab against cortactin, an intracellular protein acting downstream from agrin/LRP4/MuSK signaling pathway and promoting AChR clustering, were described in seronegative and AChR⁺ MG patients (Gallardo et al., 2014), as well as, MuSK⁺ and LRP4⁺ MG (Gasperi et al., 2014; Rivner et al.,

2020). They were also identified in other auto-immune diseases and in healthy controls (Doppler et al., 2021; Gallardo et al., 2014).

Auto-Ab prevalence, usual detection method and major clinical associations are summarized in **table 2**

Table 2. Summary of principal autoantibodies detected in MG patients

Autoantigen	Detection method	% of MG patients	% of dSN-MG patients	Other diseases	Clinical associations
AChR	RIPA	80–85%	N.A.	Rare	Thymic abnormalities, thymoma
Clustered AChR	CBA	N.T.	~20% (4–60%)	N.T.	Milder symptoms than AChR+ MG, thymic abnormalities
MuSK	RIPA	~6% (2–3% in Japanese)	N.A.	Rare	Bulbar symptoms common, no thymic abnormalities
MuSK	CBA	N.T.	13%	5%	Milder symptoms
LRP4	CBA	~2%	~19%	3.6% (10–23% in ALS)	Milder symptoms than AChR+ MG, no thymoma
Titin	ELISA	20–30% (90% in thymoma EOMG)	0–3%)	Some	Correlation with thymoma in AChR+ EOMG
Titin	RIPA	~41%	13.4%	0–3.6%	No correlation with thymoma
RyR	ELISA	~ 14% in LOMG (75% in thymoma MG)	N.T.	N.T.	Correlation with thymoma in AChR+ MG
Agrin	ELISA/CBA	2–15%	0–50%	13.8% in ALS	Mild to severe symptoms, moderate response to treatment
Kv1.4	IP and SDS-PAGE	10–20%	0%	0%	Japanese: Severe symptoms, myasthenic crises, thymoma, cardiac involvement Caucasian: Mild symptoms in LOMG
Rapsyn	Immunoblots	11%	17%	10% OND 78% SLE	Not known associations
Cortactin	ELISA, WB	5–10%	~20%	12.5%	Not known associations
ColQ	CBA	3%	3.4%	5%	Not known associations

N.T., not tested or not extensively tested ; N.A., not applicable; ALS, amyotrophic lateral sclerosis; SLE, systemic lupus erythematosus; OND, other neuroimmune diseases. (Lazaridis and Tzartos, 2020)

1.5.3.4. Seronegative MG

Seronegative MG patients are usually defined as patients presenting a similar clinical picture to seropositive MG but lacking anti-AChR, MuSK and LRP4 auto-Ab, meaning that the underlying autoantigenic target is yet unknown (Gilhus et al., 2016).

These patients now represent 2-5% of all MG cases and constitute a heterogeneous group including (1) patients with low affinity Ab, or in very low concentration below detection thresholds (e.g. the Ab directed against the clustered configuration of AChR) (Rodríguez Cruz et al., 2020); (2) patients with non-pathogenic Ab against collagen Q or agrin (Berrih-Aknin and Le Panse, 2014); (3) patients with Ab against yet unidentified antigens; (4) patients with myasthenic symptoms without Ab

mediation, including late-onset genetic forms associated with mutations in rapsyn or other muscle proteins (Gilhus et al., 2016).

Interestingly, they cover a whole spectrum of myasthenic features: they may present pure OMG or GMG forms (Berrih-Aknin and Le Panse, 2014), defects in neuromuscular transmission may be or not detected by clinical neurophysiological testing, and pharmacologic response to anticholinesterase agents is not always achieved (Doppler et al., 2021).

In terms of pathophysiological features, studies described a particular gene expression profile in the thymus of SNMG patients (Le Panse et al., 2006) and reduced plasmablast frequency in circulation (Guptill et al., 2021) when compared to AChR⁺ MG patients, as well as to controls.

1.6. Etiology

The specific underlying cause of the abnormal immune response in MG patients is not clearly defined. However, there is increasing recognition that MG is not a monolithic disease, but a multifactorial pathology that results from the combined influence of genetic factors, hormonal components and environmental factors acting in concert (Berrih-Aknin and Le Panse, 2014).

1.6.1. Genetic susceptibility

Most of the time, autoimmunity is polygenic, with genetic risk determined by the interplay of numerous genes. Several epidemiological and experimental arguments demonstrate the implication of genetic predisposition in AID as well as in MG, including polyautoimmunity, familial autoimmunity, concordance in monozygotic twins and association with genes (Berrih-Aknin, 2014).

The co-occurrence of two AIDs in individuals (poly-autoimmunity) is well documented in great majority of the spectrum of AID including MG. According to literature, the proportion of MG patients who developed other AIDs range from 13-27% (Duarte et al., 2017; Evoli et al., 2015; Green et al., 2020; Klein et al., 2013; Mao et al., 2011; Tanovska et al., 2018). Thyroid diseases were the most common, accounting for 25- 40% of all AIDs (Evoli et al., 2015; Green et al., 2020; Kubiszewska et al., 2016; Mao et al., 2011) and followed by rheumatoid arthritis (RA) and anti-phospholipid syndrome in one study (Duarte et al., 2017), and vitiligo and thrombocytopenia in another independent cohort (Evoli et al., 2015). Concomitant MG and systemic lupus erythematosus (SLE) were reported in punctual studies (García-Alfonso et al., 2020; Tanovska et al., 2018). Concomitant AID to MG can occur, both, before and after the onset of MG, and the process is not influenced by thymectomy (Ramanujam et al., 2011a).

For what concerns familial autoimmunity in MG context, higher prevalence (from 10 to 100 folds) of other AIDs was noted in individuals with affected first-degree relatives than in the general population and the closer the kin, the higher the risk (Liu et al., 2017). MG families are very rare, and few studies have focused on familial (Green et al., 2020; Liu et al., 2017; Murai et al., 2011; Nakamura et al., 2021; Salvado et al., 2016) or twin cases (Avidan et al., 2014a; Ramanujam et al., 2011b). Nevertheless, strong differences have been observed in MG inheritance. Asian cohorts reported substantially lower rates of familial disease (0,2-0,7%) (Liu et al., 2017; Murai et al., 2011) compared with North American (3.8-5.6%) or European cohorts (3,5%-7,1%) (Green et al., 2020; Salvado et al., 2016)

The twin model has been used to estimate the contribution of genetic factors to disease risk and compare disease concordance (manifestation in both twin members) between monozygotic and dizygotic twins. MG concordance was 35,5% in monozygotic twins compared to a dizygotic rate of 4–5%. Comparable results were obtained in the homozygous twins cohort recruited by the European network “FIGHT-MG” (Avidan et al., 2014a).

As in many other AID, gene polymorphism may predispose to disease development. Not surprisingly, the association of human leukocyte antigen (HLA) class I and class II genes with MG was the first one to be clearly established (Avidan et al., 2014a; Berrih-Aknin, 2014; Renton et al., 2015). The haplotype HLA A1-B8 (MHC class I) -DR3 (MHC class II) displays a strong association with EOMG patients while an association with the HLA-B7-DR2 haplotype was reported in patients with LOMG (Maniaol et al., 2012). Decades later, a study in a large Norwegian cohort showed that the DRB1*15:01 allele conferred the strongest risk in VLORG.

Other interesting gene include tumor necrosis factor α (TNF- α), which some specific alleles are associated with EOMG patients and thymic hyperplasia (Avidan et al., 2014b; Huang et al., 1999). and genes such as Forkhead Box P3 (FOXP3), Cytotoxic T cell Lymphocyte Associated Antigen-4 (CTLA-4), IL-10, IL-12 and Interferon gamma (IFN- γ) (Yilmaz et al., 2007; Zagoriti et al., 2013). These results highlight the important role of an individual’s genetic background in susceptibility to MG.

1.6.2. Epigenetics signatures

These mechanisms link environment and genetics, and include, specially, DNA methylation and microRNA. Epigenetic modifications could influence AID development (Berrih-Aknin, 2014). Differences in the epigenetic signatures were observed not only between AID patients and healthy controls, but also among different AIDs.

A few studies have been done regarding DNA methylation. One characterized peripheral monocytes methylation profile of MG discordant monozygotic twins, and found modest changes in DNA methylation, same as observed between MG and control cases (Mamrut et al., 2017). Another study showed that CTLA-4 methylation was significantly higher in PBMC of MG patients when compared to controls and was associated with thymus abnormalities (Fang et al., 2018).

microRNA (miRNA) are small non-coding RNA acting as post-transcriptional regulators of gene expression. Their specific interactions with coding mRNA lead to decreasing protein expression (Cron et al., 2018). Several miRNA have been related to MG pathogenesis (Wang and Zhang, 2020) and their deregulated expression have been described in the thymus of AChR⁺ MG patients (Cron et al., 2018), in PBMC, or in both compartments (Cron et al., 2019). and have been involved in thymic inflammation and development of ectopic GC (Bortone et al., 2020; Cron et al., 2020). Some circulating miRNA have biomarkers potential, since MG subtypes have revealed specific miRNA profiles in patients' sera (Punga and Punga, 2018; Sabre et al., 2020).

1.6.3. Environmental risk factors

In MG, as in other AID, nature and nurture are necessary. Environmental factors have their role in disease development. The list includes both predisposing factors, such as hormones, low vitamin D, diet and lifestyle as well as triggering factors, such as infections and drugs (Berrih-Aknin, 2014). Here, only the most studied factors in literature are detailed.

1.6.3.1. Sex hormones and gender influence

Gender is a deciding epidemiological risk factor for the development of AID. Several observations sustain this statement and raise the question of estrogens as mediators of sex differences in autoimmunity (Bubuioc et al., 2021). As many AID, MG is more prevalent in women than in men (Bubuioc et al., 2021) and female MG patients complained about symptoms worsening before and during menstrual period, when progesterone levels are lowering (Leker et al., 1998). Additionally, acquired MG may manifest for the first time during pregnancy (Keesey, 2004).

Estrogens are the primary female sex hormone and act through estrogen receptors alpha and beta (ER α and ER β). In MG, a clear dysregulation of ER expression was shown in thymocytes (with up-regulation of ER α) but also in patients' PBMC (up-regulation of both ERs) when compared with healthy controls (Nancy and Berrih-Aknin, 2005). Furthermore, estrogen stimulation down-regulated the expression of α -AChR and MHC-II in medullar thymic epithelial cells (mTEC), inducing a possible gender-defective tolerization to AChR (Dragin et al., 2017a). Estrogen stimulation also reduced

expression of autoimmune regulator (AIRE) in thymic cells, AIRE is a key factor in immune central tolerance. Impairment in its expression, below a threshold, increases female susceptibility to AID (Dragin et al., 2017b).

1.6.3.2. Vitamin D

Vitamin D plays a role in immune system modulation (Alhassan Mohammed et al., 2017) and low levels are related to AID such as MS and psoriasis (Berrih-Aknin, 2014). Regarding MG, studies on Vitamin D status in different MG cohorts agreed to show lower serum or plasma vitamin D levels (Askmark et al., 2012; Chroni et al., 2016; Guan et al., 2017c; Kang et al., 2018) or higher proportion of MG patients with insufficient levels (Justo et al., 2021) when compared to healthy controls. Moreover, fatigue improvement could be observed after vitamin D3 supplementation (Askmark et al., 2012) and even MG remission. One study demonstrated the role of vitamin D on the immune system in MG modulation and suppression (Alahgholi-Hajibehzad et al., 2015).

1.6.3.3. Gut microbiota and fecal metabolome

The human gastrointestinal tract harbours a very complex and dynamic microbial community that is critical to the development and maintenance of adequate metabolic and immune homeostasis in the host (Moris et al., 2018). Alterations in this microbiota have been described in several AID, including nervous system AID (De Luca and Shoenfeld, 2019).

Gut microbial disorders may contribute to the onset of MG. Germ-free mice colonized with MG microbiota showed substantially impaired locomotion ability, when compared to mice colonized with healthy microbiota (Zheng et al., 2019). Furthermore, studies in MG patients (mostly AChR⁺) have shown decrease diversity, and significantly altered gut microbiota and fecal metabolome in comparison with age- and sex- matched healthy controls (Moris et al., 2018; Qiu et al., 2018; Tan et al., 2020; Totzeck et al., 2021). While severe dysbiosis in MG was a common hallmark described by all authors, no real consensus exists among the studies regarding the bacterial genera or families that differ between MG and healthy controls. Punctual studies looked-up for links between disease severity, or disease course type and microbiota richness and evenness. Decreased diversity indexes were found in GMG patients (Tan et al., 2020) and in patients with high MG scores (Zheng et al., 2019). In this latter study, some microbes (19 operational taxonomic units) were specifically correlated with MG severity.

1.6.3.4. Infections

Infections, especially those of viral origin, have been proposed as the major external cause in almost all AID. Involved mechanisms include molecular mimicry, superantigens, bystander damage, or polyclonal lymphocyte activation (Berrih-Aknin, 2014).

An article looking for potential implication of infectious agents in MG reviewed 48 topic-related papers, and retrieved 21 different pathogens in the 196 MG patients documented in literature. MG most related pathogen was Epstein–Barr virus (Leopardi et al., 2021). EBV has for many years been a major candidate for induction of AID (Gilhus et al., 2018). EBV viral molecules have been found in the MG thymus (Cavalcante et al., 2010) and anti-EBV Ab serum levels in MG patients were increased when compared to controls (Csuka et al., 2012). Other candidate virus include Cytomegalovirus, Poliovirus, Papillomavirus and more recently West Nile virus, Dengue, Zika virus infection (reviewed in (Gilhus et al., 2018; Leopardi et al., 2021) and even COVID-19 (Restivo et al., 2020; Sriwastava et al., 2021). Additionally, polyinosinic-polycytidylic acid (poly(I:C)), a substitute for viral dsRNA, was shown to increase AChR thymic expression and induced anti-AChR Ab in the sera of mice (Cufi et al., 2013), reinforcing the role of dsRNA signaling in the etiology of MG.

1.6.4. Drug induced MG

A number of medications precipitate autoimmunity (Acosta-Ampudia et al., 2019). MG is not the exception, as certain drugs are known to cause *de novo* forms by inducing alterations in immune homeostasis (Dresser et al., 2021). The principal categories are listed and briefly described below.

1.6.4.1. Rheumatoid Arthritis Drugs

D-penicillamine has been used for the treatment of AID such as RA, Wilson's disease, cystinuria, among others (Sheikh et al., 2021). In MG, it has been described as inductor of mild and predominantly ocular form of the disease, with seropositivity to AChR in more than 75% of the cases and rapid recovery with medication suspension (Jay Katz et al., 1989). The exact mechanism of D-penicillamine-induced MG has not been elucidated (Penn et al., 1998) but it has been suggested to cause autoimmunity against AChR due to direct modification of pre-existing MHC molecules and/or peptides on the surface of antigen-presenting cells (Hill et al., 1999).

Hydroxychloroquine, widely used in the management of RA but also lupus nephritis as well other systemic rheumatic diseases (sarcoidosis, Sjögren's syndrome, etc.), was associated with MG onset (Elavarasi and Goyal, 2020; Varan et al., 2015). Patients presented mild symptoms with positive

AChR auto-Ab, and symptomatic recovery and auto-Ab titers reduction after drug withdrawal (Varan et al., 2015).

1.6.4.2. Tyrosine kinase inhibitors

These drugs are used in the treatment of different hematological cancers and solid tumors, and have been associated with emergence of MG (Sheikh et al., 2021). Patients treated with nilotinib (Sanford et al., 2014), imatinib (Kopp et al., 2019), dabrafenib, trametinib (Zaloum et al., 2020) or tandutinib (Lehky et al., 2011) developed, after several weeks, clinical and electrophysiological abnormalities that improved with treatment withdrawal or reduction in the dose. Tyrosine-kinase inhibitors cause MG either through immune dysregulation, or through a direct effect on neuromuscular transmission (in the case of tandutinib) (Sheikh et al., 2021).

1.6.4.3. Immune checkpoints inhibitors (ICI)

Immune checkpoint inhibitors (ICI) such as CTLA-4 and programmed cell death-1/programmed cell death-ligand 1 (PD-1/PD-L1) blocking agents are indicated as a standard of care in several cancers.

The growing use of ICIs has led to the discovery of varied and sometimes fatal immune-related adverse events. Immune-related MG is rare but life-threatening (Huang et al., 2020). A recent study reported that 14 of 5898 (0,24%) patients receiving ICI treatment developed MG (Safa et al., 2019). The pathophysiology of ICI-triggered MG is not well understood but is proposed to involve changes in T cell response such as increased ratio of effector to regulatory T cells, T helper cells and various cytokines such as IL-17 (Sheikh et al., 2021).

1.6.4.4. Interferons

In clinics, IFN are used to treat viral infections such as hepatitis C, a variety of cancers, and AID such as MS There are three major classes of IFN: type I (including 13 subtypes of IFN α , and IFN β , ω , κ , ϵ), type II (IFN γ) and type III (4 subtypes of IFN λ) (Travar et al., 2016)

De novo onset of MG have been associated with the use of IFN-2 α in the context of chronic hepatitis C treatment (Batocchi et al., 1995; Bora et al., 1997; Gurtubay et al., 1999), and of IFN-1 β for treating MS (Blake and Murphy, 1997; Dionisiotis et al., 2004) and neuromyelitis optica (NMO) (Etemadifar et al., 2011). Induction of the autoimmunity profile is explained by the establishment of a general immune deregulated context with pro-inflammatory cytokines enhancement, and lymphocyte subtypes alterations (Baik et al., 2016).

1.6.4.5. Other drugs

Additionally, statins (such as atorvastatin, fluvastatin, simvastatin and bezafibrate), lithium, anticonvulsants (such as carbamazepine), and biological drugs used in cancer treatment (such as Alemtuzumab) have been reported as MG inducers (Sheikh et al., 2021) and are shown in **Table 3**. Other drugs, especially certain antibiotics, antiarrhythmics, anesthetics and neuromuscular blockers, have deleterious effects on neuromuscular transmission, resulting in increased weakness in MG or MG-like symptoms in patients who do not have MG.

Table 3. List of the most important drugs described in literature for de novo MG induction

Drug	Mechanism	ADR Probability	Comments
Immune Checkpoint inhibitors	T cell activation Increased ratio of T effector to T regulatory cells, B cell activation, autoantibody production, cytokines such as IL-17	Definite	Avoid after emergence of life-threatening MG If to be used in MG patients, pre-treat with steroids, IVIG, or plasmapheresis
D-Penicillamine	Modification of MHC or other molecules on the surface of antigen-presenting cells	Definite	Discontinue and avoid if MG occurs
Tyrosine kinase inhibitors *	? Unspecified immune dysregulation Inhibition of neuromuscular transmission (with tandutinib)	Doubtful (probable with tandutinib)	Not contraindicated, association rarely reported
Interferons	Immune dysregulation through changes in cytokines, natural killer cells, alteration of lymphocyte profiles	Possible	Not contraindicated, association rarely reported
Statins *	Shift in T cell polarization Superimposed myopathy Mitochondrial toxicity	Probable	Discontinue and avoid in rare cases of emergence or exacerbation of MG

(Sheikh et al., 2021)

*: also may affect neuromuscular transmission; ADR: adverse drug reaction.

1.7. Immunopathology of AChR⁺ MG

To understand the therapeutic strategy presently proposed to patients, and to conceive new ones, it is necessary to consider the immune deregulation context in MG and the involved actors.

1.7.1. Thymus implication

MG is a B cell-mediated, CD4⁺ T cell-dependent AID that attacks the skeletal muscle, and the thymus is assumed to be the initiation site (Balandina et al., 2005). Introducing normal tissue physiology and function are necessary to prevent deregulations afterwards.

1.7.1.1. Physiology of the healthy thymus

The thymus is a primary lymphoid organ essential for the maturation and education of T lymphocytes via positive and negative selection, fostering the establishment of central tolerance (Pearse, 2006). This bi-lobar gland is predominantly located in the anterosuperior mediastinum,

directly behind the sternum and between the lungs (Le Floch et al., 2013). It presents two well-defined compartments, the cortex and the medulla (Nitta and Takayanagi, 2021), and harbours two major categories of cells: thymocytes (T cells developing progenitors within the thymus) and TEC that can be sub-classified into cortical (cTEC) and medullary (mTEC), according to their spatial, morphological, transcriptomic and functional characteristics (Handel et al., 2018).

The cortex is the outer region of the thymus; it contains T cells in the early stages of development and cTEC. cTEC are responsible of T cell lineage commitment and positive selection, a process that allows the suppression of thymocytes expressing T cell receptor (TCR) that are not able to interact with the cognate peptide–MHC complex expressed by cTEC (Handel et al., 2018).

The later stages of thymic T cell differentiation take place in the inner region, known as the medulla, with the help of mTEC. In this compartment, mTEC expressing tissue-restricted antigens and high levels of costimulatory molecules challenge positive selected thymocytes for their recognition of body's self-antigens (Kadouri et al., 2020). According to TCR and tissue restricted antigens-MHC interaction's strength, thymocytes will either undergo apoptosis (autoreactive thymocytes), be diverted into FOXP3⁺ CD25⁺ regulatory T (Treg) cell lineage (agonist selection), or be retained in the naive T cells pool that will migrate to peripheral (lymphoid) tissues (Kadouri et al., 2020). A simplified representation of the mechanisms involved in thymic central tolerance is shown in **Figure 14**.

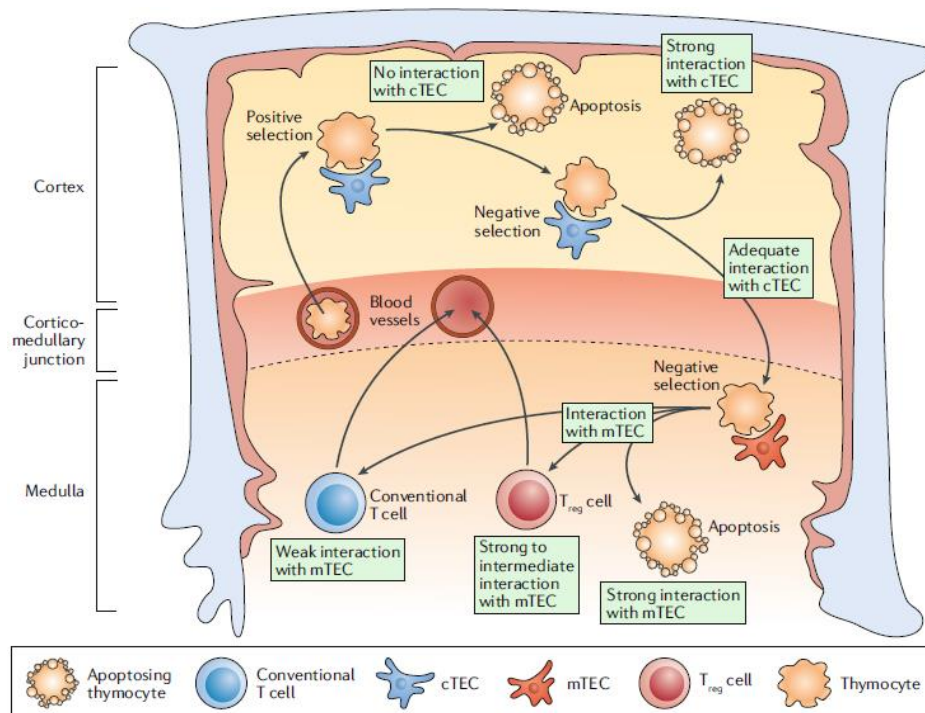


Figure 14. Schematic representation of positive and negative selection of thymocytes

Steps involved in thymic T cell differentiation in the different thymic compartments. cTEC, cortical TEC; mTEC, medullary TEC; Treg cell, regulatory T cell. (Handel et al., 2018)

In addition to TECs and thymocytes, the thymus contains a variety of other cell types. They include myoid and neuroendocrine cells (Park et al., 2020a) and other stromal cell types of mesenchymal origin such as fibroblasts and endothelial cells that are not only components of the parenchymal and vascular architecture, but also have a critical role in controlling TEC development (Nitta and Takayanagi, 2021).

Among the immunological cell types, there are:

- Dendritic cells (DC) that are found at the corticomedullary junction, as well as, in the medulla. They are the antigen presenting cells (APC) involved in thymocytes' maturation (Le Floch et al., 2013)
- Monocytes and macrophages that clear the gland after the massive apoptotic event (Pearse, 2006)
- Few immature and B cell progenitors mostly identified in the cortex, while mature B cells with an APC phenotype are located in the medulla (Gies et al., 2017)
- Natural killer (NK) cells and innate lymphoid cells that were reported in a recent study aiming to map the complex landscape of human thymic cells through single-cell RNAseq (Park et al., 2020a).

The thymus is functionally dynamic till puberty (Le Floch et al., 2013), from then on it undergoes a significant involution, starts to slowly shrink and stromal cells become replaced by adipose tissue (AT). With aging, the number of thymocytes decreases significantly mainly due to qualitative and quantitative degeneration of thymic stromal cells (Nitta and Takayanagi, 2021).

1.7.1.2. Thymus abnormalities in AChR⁺ MG

In AChR⁺ MG patients, 2 thymic abnormalities are usually found: thymoma and thymic hyperplasia.

1.7.1.2.1. Thymoma

Thymoma is often associated with AID and the paradigm disorder is MG (Yano, 2020). Literature reports that 30% of thymoma patients develop MG, a form usually called thymoma-associated MG or TAMG (Marx et al., 2018), while 10 to 15% of MG patients develop a thymoma (Dresser et al., 2021) as described previously on section 1.5.1.3. TAMG typically occurs after age 40 but may affect children (Marx et al., 2018) and are predominantly cortical (Berrih-Aknin, 2016).

Thymomas are thymic tumors derived from epithelial cells. The World Health Organization classification (Marx et al., 2022) subdivides it into the malignant A, AB, B1, B2, and B3 types according to their histological characteristics and the presence of infiltrating thymocytes. These tumors present functional cortical epithelial cells and a large number of immature T cells. However, their small or completely absent medullar zone compromises the medullary epithelial cell function, and negative selection of thymocytes, increasing the frequency of autoreactive T cells that are able to reach the periphery (Yano, 2020). Other physiopathological characteristics include (Marx et al., 2018):

- Absence of myoid cells.
- Changes in their gene/protein expression profile including variable levels of striational antigens (titin and RyR), and reduced levels of HLA-class II and AIRE which compromises the efficacy of selections.
- FOXP3 dysregulation impairing Treg generation.

All these features associate thymoma with high autoreactivity and reduced tolerance mechanisms. MG overcoming may be due to deficient tolerance mechanisms. This increased self-reactivity might explain the frequent presence of auto-Ab detected in TAMG patients which can target titin, RyR, and cytokines such as IL-12 and type I IFN (Berrih-Aknin, 2016).

1.7.1.2.2. Thymic Hyperplasia

Thymic hyperplasia is a condition in which the thymus gland is inflamed and presents ectopic GC (Berrih-Aknin and Le Panse, 2014) (**Figure 15**), comparable to what has been described in other AID, where GC are located in the pathogenic tissues (Berrih-Aknin, 2016).

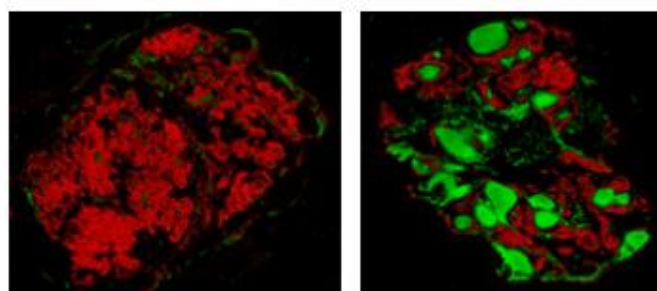


Figure 15. Immunofluorescence images comparing the microscopic structure of a control and a AChR⁺ MG thymus

MG thymus is characterized by the abnormal infiltration of B-cells organized in ectopic germinal centers. Here, epithelial network is stained by an anti-keratin Ab in red, and the germinal centers are stained by anti-CD21 Ab that recognizes B and follicular dendritic cells in green. (Berrih-Aknin and Le Panse, 2014)

These highly organized structures may be responsible of AChR auto-Ab production, as patients with hyperplastic thymus usually present elevated AChR Ab titers while most patients with involuted/normal thymus present low or negative Ab titers (Berrih-Aknin et al., 2013). Indeed, the thymus has been shown as one of the sites of anti-AChR production. Several arguments sustain this statement, such as the detection of AChR auto-Ab (Mittag et al., 1976), the identification of thymic cells able to produce these auto-Ab *in vitro*. Further on, confirmation was given through AChR auto-Ab detection in sera of immunodeficient mice grafted with MG thymic fragments (Schönbeck et al., 1992; Sudres et al., 2017).

Changes that take place in MG hyperplastic thymus include neoangiogenic processes with formation of lymphatic endothelial vessels expressing CCL21 (Berrih-Aknin et al., 2009) and high endothelial venules expressing CXCL12 (Weiss et al., 2013). Both of these cytokines are overexpressed in MG thymus favoring the recruitment of peripheral immune cells into this organ to develop GC (Berrih-Aknin, 2016). Modified chemokines expression, namely CXCL13, CCL21 and CXCL12 detected in MG thymus was later shown to be triggered by IFN- β (Cufi et al., 2013, 2014). IFN-I signature is typically detected in hyperplastic MG thymus (Payet et al., 2022) and depicted by the overexpression of IFN-stimulated genes (ISG) (Poëa-Guyon et al., 2005) and an increased expression of IFN- β (Cufi et al., 2013). IFN- β has pleiotropic actions over MG thymus, it was shown to induce overexpression of α -AChR in TEC, the main antigenic target in MG, and of the B-cell activating factor (BAFF), which favors autoreactive B-cells (Cufi et al., 2013, 2014). It also activates the IL-23/ Th17 pathway in TECs, leading to the overexpression of podoplanin by Th17 pathogenic cells, which are crucial for sustained formation of ectopic GC (Villegas, Bayer et al., 2019).

Other deregulated chemokines described in literature are CCL17 and CCL22 (Zhao et al., 2021), they are mainly produced in the medullar zone by Toll-like receptors (TLR) 4 expressing TEC and co-localized with CCR4⁺ DC that were present in higher number in MG, denoting the potential role of TLR4 activation in DC recruitment into the MG thymus. Therefore, accumulating data show that innate immunity activation via TLRs, INF-I signature and chronic inflammation are hallmarks of follicular hyperplastic MG thymus (Cavalcante et al., 2011; Payet et al., 2022; Zhao et al., 2021).

The anti AChR Ab produced by GCs will target AChR at the NMJ but a complementary hypothesis postulates that it may also target AChR in the thymus, initiating a complement cascade (Zhao et al., 2021). This is comforted by detection of both C1q and C3 in the TECs from thymus with GC (Leite et al., 2007). Complement cascades result in AChR antigen release from AChR specific TECs and the generation of GC through a positive feedback loop in thymus (Zhao et al., 2021). Additionally, these autoreactive B cell and plasma cells that originated and matured in the thymus are able to

emigrate to the periphery and persist in the circulation. This postulate is comforted by a recent study showing that Thymus-associated B cell clones were detected in the circulation even 12 months after thymectomy (Jiang et al., 2020).

1.7.2. Immune cells role in MG

1.7.2.1. Innate immunity

NK cells participate in the development of AID such as RA, SLE and inflammatory bowel disease by the production of numerous cytokines and chemokines (Zhao et al., 2021). In EAMG animal models, NK cells usually foster the immune response of Th1 cells by promoting the secretion of IFN- γ and suppressing the production of transforming growth factor beta (TGF- β 1). NK cell-deficient mice have shown lower anti-AChR levels, defective Th1 responses and resistance to EAMG symptom development (Shi et al., 2000). Moreover, in the clinical practice, MG patients that are good responders to plasmapheresis therapy, show significantly decreased NK cell cytotoxicity compared to non-responders MG (Chien et al., 2011).

Natural killer T (NKT) cells produce immunomodulatory cytokines regulating the immune response. Activation of NKT cells by a synthetic glycolipid agonist of NKT cells, α -galactosylceramide (α -GalCer), led to the increased production of IL-2, which acts as a critical cytokine for Treg cells (Zhao et al., 2021). Liu and colleagues have shown that α -GalCer administration in EAMG mice increased the size of the Treg cell compartment, and augmented their regulatory function on autoreactive T cells by higher expression levels of anti-apoptotic FOXP3 and B-cell lymphoma 2. Therefore the NKT cells activation protected mice from the induction of EAMG (Liu et al., 2005).

DC are central antigen presenting cells operating at the interface of innate and adaptive immunity via cellular interactions and secretion of cytokines such as IL-1 β . *In vivo*, an EAMG study showed that injection of caspase-1 inhibitor was able to impair maturation of DC and reduce intracellular production of IL-1 β . This effects resulted in reduced number of Tfh cells and decreased IL-17 production by $\gamma\delta$ T cells and CD4⁺ T cells and ameliorated EAMG symptoms (Wang et al., 2015a)

1.7.2.2. Adaptive Immunity

T cells are widely distributed in both animal and human tissues and are classically distinguished between 2 types: cytotoxic T cells or T CD8⁺ and helper T cells or T CD4⁺. Helper T cells, once activated, can differentiate into T helper (Th) 1, Th2, Th9, Th17, Th22, T follicular helper (Tfh) or regulatory T cell (Treg) subsets, as shown in **Figure 16**. Commitment depends on the intensity of stimulation and the

cytokine microenvironment, and it is not always permanent making functional plasticity of T cell subsets possible (Bluestone et al., 2009; Geginat et al., 2014)

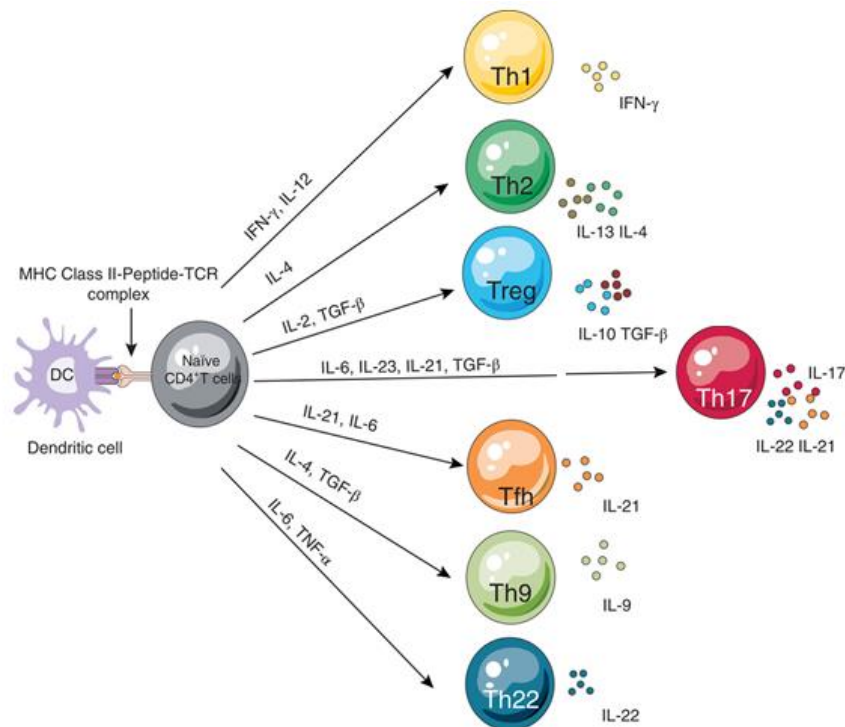


Figure 16. Differentiation of naïve T CD4⁺ cells in the different Th cell subsets

Driven by the inflammatory environment, naïve CD4⁺ T cells could be polarized to functionally distinct Th cells, such as Th1, Th2, Th9, Th17, Th22, follicular Th, and regulatory T cells. Adapted from (Yang et al., 2019)

T helper subsets

Although MG is a B cell-mediated disease, CD4⁺ T cells and their cytokines contribute to the development of the disease. Animal studies have shown that mice with depletion of CD4⁺ T cells or class II major histocompatibility complex (MHC II) fail to develop EAMG after immunization with AChR (Kaul et al., 1994). Th1 cells, Th2 cells, and Th17 cells are important subtypes of CD4⁺ T cells characterized by the different patterns of cytokines they secrete. These cells, under physiological conditions, protect the organism from the attack of foreign antigens; but under pathological conditions, Th1 and Th17 cells are associated with autoimmunity, and Th2 cells are implicated in allergic responses (Wang and Yan, 2017).

Patients with MG have autoreactive Th1 and Th2 cells (Yi et al., 1994) and display increased numbers of IFN-γ or IL-4-expressing cells in PBMCs (Link et al., 1994), suggesting that both Th1 and Th2 cells are involved in MG. IFN-γ is known to induce MHC II and costimulatory molecules in adjacent tissues, such as myocytes, prompting them to present antigens and promote Ab response (Dresser et

al., 2021). IL-4 as other Th2 cytokines is known to play a role in the induction of B cells; therefore, it is believed that a humoral Th2 response has also a direct role in the immunopathogenesis of the disease (Balasa and Sarvetnick, 2000). A study assessing the percentage of Th1 and Th2 cells among CD4⁺ T cells in differently treated MG patients, found a higher Th1/Th2 ratio in the glucocorticoid-treated group, which correlated positively with clinical severity (Masuda et al., 2010).

Other cytokines that were reported to be modulated in MG context include IL-17 and IL-21 (Çebi et al., 2020). IL-17 is a Th17-activated cell-related cytokine that indirectly promotes Ig production by affecting the balance of the cytokine profile of Th1 and Th2 cells in MG PBMC (Masuda et al., 2010). Several studies have shown that patients with MG have elevated levels of Th17 cells and IL-17, which correlate with disease severity and Ab titers (Roche et al., 2011; Wang et al., 2012; Xie et al., 2016). Villegas and colleagues showed that MG patients displayed an overexpression of IL-17 in the thymus. This activation was sustained by high levels of IL-23 secreted by TEC. IL-23 overproduction was due to a dysregulation of IFN type I pathway and was maintained by IL-17, forming a chronic loop of inflammation. Additionally, Th17 cells were localized around thymic ectopic GC and expressed podoplanin, a protein involved in B-cell maturation and antibody secretion, implying that IL-17 may participate in ectopic GC maintenance (Villegas, Bayer et al., 2019). These results are in line with experimental data showing a critical role of IL-17 secreting CD4⁺ T cells in EAMG induction (Schaffert et al., 2015).

On the other hand, IL-21 is reportedly produced mainly from activated T follicular helper (Tfh) Tfh1 and Tfh17 cells (Zhang et al., 2016a). Tfh cells are necessary for the generation of GC in secondary lymphoid organs and IL-21 which promotes B cell differentiation, antibody production, and Ig isotype switching, resulting in long-lasting antibody responses (Çebi et al., 2020). Both Tfh and IL-21 have been reported to be increased in MG patients' blood (Ashida et al., 2021; Çebi et al., 2020; Li et al., 2014b; Wen et al., 2016; Zhang et al., 2016a, 2016b) and this correlated with disease severity. Authors also reported a higher percentage of thymic Tfh cells in thymoma-associated MG, suggesting that Tfh cells might be involved in the pathogenesis of MG (Song et al., 2016).

Finally, MG patients showed higher frequency of memory AChR-specific CD4⁺ T cells with an inflammatory phenotype in the periphery when compared to controls. Inflammatory profile was characterized by an increased production of IL-17, TNF γ and GM-CSF with negligible expression of IL-10 (Cao et al., 2016).

Treg cells

Treg cells suppress the function of other effector T cells and APC by releasing anti-inflammatory cytokines, such as IL-10 and TGF- β , and through the expression of FOXP3, among other mechanisms (Dresser et al., 2021). In MG, the Treg cell count reported in literature are not unanimous, with authors showing lower frequencies when compared to normal controls (Jing et al., 2019; Kohler et al., 2017; Li et al., 2008; Xu et al., 2012b) and others showing normal counts (Balandina et al., 2005; Thiruppathi et al., 2012; Villegas et al., 2018). Although there is no consensus about Treg frequencies, most studies report that these cells have reduced suppressive activity in MG and that this impairment is associated with a reduced expression of FOXP3 (Gradolatto et al., 2014; Thiruppathi et al., 2012).

Natural Treg cells can differentiate into Th17 cells in the appropriate cytokine environment, including IL-1, IL-6 and IL-21 (Berrih-Aknin, 2016). These cytokines are increased in MG thymus, and the changes in the signature of Treg cells towards Th17 cells strongly suggest that Treg/Th17 balance is altered in MG thymus in favor of Th17 cells (Gradolatto et al., 2014; Villegas et al., 2018). A detailed microarray analysis of thymic Treg from MG patients compared with control thymuses revealed a Th1/Th17/Tfh signature, characterized by increased expression of IFN- γ , IL-21, TNF- α and IL-17-related genes (Gradolatto et al., 2014). In induced EAMG models, Treg cells are defective when rats are immunized with AChR in the presence of adjuvant, showing that Treg cells could become malfunctioning in response to an immune challenge (Aricha et al., 2008). These data suggest that the defect of Treg cells in MG patients is a consequence of the perturbation of the immune system rather than the cause of the disease.

B cells

As discussed earlier, B cells play crucial roles in MG, including auto-Ab production, complement activation and cytokine release. B cell profile in MG patients differ according to disease status (Min et al., 2019) and studies suggest that BAFF signaling is enhanced in MG (Uzawa et al., 2021). BAFF signaling through interaction with BAFF-receptor (BAFF-R) is essential for B cell survival, maturation, and their development into Ab secreting cells (Thompson et al., 2001), and an excess of BAFF acts favorably on autoreactive B cells (Ragheb and Lisak, 2011). MG patients not only have increased levels of circulating BAFF in their serum (Kang et al., 2016; Ragheb et al., 2008) which correlated with AChR⁺ Ab titers, but also increased CD19⁺BAFF-R⁺ B cells frequency when compared to healthy controls (Li et al., 2008), supporting a role for dysregulated BAFF signaling in MG pathogenesis.

Patients also present alterations in Breg cells subsets. These cells, responsible of the inhibition of CD4⁺, of Th differentiation and the promotion of Treg cell expansion predominantly through IL-10

secretion, are decreased in AChR⁺ MG patients with moderate to severe presentation, as well as, their IL-10 production in comparison with controls (Karim et al., 2017; Sheng et al., 2016; Yi et al., 2017; Yilmaz et al., 2018). A dysfunction of B reg may play a role in the installation of MG (Dresser et al., 2021).

Figure 17 synthesizes the role of innate and adaptive immune systems in MG pathogenesis

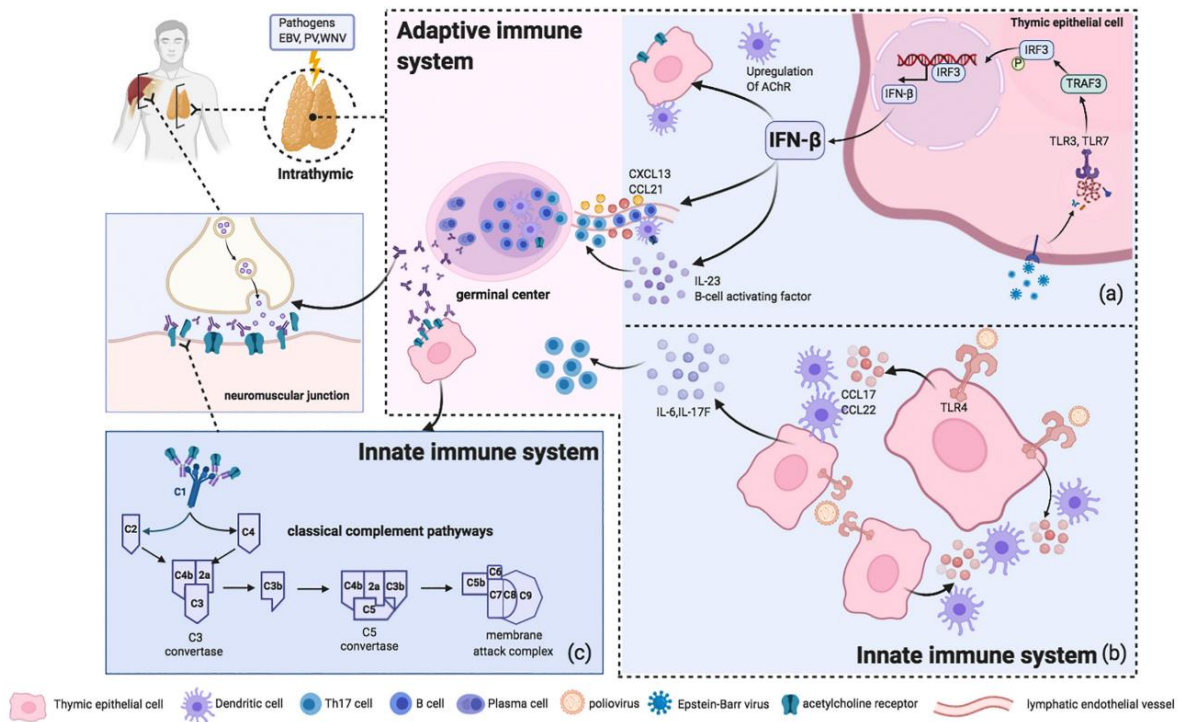


Figure 17. Role of innate and adaptive immunity in MG pathogenesis

Both, innate and adaptive immune systems play a role in pathogenesis in MG. In the context of genetic background predisposing and environmental risk factors to MG, the invading pathogens trigger the aberrant activation of TLRs pathways in the thymus. (a). EBV binds to TLR3 or TLR7, resulting in the overproduction of pro-inflammation cytokines such as IFN- β and chemokines to recruit peripheral B cells and Th17 cells to generate GCs in thymus. IFN- β also promotes TECs to express AChRs and uptaken by APC, leading to the autosensitization against AChR and the production of autoantibodies. (b). TLR4 pathway is activated by poliovirus, resulting in the expression of chemokines to attract DC and the production of Th17-related cytokines to alter the effector T cell/regulatory T cell balance. (c). The AChR antibodies play their role at both thymus and peripheral NMJ via activating the classical complement pathway causing damage to the TECs and postsynaptic membrane, respectively. (Zhao et al., 2021)

1.8. Animal models of AChR⁺ MG

As indicated above, the first model was established by serendipity almost 50 years ago by Patrick and Lindstrom. By immunizing rabbits with AChR protein purified from the eel's electric organ emulsified with complete Freund's adjuvant (CFA), they observed that animals recapitulate MG-like symptoms and that this symptoms were abrogated using an inhibitor of acetylcholinesterase (Patrick and Lindstrom, 1973), as shown in **Figure 18**. Symptoms onset was due to the Ab rabbits raised against the xenogeneic fish AChR, and that were able to cross-react with the own rabbit AChR, impairing neuromuscular transmission.

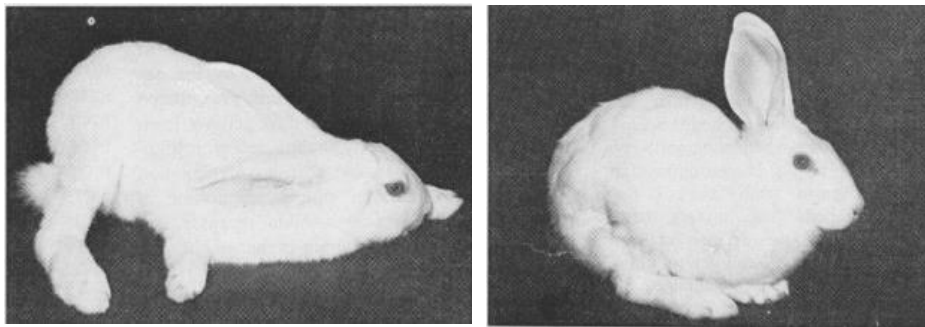


Figure 18. Photographs of experimental autoimmune myasthenia gravis (EAMG) rabbits, before and after treatment

The left photograph shows a rabbit, 5 days after the third subcutaneous injection of AChR, with extreme paralysis. The right photograph is the same animal 1 minute after receiving 0.3 mg of edrophonium intravenously. (Patrick and Lindstrom, 1973)

This model constituted the first description of an EAMG model. Since, alternative strategies have been developed to address different physiopathological questions and they can be classified in the main categories described below.

1.8.1. Models obtained by active immunization

Active immunity refers to the process of exposing the individual to an antigen, challenge its immune system and generate an adaptive immune response. In active EAMG context, induction is mainly done by injection of emulsions containing an adjuvant plus complete AChR extracted from Torpedo fish or electric eel (Link and Xiao, 2001) or a rat specific epitope corresponding to the MIR of AChR (Baggi et al., 2003, 2004). As shown in **Table 4** AChR obtained from other sources have also been tested in literature (Losen et al., 2015).

EAMG can be induced in various animal species (Losen et al., 2015) as shown in **Table 4**, but privileged species are rats and mice, mainly due to the high incidence of clinical EAMG signs (Mantegazza et al., 2016). Common strains used in EAMG models include Lewis rat (Berman and Patrick, 1980) and C57Bl/6, SJL, and AKR mice (Biesecker and Gomez, 1989).

Table 4. AChR sources and animal species described in literature in EAMG models

Source of AChR	Recipient animal
<i>Torpedo californica</i> (electric organ)	Rat (<i>Rattus norvegicus</i>) Mouse (<i>Mus musculus</i>) Pig (<i>Sus scrofa domesticus</i>) Rhesus monkey (<i>Macaca mulatta</i>) Frog (<i>Rana ripiens</i>) Guinea pig (<i>Cavia porcellus</i>)
<i>Torpedo marmorata</i> (electric organ)	Rat (<i>Rattus norvegicus</i>) Rabbit (<i>Oryctolagus cuniculus</i>)
<i>Electrophorus electricus</i> (electric organ)	Rabbit (<i>Oryctolagus cuniculus</i>) Rat (<i>Rattus norvegicus</i>) Guinea pig (<i>Cavia porcellus</i>)
Rat AChR (syngeneic muscle)	Rat (<i>Rattus norvegicus</i>)
Cat (denervated muscle)	Rabbit (<i>Oryctolagus cuniculus</i>)
Chicken (denervated muscle)	Rabbit (<i>Oryctolagus cuniculus</i>)
Human AChR (denervated muscle)	Rat (<i>Rattus norvegicus</i>)
1–210 sequence of the human AChR- α 1 subunit (<i>Escherichia coli</i>)	Rat (<i>Rattus norvegicus</i>)
97–116 sequence of the rat AChR- α 1 subunit (synthetic)	Lewis Rat (<i>Rattus norvegicus</i>)
Chimeric Aplysia ACh-binding protein (AChBP)/human muscle AChR	Lewis Rat (<i>Rattus norvegicus</i>)

(Losen et al., 2015)

Literature describes several sites for antigen injection: foot pads, base of the tail, hip and shoulder regions. Footpad injection is widely used but ethical considerations (animal suffering) push to favor injection at the base of the tail (Losen et al., 2015) or to develop new protocols.

Mice and rat models of EAMG present, each, particular features. In rats, EAMG usually requires a single immunization with purified AChR in CFA for disease induction. MG develops in 2 phases: (1) an acute transient phase with IgM antibodies and extensive phagocytic invasion at the NMJ and (2) a progressive chronic late phase with high titer of IgG antibodies and complement attack of the NMJ (Link and Xiao, 2001). In mice, EAMG induction needs several injections; a first one with AChR purified receptor in CFA, and 2-3 boosts with AChR plus incomplete Freund's adjuvant. MG symptoms appear 7–14 days after the last injection (Lindstrom, 1980). This resistance to develop clinical manifestations of the disease may be due to the existence of a higher safety factor in mice, *i.e.* a higher ratio of AChR per surface unit at the NMJ. Both species present comparable MG symptoms (Mori and Shigemoto, 2019) and Ab against foreign and self AChR. These auto-Ab are different from humans in their subtype, while murine AChR Ab are IgG1, IgG2a, and IgG2b types, human Ab are mainly IgG1 and IgG3 (Christadoss et al., 2000).

1.8.2. Models obtained by passive immunization

This is the simplest protocol for studying the pathogenic effects of auto-Abs *in vivo*. Passive immunization involves the production of Ab in one animal and transfer to another (Mantegazza et al., 2016). It was first accomplished via daily intraperitoneal injections into healthy BDF mice of serum IgG fraction isolated from MG patients (Toyka et al., 1975). It may result from administration of monoclonal Ab (IgG1 or IgG2a) directed against the AChR α -subunit, either derived from AChR-immunized animals (Tzartos et al., 1987) or cell line culture supernatants. Compared to active immunization, the passive strategy presents faster onset but shorter-lasting MG symptoms.

Active and passive immunization models suit perfectly for immunopathogenesis characterization of AChR auto-Ab, for the study of the pathogenicity of new antigens and for testing the therapeutic potential of drugs targeting the reduction of pathogenic Ig (Mori and Shigemoto, 2019). However they do not mimic the complex pathogenesis encountered in human disease, neglect the key role of the thymus, and induce a strong inflammatory bias (by the use of adjuvants, in the case of EAMG) (Christadoss et al., 2000). To overcome these limitations, other models based on transfer of MG tissues transfer have been developed.

1.8.3. Adoptive transfer of MG tissues

To examine the role of the thymus in the immunopathogenesis of MG, there are 2 possible strategies: (1) transplantation of fragments of the organ itself, or (2) transplantation of the thymus cellular content after cell selection. Because of well described issues of xenograft rejection, both strategies are only suitable in severe immunodeficient mice models lacking mature B- and T-cells which are permissive for human cell grafts (Mantegazza et al., 2016). These transplantations led to partial immunological humanization of the mice.

Severe combined immunodeficient mice (SCID) mice transplanted with MG patients' peripheral blood mononuclear cells (PBMC) or thymic lymphocytes were able to reproduce MG patients' features. Intraperitoneal (IP) injection of MG thymocytes in SCID mice induced rapid onset of anti-AChR Ab that were not long-lasting (Schönbeck et al., 1993). IP injection of MG PBMC induced typical signs of human MG and the raise in AChR Ab titers (Martino et al., 1993; Wang et al., 1999). Similar results were obtained in SCID mice receiving controls' PBMC and immunized with AChR, indicating that normal human immune repertoire can generate *in vivo* anti-self-reactions (Martino et al., 1993).

Regarding tissue transplantation, SCID mice engrafted with small MG thymus fragments beneath the kidney capsule presented human Ab targeting mice AChR (Schönbeck et al., 1992). When compared to dissociated cell transplanted models, AChR Ab production started later (2-3 weeks after transplantation) but lasted longer, reaching a peak at 11 weeks before decreasing (Schönbeck et al., 1993). These studies indicated that MG thymus constitutes a potent autoimmune microenvironment. However, the absence of clinical weakness and failure to detect human cells were important limitations of these models. Explanations to these observations may be related to the small number and size of the grafts.

Engraftments of MG thymic fragments have also been performed subcutaneously in NOD-*scid* IL-2R γ^{null} (NSG mice) using a different protocol (Sudres et al., 2017). NSG mice do not present T or B cells, neither NK, and do not develop T/B cells leakiness (Takata et al., 2019). Thus, NSG mice allow engraftment with human hematopoietic stem cells and/or human tissues and the subsequent development of a functional human immune system. The transplantation of 3-4 MG thymus fragments into the lower back of NSG mice was able to recapitulate most of the human MG features. In this model, 90% of the animals displayed human anti-AChR Ab in the serum, and most displayed MG-like symptoms with loss of AChR at the muscle endplates. Human cells were detectable not only in blood but in spleen of mice. Interestingly, the overall clinical picture observed in animals resembled that of the donor of MG thymus fragment (*i.e.* mild, severe...) [Sudres et al., 2017].

Advantages of the NSG-MG model over the other humanized SCID models include: NSG mice are more permissive for xenogeneic engraftment (Lepus et al., 2009; McDermott et al., 2010), potentially explaining the detection of human cells in mice spleen, and the subcutaneous location allows the transplantation of relatively larger fragments of thymus in comparison with the renal capsule, leading to the transfer of higher amounts of potentially pathogenic cells and We have used this model during the present study.

1.8.4. Variants of classical models

Alternative models have been used in MG for the elucidation of biological mechanism or determination of cytokine and cell types' role in disease pathogenesis (Tüzün et al., 2012). These models are set mostly in mice due to the availability of transgenic, knockout, and mutant mice that are optimal for establishing different proofs of concept (Mantegazza et al., 2016).

Examples of novel mice model include the development of an ocular EAMG model presenting clinical ocular signs (ptosis of the palpebra) which allows the study of pathogenic mechanisms. Several

Class II MHC transgenic and deficient mice strains were immunized subcutaneously with purified human AChR α subunit and tested for their susceptibility to OMG (Yang et al., 2007).

By the same token, different adjuvants were considered. As aforementioned, the majority of EAMG models use CFA, which stimulates immune response through TLR activation. However, due to severe side effects such as granulomas or adjuvant arthritis, as well as, its impact on animals welfare, new alternatives should be considered (Grumstrup-Scott, 2022). A combination of poly(I:C) (a TLR3 agonist) with Lipopolysaccharides (LPS) (a TLR4 agonist) could be an effective alternative to CFA in EAMG induction (Robinet et al., 2017b, 2017a). Finally, the advent of recent new efficient vaccination strategies based on the use of RNA may ease the creation of new versatile models.

1.8.5. Clinical and biological evaluations of the models

The course of MG animal models is evaluated by monitoring mice behavior, loss of body weight and muscular strength of the immunized animals. Myasthenic symptoms, assessed after exercise, include tremor, hunched posture, muscle weakness, and fatigue.

Muscular strength is regularly assessed by Grip test and inverted grid/screen test after exercise (Tuzun et al., 2015). In grip test, the inspector horizontally pulls the tail of a rodent that grips a bar connected to a monitoring device, as shown in **Figure 19A**, the maximal value is recorded as the forelimb grip strength. A modified version of this test does the measure with the system in vertical position instead of horizontal position (Takeshita et al., 2017) as shown in **Figure 19B**, which seems to present lower variability. In both apparatus configuration, sick MG mice show lower values than control mice.

In the inverted screen test, the muscle strength of all four limbs is assessed. Mice are placed in the center of stainless-steel wire mesh. Then, the screen is rotated 180° and the time the mouse resist clinged to the wire is measured (Deacon, 2013). Mice resistance to falling is usually diminished in sick EAMG mice. The test is exemplified in **Figure 19C**.

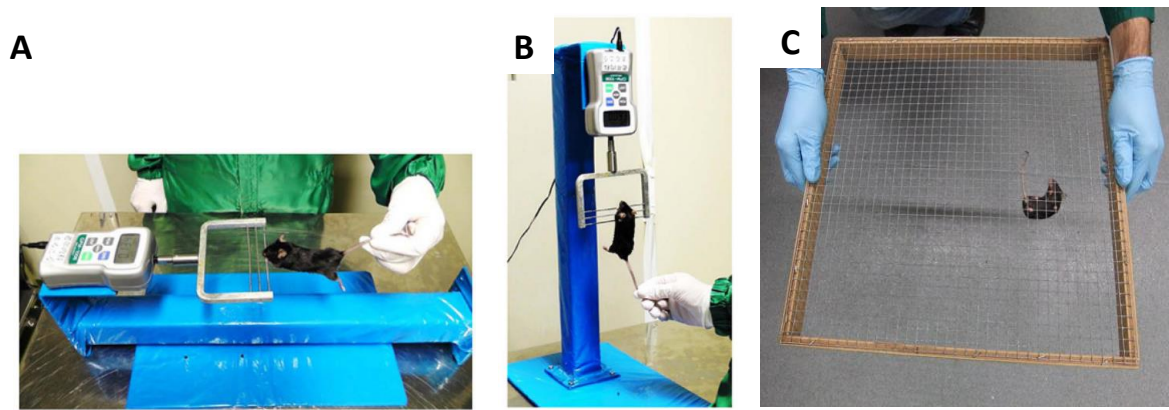


Figure 19. Tests for strength measures in mice models

Experimental apparatuses of: (A) the conventional forelimb grip strength test, (B) the modified forelimb grip strength test and (C) the inverted screen test (Takeshita et al., 2017)

EMG studies are also helpful tools in MG mice models evaluation, especially for those in which NMJ are highly affected showing myasthenic decrements (Losen et al., 2015). The examinations are similar to the ones performed in human and are done with the same instruments (repeated stimulations with electrodes, calculation of the decrement, analysis of the jitter).

Finally, response to voluntary exercise can also be assessed in mice through voluntary wheel running. Compared to forced treadmill exercise, voluntary wheel running presents several advantages: (1) it is a more physiological approach to natural running behavior of mice; (2) it is performed in non-stressed conditions as no human pressure is imprinted on animal's performance (3) it can be easily applied in long-term studies (Manzanares et al., 2019). The interpretations of the results, however, are somewhat difficult because of large variations in animals' performance. Moreover, disease induction methodology may introduce biases (injection in the foot pad may impair the walking behavior). This examination has never been applied to MG preclinical studies.

1.9. Therapeutic strategies

Therapeutic strategies for MG aim to improve patients' condition, rendering them minimally symptomatic, if not to cure them, while minimizing side effects from medications (Sanders et al., 2016). Ideally, it should help patients to achieve complete stable remission, a status in which they have not presented myasthenic symptoms or signs for at least 1 year without any therapy (Jaretzki et al., 2000).

1.9.1. Symptomatic drug treatment

1.9.1.1. Acetylcholinesterase inhibitors (AChE-I)

AChE-I improve NMJ transmission by blocking AChE enzymes and prolonging the availability of ACh at NMJ. They have no effect over the immune processes that cause and perpetuate MG. Oral AChE-I are the first-line treatment for MG. In more severe cases they are usually adjunctive to more definitive treatment in MG (Evoli, 2017). Their use is also recommended for neonatal MG till spontaneous resolution of symptoms (Mantegazza et al., 2011).

The list of pharmacological agents includes: physostigmine, neostigmine, and pyridostigmine, ambenonium chloride and edrophonium. These drug present short- or long-acting effects and the onset of their therapeutic effect vary, defining the context in which each one may be used (Colovic et al., 2013). Adverse effects are mainly due to cholinergic stimulation of muscarinic AChR present on smooth muscle and autonomic glands. One-third of patients, mainly elderly, present abdominal pain and diarrhea, bradycardia and hypotension (Rostedt Punga et al., 2008).

1.9.1.2. Other drugs

Ephedrine and salbutamol are β 2-adrenergic receptor agonists that have important therapeutic benefit in CMS patients (Lashley et al., 2010; Liewluck et al., 2011), including those with severe AChR deficiency (Rodríguez Cruz et al., 2015). Ephedrine showed mild but significant reduction of quantitative MG score in a small randomized trial (Lipka et al., 2017) and salbutamol may also have beneficial effects on muscle strength in patients with autoimmune MG but needs confirmation in clinical trials (Tannemaat and Verschuuren, 2020).

Tirasemtiv is a, fast, skeletal troponin activator that sensitizes the sarcomere to calcium and increases muscle force. In preclinical studies, it was able to decrease muscle fatigability and increase muscle force and grip strength (Russell et al., 2012). A small randomized clinical trial reported that tirasemtiv was well-tolerated and had modest effects (Sanders et al., 2015). The role of these agents in MG treatment requires further confirmation (Evoli, 2017)

1.9.2. Immunomodulating therapies

1.9.2.1. Glucocorticoids

Corticosteroids induce immunosuppression through different mechanisms, such as inhibition of cytokines transcription or blockade of transcription factors (Rhen and Cidlowski, 2005). These molecules are usually used as chronic therapeutic regimen when MG patients do not meet treatment goals after adequate trial of AChE-I (Sanders et al., 2016). They have good therapeutic effect, at least 75% of patients respond to corticosteroids, being beneficial for both OMG and GMG forms (Barnett et al., 2019).

The most commonly used glucocorticoid is prednisone, a prodrug form of prednisolone. It has a potent immunosuppressive activity, low anti-edemigen activity and half-life compatible with an alternate-day schedule. However, therapy initialization may require hospitalization due to risks of drug-induced exacerbation (Mantegazza et al., 2011), and establishing an optimal minimal dosage is usually a long and difficult process

The drawbacks to chronic steroid therapy are many, and other options are preferable. Chronic usage of these drugs are associated with adverse effects on virtually every system in the body (osteoporosis, cataract, glaucoma, obesity and diabetes mellitus, hypertension, mood disorders, alopecia among others)(Barnett et al., 2019).

1.9.2.2. Non-steroidal immunosuppressive agents

They can be used with corticosteroids for 2 aims: (1) as “steroid-sparing agents” or (2) as an add-on option to ameliorate poor corticosteroids response. They may also be used as a single drug when corticosteroids are contraindicated or refused (Sanders et al., 2016). Compared to steroids they have a delayed-onset effect (Tannemaat and Verschuuren, 2020). The most common pharmacological agents of this group include:

Azathioprine (Imurel)

Azathioprine is an antimetabolite which inhibits purine synthesis and blocks CD28 costimulatory signaling, leading to T cells suppression (Tiede et al., 2003). Azathioprine is the most frequently immunosuppressor (IS) used for MG treatment (Farmakidis et al., 2018). Retrospective studies suggested response rates to this drug ranging from 70% to 91% (Farmakidis et al., 2018). Its efficacy has been confirmed in a randomized clinical trial (Palace et al., 1998). Azathioprine is well tolerated,

however, liver and bone marrow (BM) toxicity, as well as, systemic reactions may occur (Kissel et al., 1986).

Mycophenolate mofetil (CellCept)

Mycophenolate mofetil is a prodrug of mycophenolic acid that inhibits purine synthesis and depletes guanosine nucleotides preferentially in T and B lymphocytes, impairing their proliferation (Hartono et al., 2013). Its potent immunosuppressive properties made it useful in organ rejection prophylaxis. Although two controlled trials failed to show mycophenolate mofetil efficacy in MG (Sanders and Siddiqi, 2008), it is still used and recommended in several national MG treatment guidelines (Sanders et al., 2016). Chronic use of this molecule may produce gastrointestinal effects and teratogenicity (Zwerner and Fiorentino, 2007)

Calcineurin Inhibitors

This category includes cyclosporine and tacrolimus; both are useful to prevent rejection of solid organ transplant, as they suppress T cells and NK cells. Mechanism of action of calcineurin inhibitors implies suppressing synthesis of pro-inflammatory cytokines (Gutfreund et al., 2013). Tacrolimus have some advantages when compared to cyclosporine: it is effective at a much lower dose and can be given in a once-daily formulation improving compliance. However, it is more expensive than cyclosporine (Barnett et al., 2019) and the side effects are different.

Although randomized clinical trials available evidence is not convincing, in Japan it is used as the first line or second line of treatment for MG (Kanai et al., 2017), and the international consensus guidance for management of MG includes tacrolimus as a possible therapeutic option (Sanders et al., 2016). Regarding cyclosporine, evidence from randomized trials supports its use in MG but potential serious adverse effects and drug interactions limit its use (Sanders et al., 2016). Calcineurin inhibitors have severe side effects including nephrotoxicity, hypertension, fibrosis and, as immunosuppressive agents, increased sensitivity to infections and malignancies.

Methotrexate

Methotrexate is an antimetabolite that inhibits the dihydrofolate reductase enzyme. It impairs the synthesis of purines and therefore DNA, resulting in cell-cycle arrest (Heckmann et al., 2011). It is used in high doses as chemotherapy in oncology patients, and in lower doses it is proposed as AID treatment; in both cases, the mechanism of action may differ (Brown et al., 2016). Studies on the use of methotrexate in MG are limited, and the available data do not provide convincing evidence of efficacy (Pasnoor et al., 2016). This drug could be a therapeutic option for GMG patients who have not

tolerated or responded to other steroid-sparing agents, however, long-term toxicity should be always considered (Narayanaswami et al., 2021).

Cyclophosphamide

Cyclophosphamide is an alkylating agent exhibiting cytotoxic effects and thus stopping B and T cells proliferation (Souto et al., 2019). It is used as part of the regimen for autologous BM transplantation (Bryant et al., 2016). Due to its severe side effects, including late development of malignancies, BM depression and infertility, the use of cyclophosphamide is restricted to the most severe cases of MG (Sanders et al., 2016)

1.9.3. Thymectomy

Thymectomy is the surgical removal of the thymus gland. It is mandatory in patients with thymoma and is recommended as an option for non-thymomatous adult AChR⁺ GMG patients, in particular those younger than 50 years, according to the last update of the International Consensus Guidance of Management of MG (Narayanaswami et al., 2021), **Table 5**.

Table 5. Clinical benefit of thymectomy in the different MG subgroups

Subgroup	Antibody	Additional antibodies	Age at onset	Proportion of patients (%)	Thymus	Clinical benefit of thymectomy
Early-onset	Anti-AChR	Rare	<50 years	15–25	Hyperplasia common	Proven
Late-onset	Anti-AChR	Common	>50 years	35–45	Atrophy common	Not proven (but possible)
Thymoma	Anti-AChR	Very common	Any	10	Lymphoepithelioma	Proven
MuSK	Anti-MuSK	Rare	Any	1–10	Normal	None
LRP4	Anti-LRP4	Rare	Any	1–5	Normal	None
Seronegative	None of the above detected	Variable	Any	10–15	Variable	None
Ocular	Variable	Rare	Any	15	Variable	None

AChR, acetylcholine receptor; LRP4, low-density lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase.

(Gilhus et al., 2016)

The rationale for thymectomy in patients without thymoma lies on the accepted role of thymus hyperplasia in MG pathogenesis (Truffault et al., 2017a). Early consideration of thymectomy in the disease improves clinical outcomes and minimizes need for long-term IS therapy and hospitalizations for disease exacerbations (Barnett et al., 2014; Wolfe et al., 2016, 2019). However, as thymectomy is not “curative” and patients have ongoing production of pathogenic Ab and impaired NMJ transmission, IS is required in more than 90% of patients, although, at reduced doses (Barnett et al., 2019). The

persistence of these Ab for some time, or perhaps indefinitely, relates to the persistence of the Ab-producing cells in the peripheral immune compartment (Lisak and Richman, 2020)

1.9.4. Short-term immunomodulation procedures

1.9.4.1. Classical approaches

This category includes aphaeretic therapeutic methods such as plasma-exchange (PLEX), immunoadsorption and Ig administration, either IV (IVIg) or most recently subcutaneously (SCIg). These interventions aim, through different forms, to rapidly remove Ab from patients' circulation or to modulate their effective functions; thus, modulating the immune system (Barnett et al., 2019). In PLEX and immunoadsorption, a patients' blood volume is taken out and separated into the cellular components and plasma. In the case of PLEX, the whole plasma is then discarded and replaced with a colloid fluid, combined back with the cellular components, and returned to the same patient. In the case of immunoadsorption, Ig (including the pathogenic Ab) are absorbed and retained, while the "purified" plasma is returned to patients body (Kronbichler et al., 2016). Procedures are shown in **Figure 20**. In the case of Ig administration, the approach is prepared from a pool of Ig from the blood of healthy donors, and infused in patients. Ig neutralize and block the production of auto-Ab, however the exact mechanisms are not completely understood (Zinman et al., 2007).

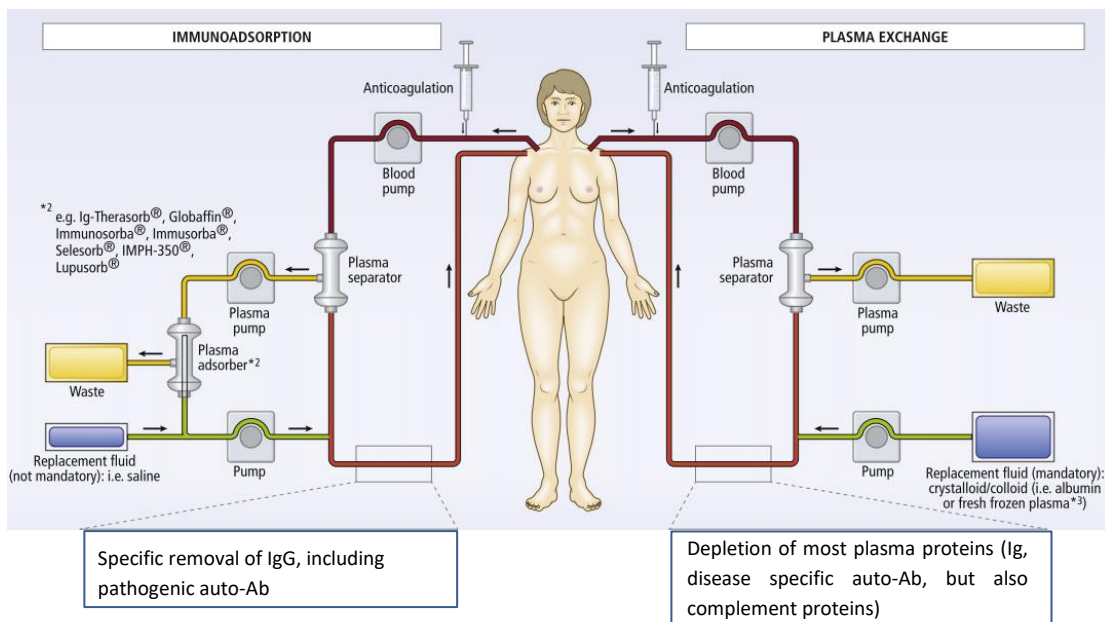


Figure 20. Technical aspects and molecular changes exerted by immunoadsorption and plasma exchange

Didactic representation of the immunoadsorption (left side) and plasma exchange (right side) procedures and effects (Kronbichler et al., 2016)

The most studied methods in literature are PLEX and IVIg. Due to their reported efficacy, IVIg and PLEX are recommended in myasthenic crisis (Karelis et al., 2019), worsening MG (Barth et al., 2011; Dhawan et al., 2015; Zinman et al., 2007) and as maintenance therapy for patients with refractory MG cases or with IS agents contraindication (Sanders et al., 2016). Their beneficial effect is rapid, but transient, lasting for 4 to 5 weeks. As preoperative therapy, IVIG was shown to be more effective in comparison to PLEX (Alipour-Faz et al., 2017). Choice between PLEX and IVIg depends on individual patient factors, including presence of respiratory distress, medical comorbidities, access to medication, and cost. PLEX will likely remain the treatment of choice in true myasthenic crisis because of faster onset of action (Dhawan et al., 2015; Souto et al., 2019).

Regarding SCIg, recently, several studies assessed its tolerability, and safety in MG. Literature reports included cases reports, a retrospective study and a prospective open label study, all reviewed by Adiao et al. The analysis of available data concluded improvements in functional disability in patients with MG using SCIg (Adiao et al., 2020).

Limitations of these procedures include cost, resource use, limited supplies and side-effects such as headache, hemolytic anemia and possible thrombotic events. The latter ones are less frequent in SCIg (Beecher et al., 2017; Bourque et al., 2016).

1.9.4.2. Novel specific AChR immunoadsorption approach

An emerging therapeutic option in this category is specific AChR auto-Ab removal using recombinant AChR extracellular domain coupled to insoluble carriers as immunoadsorbents (Lazaridis et al., 2015). In this apheretic approach only the specific auto-Ab involved in the pathogenesis of the disease are removed leaving all the other plasma components unaltered. *In vivo* studies of these immunoabsorbents showed effective Immunoadsorption, resulting in a marked auto-Ab titer decrease and dramatic symptom improvement in EAMG rat model (Lazaridis et al., 2017).

1.9.5. Biological drugs

MG worsening or not responding to symptomatic and IS treatments is called refractory MG, and it represents 10% of cases. Biological drugs, specifically Ab-based medicines, are potential treatment options for people with refractory MG. Several soluble or membrane bounded molecules, involved in different functions and pathways, have been targeted, as shown in **Figure 21**.

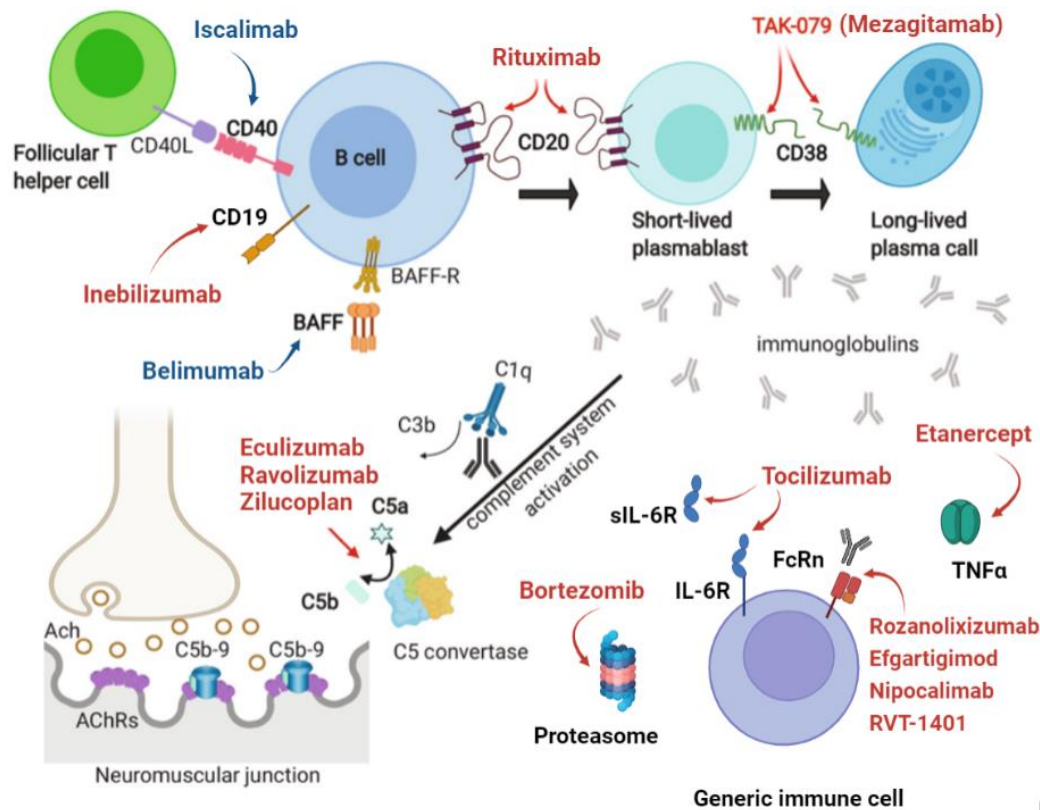


Figure 21. Targets of monoclonal antibody therapies in MG

Schematic summary of the principal cellular and molecular actors targeted by biological drugs tested in MG context. In red, the molecules that have shown beneficial results for MG or are under current study, and in blue, molecules that have been tested but were abandoned due to lack of clinical results. Adapted from (Alabbad et al., 2020), using Biorender. FcRn: neonatal Fc Receptor, IL-6R: IL-6 Receptor, s: soluble

Some of these biological drugs seem to be effective, other need to be further studied so to conclude over their efficacy, some molecules are still in earlier phases of study and some have not produced the expected amelioration after testing. In this section, encouraging molecules and the ones that are still under study are described, as well as their reported testing outcomes.

1.9.5.1. Targeting B cells

CD20-targeting mAb

Rituximab is a human-mice chimeric anti-CD20 monoclonal Ab that targets pro-B cells and all mature B cells, but not long-lived plasma or plasmablast cells (Huda, 2020). It causes prolonged B-cell depletion and is currently used in B-cell lymphoma adult treatment. It can induce the killing of CD20⁺ cells via multiple mechanisms such as complement-mediated cytotoxicity, Ab-dependent cellular cytotoxicity, and other indirect effects (Weiner, 2010).

A recent systematic review evaluated the efficacy and safety of the different doses of RTX used in 21 uncontrolled observational studies (from 2008 to 2020), gathering information of 260 refractory AChR⁺ MG patients. Meta-analysis showed that standard and lower-to-standard doses of RTX were able to induce improved clinical status in around 77% of patients and no significant differences in adverse reactions between both groups were observed. This was debated by the CADTH Canadian Drug Expert Committee (Young and McGill, 2021) concluding that studies that directly compare RTX clinical effectiveness to other MG treatment options are needed (Young and McGill, 2021). Adverse events associated with the use of RTX were relatively common, occurring in approximately 25% to 45% of patients, including: infection, hematological disorders (*i.e.*, thrombocytopenia, cytopenia or hypogammaglobulinemia), cardiologic disorders (*i.e.*, arrhythmia), psychiatric disorders and rarely death (Li et al., 2021b; Dos Santos et al., 2020)

CD38-targeting mAb

A limitation of the CD20-targeted approaches is that they do not eliminate the non-CD20 expressing, long-lived plasma cells. These cells can be targeted through CD38 which is expressed on plasma cells, but also T and NK cells, as well as, a range of non-immune cells (Alabbad et al., 2020). Mezagitamab (TAK-079) is a high-affinity antibody directed against CD38, originally developed for multiple myeloma (Krishnan et al., 2020). It is under evaluation in a phase II trial for MuSK and AChR⁺ MG.

CD19-targeting mAb

Inebilizumab is a humanized IgG monoclonal ab targeting the CD19 surface antigen on B cells. In contrast to rituximab, inebilizumab depletes a broad spectrum of B cells including plasmablasts and some plasma cells (Cree et al., 2019). A multicenter study in AChR⁺ and MuSK⁺ MG is ongoing.

1.9.5.2. Targeting tumor necrosis factor- α (TNF- α)

Etanercept is a fusion molecule containing the ligand-binding domain of human TNF receptor 2 linked to an Fc region. This TNF α antagonist decoy was originally developed to treat RA and has been shown to suppress ongoing EAMG (Christadoss and Goluszko, 2002; Duan et al., 2002). A pilot trial in corticosteroid-dependent MG patients showed clinical. However, cases of MG onset after a long-term treatment with etanercept were reported in RA (Fee and Kasarskis, 2009; Sawada et al., 2013) or psoriatic arthritis (Bruzese et al., 2015)

1.9.5.3. Targeting the complement

The role of complement in AChR⁺ MG is well established. In fact, complement activation represents one of the main effector functions of anti-AChR Ab. Therefore, targeting the complement is not counter-intuitive. Many EAMG trials investigating different complement inhibitors have been conducted; therapeutic strategies included recombinant proteins, chemicals, soluble isoforms of the complement receptor, monoclonal Ab, and small interfering RNA (siRNA). These studies focused on either inhibiting a terminal (C5, C6) or a classical pathway (C1q, C2) component or acting on complement regulators. C5 targeting molecules reached the stage of clinical trials and are presented below.

Eculizumab is a humanized monoclonal Ab that binds to C5 with high affinity, inhibiting its cleavage into C5a and C5b, thereby blocking MAC formation and preserving the post-synaptic membrane from damage. Eculizumab has been approved for the treatment of paroxysmal nocturnal haemoglobinuria and atypical hemolytic uremic syndrome (Rother et al., 2007; Wong and Kavanagh, 2015). Regarding MG, an international phase III trial (REGAIN) showed significant benefits in refractory generalized nonthymomatous AChR⁺ MG patients (Howard et al., 2017). These convincing results led to its approval for severe, refractory generalized AChR⁺ MG in the US, Canada, Europe, and Japan (Barnett et al., 2019). The latest MG international guideline recommends considering eculizumab after trials of other unsuccessful immunotherapies. Vaccination against *Neisseria meningitidis* is mandatory in patients, as the inhibition of C5 increases the risk of infection with this bacteria (Narayanaswami et al., 2021). Reported adverse effects to this treatment included headache, upper respiratory tract infection, nasopharyngitis and bacteraemia (Howard et al., 2017).

Ravolizumab is a re-engineered version of eculizumab with enhanced therapeutic efficacy and similar safety profile (Mantegazza et al., 2020). A phase III multicenter trial to evaluate its safety and efficacy in GMG patients has demonstrated rapid and sustained clinical improvement (Vu et al., 2022).

Zilucoplan is a synthetic macrocyclic peptide that allosterically inhibits C5 cleavage. The binding site of zilucoplan on C5 is distinct from eculizumab, patients genetically resistant to eculizumab demonstrated response to zilucoplan (Mantegazza et al., 2020). Zilucoplan has the advantage of subcutaneous administration; therefore, therapy can be self-administered by patients at home, which greatly improves its convenience (Barnett et al., 2019). A phase II study with GMG patients showed clinically meaningful and statistically significant improvements. Common adverse events were upper-tract infections and headaches (Howard et al., 2020). A phase III study for patients with GMG, named RAISE, was recently completed and UCB Pharma has disclosed positive topline results. Zilucoplan also requires *N. meningitidis* vaccination.

1.9.5.4. Inhibition of the neonatal Fc receptor

A novel treatment approach targets the neonatal Fc receptor (FcRn). FcRn function is to rescue IgG and albumin from lysosomal degradation by binding them and allowing their return unharmed, to extracellular space (Liu, 2018). Antagonists of FcRn bind to it impairing binding to native IgG. As a result, native IgG are delivered to the lysosome and degraded. The current primary safety considerations with anti-FcRn therapies is the reduction in serum albumin levels, but to date there has been no demonstrable adverse clinical effects in the human clinical trials (Gable and Guptill, 2020).

Four approaches are currently in clinical testing for MG: one is based on Fc fragment (Efgartigimod) and 3 on anti-FcRn monoclonal Ab (Nipocalimab, Rozanolixizumab, RVT-1401). Other FcRn targeted strategies such as recombinant Fc multimers, Fc γ R targeted therapeutics, and additional FcRn monoclonal Ab are at preclinical stage in MG (Liu, 2018).

Efgartigimod is a Fc fragment derived from an IgG1 and engineered to increase Fc/FcRn binding at neutral and acidic pH (Bayer and Vilquin, 2020). Recently the results of a phase III multicentric trial (ADAPT) were published, and showed tolerance and efficacy in patients with GMG. Additionally, an open-label extension is ongoing (Howard et al., 2021).

Nipocalimab is a human anti-FcRn deglycosylated IgG1 complete monoclonal Ab lacking effector function. Nipocalimab binds with high affinity to FcRn at both endosomal pH and extracellular pH allowing occupancy of FcRn throughout the recycling pathway. It is not expected to cross the placenta (Ling et al., 2019). A phase II study was completed recently in GMG patients with poor response to standard of care therapy. So far, an official press release announced that 52% of patients who received nipocalimab had a significant and durable clinical improvements. Nipocalimab was well-tolerated and severe or serious treatment-related adverse events were not reported (Keller et al., 2021).

Rozanolixizumab is a humanized anti-FcRn, high-affinity, IgG4 monoclonal Ab (Gable and Guptill, 2020). In phase II study in GMG patients, beneficial changes from baseline were not statistically significant when compared to placebo, however, a second treatment phase led to further clinical improvements (Bril et al., 2021). In the recent phase III study, UCB Biopharm officially reported achievement of primary and all secondary endpoints with statistical significance and no new observed safety issues (UCB, 2021).

RVT-1401 is a human recombinant anti-FcRn monoclonal IgG1 Ab, which was developed for IV or subcutaneous administration (Collins et al., 2019). So far, it was well tolerated and it may provide a good therapeutic option with less side-effects than other existing therapies (Barnett et al., 2019). The

results of phase II clinical trial evaluating safety and pharmacodynamic effects of subcutaneous RVT-1401 in AChR⁺ MG patients are pending.

1.9.5.5. Other inhibitors

Bortezomib is an inhibitor of proteasome activity in plasma cells; it interrupts the proteolytic pathway and induces cell death. It is used in cancer treatment and can induce clinical improvements in SLE (Kohler et al., 2019). In EAMG, bortezomib exhibited a reduction of AChR Ab titers and a clinical improvement (Gomez et al., 2014). The results of a phase IIa trial on patients with active refractory MG are awaited (Huda, 2020).

Tocilizumab is a recombinant humanized mAb against the IL-6 receptor (IL6-R) (Sebba, 2008) that demonstrated clinical effectiveness in other AID (Huda, 2020). Of various cytokines that mediate Th1 and Th2 responses, IL-6 plays a prominent damaging role in MG (Deng et al., 2002). Tocilizumab binds both cell-surface-bound and soluble IL-6R and prevents the proinflammatory effects of IL-6. Clinical benefits of tocilizumab treatment include a published case report of two MG refractory patients ameliorated. However, no effect on auto-Ab titer was detected (Jonsson et al., 2017).

1.9.6. Cell therapy

Autologous hematopoietic stem cell transplantation (aHSCT) has been used since 1996 in the treatment of severe AID, refractory to conventional therapy, with good results (Farge et al., 2010) including MG (Bryant et al., 2016). This and novel cell therapy approaches, in MG context, are discussed below.

1.9.6.1. Hematopoietic stem cells transplantations (HSCT)

This treatment aims to wholly replace an autoreactive immune system by one that is protective and self-tolerant. Autoimmune neurologic conditions that have tested autologous HSCT include MS, chronic inflammatory demyelinating polyneuropathy, NMO, MG and others (Sharrack et al., 2020).

Ten multiresistant MG patients have been reported in literature for having received HSCT. Seven were treated at a single centre with intensive conditioning chemotherapy regimens to destroy the autoreactive immune system, and showed good tolerance with achievement of consistent, durable, symptom-free, and treatment-free remission in all patients after a median follow-up of 40 months (Bryant et al., 2016). Cited adverse effects related to HSCT included transient viral reactivations and development of another AID in a single patient. HSCT reported similar outcomes in 3 independent

case reports using cyclophosphamide-based conditioning, 2 of them received autologous HSCT (Håkansson et al., 2017; Sossa Melo et al., 2019) and one patient was transplanted with peripheral blood stem cell infusion from his HLA-matched sibling (Strober et al., 2009). Despite good results, this type of treatment is extremely aggressive and risky (Barnett et al., 2019).

1.9.6.2. Modified chimeric antigen receptors (CAR) T cells

CAR are engineered receptors that are expressed by immune effector T cells, enabling them to recognize specific antigens and destroy cells expressing them (Zhang et al., 2017). DNA engineered can be performed using patient's (autologous) or donor's (allogeneic) T cells. However, cytokine release syndrome and neurotoxicity induced by excessive CAR T cells activation in patients prohibits their use outside of oncology (Bonifant et al., 2016). An option to bypass unchecked CAR T-cell activation is mRNA modified CAR T cells. Descartes-08 is an mRNA-modified, autologous CAR T-cell product directed against B-Cell Maturation Antigen (BCMA), tested for GMG and other AID. Descartes-08 is intended to halt production of auto-Ab by targeting pathogenic long-lived plasma cells with the potential to survive for decades within the body. There is an ongoing Phase 1b/2a study in GMG at multiple centers in the US and Canada. Late-breaking interim data shared at the 14th MGFA Conference (Miami, 2022) indicated safety and tolerance; justifying the advance of Descartes-08 to larger controlled studies in MGAID.

1.10. Final remarks to conclude this chapter

Since MG first description, considerable progress has been made in MG understanding throughout these centuries. MG is one of the few AID for which detailed knowledge is available regarding both the target antigens and contributing factors. But there is still a lot to be done in the field. To date, the major novel challenges for MG research are: to identify the primary cause of the disease, to develop new sensitive and specific diagnostic tools, to work on biomarkers to predict the course of the disease, and to improve the therapeutic options. This last point is essential taking into account that, despite the current therapeutic options and the advances in novel immune-targeted therapies, there is still a subset of patients presenting refractory disease with severe or life-threatening symptoms (Silvestri and Wolfe, 2014), and finally a large proportion of MG patients do not feel satisfied with their quality of life and their treatment plan.

2. Mesenchymal Stromal Cells (MSC)

2.1. Generalities

Stem cells can be distinguished according to their source and differentiation potential. Embryonic stem cells, coming from the inner cell mass (30–50 cells) of the pre-implantation embryo, are able to form all cellular structures, except the placenta, when the appropriate information for differentiation are provided (Brignier and Gewirtz, 2010). Furthermore, embryonic stem cells can be expanded continuously in culture and retain the ability to differentiate into derivatives of all three embryonic germ layers (Rippon and Bishop, 2004). In contrast, adult stem cells are undifferentiated multipotent stem cells obtained from adult individuals, that can differentiate into the cell types that constitute their respective source tissues (Berebichez-Fridman and Montero-Olvera, 2018) (**Figure 22**).

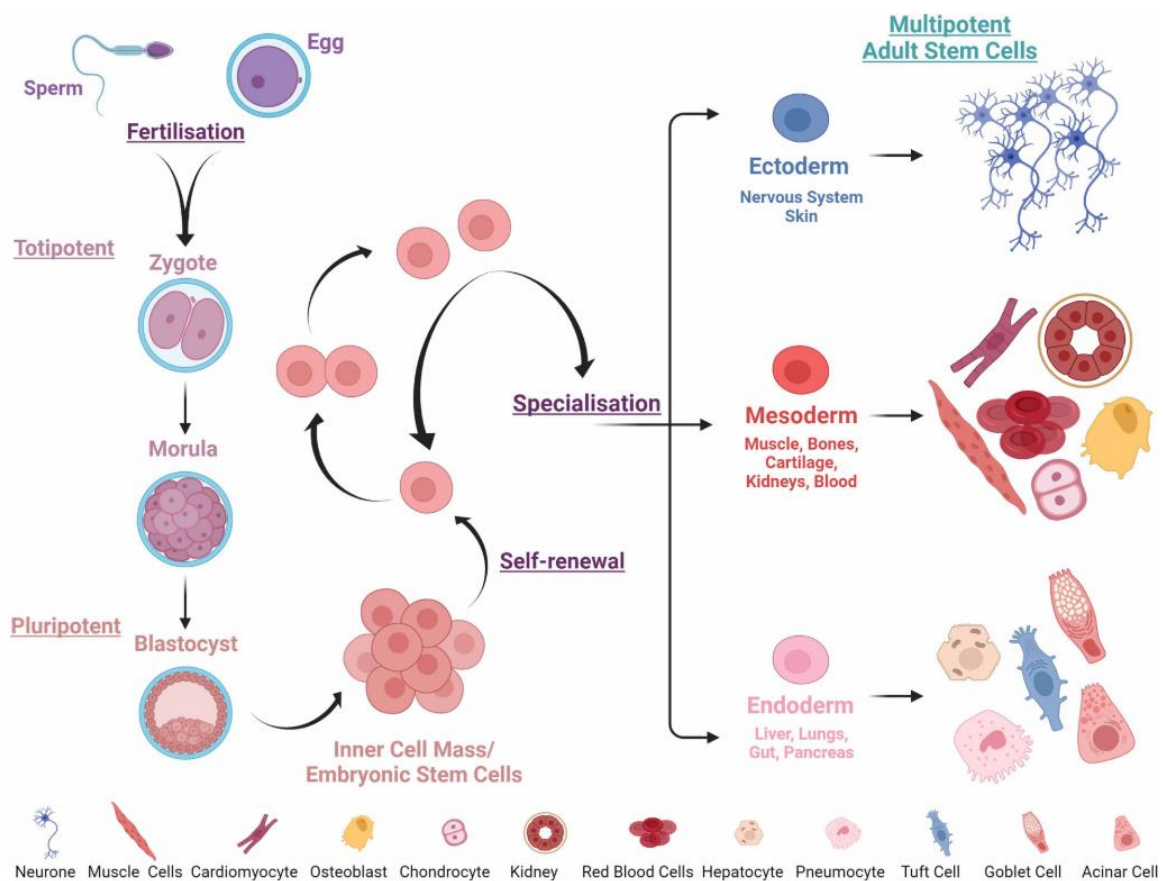


Figure 22. Illustration of the potential and the potency of embryonic and adult stem cells

Totipotent stem cells are derived from the fertilized egg and can form all cells of the developing organism. Like totipotent stem cells, pluripotent stem cells or embryonic stem cells (ESCs) can form all tissues of the body, except the placenta. Multipotent stem cells, found within adult tissues are more specialized and are lineage restricted, differentiating into cell types linked to their tissue of origin. (Stewart, 2021)

Among the adult “stem” cells we found the mesenchymal stromal cells (MSC). The term “mesenchymal” refers to a group of cells derived mainly from the third germ layer (the mesoderm) and that are responsible of building connective tissue in adult organisms such as bone, cartilage, tendons, ligaments, etc. They can also differentiate into adipocytes, and more controversially, into endothelial cells, muscle cells or neurons under physiological or specific experimental conditions (Andrzejewska et al., 2019).

Ontogenically, the true origin of MSC is unknown (Isern et al., 2014). MSC may be related to pericytes or adventitial cells, which parallel or enwrap the vessels, and this would explain their presence in all organs (Pittenger et al., 2019). Being present at low frequency in most adult connective tissues, they are responsible for tissue growth, maintenance, repair, and trophic support. They are also implicated in wound healing and cell substitution in physiological or pathological contexts (Berebichez-Fridman and Montero-Olvera, 2018). All MSC may not be equivalent, and their specific differentiation ability may be guided by their tissue of residence (Sacchetti et al., 2016).

MSC have been extensively studied in experimental medicine, given their unique properties in terms of immunomodulation, soluble factors secretion and homing to injured tissue sites (Stefańska et al., 2020). These are key features for different disease treatments including tissue regeneration, cancer, and immunological/inflammatory disorders (Augello and De Bari, 2010). MSC were first isolated from BM in 1960-1970’s and since, remarkable progresses have been done in phenotypic and functional characterization of these cells. Description of relevant aspects of their nature, obtention, production and applications are described in the following sections.

2.2. *Historical aspect*

The presence of nonhematopoietic stem cells in the BM was first proposed by Cohnheim in 1867 (Stefańska et al., 2020). His work suggested the possibility of BM being a potential source of fibroblasts implicated in the normal process of wound repair (Chamberlain et al., 2007). But it was only 100 years later that Tavassoli and Crosby, through heterotopic transplantation of intact pieces of marrow into animals, clearly established proof of osteogenic potential associated with BM (Tavassoli and Crosby, 1968). These transplanted boneless fragments of marrow resulted in the generation of ectopic “ossicles”, shells of bone enveloping a marrow cavity with hematopoietic cells lodging inside (Bianco et al., 2010). However, their work could not decipher which exact cell type served as progenitor of differentiated bone cells. Friedenstein and colleagues demonstrated that the osteogenic potential was associated with a minor subpopulation of BM cells.

These cells were distinguishable from the majority of hematopoietic cells by their rapid adherence to tissue culture, their fibroblast-like shape and their ability to grow rapidly *in vitro* in the form of clonogenic colonies (colony-forming unit fibroblast) (Friedenstein et al., 1970). *In vivo* transplantation demonstrated that bone, cartilage, AT, and fibrous tissue could be experimentally generated by the progeny of a single BM stromal cell (Ashton et al., 1980; Bab et al., 1986; Friedenstein et al., 1974).

Because of Friedenstein, these cells were known as “osteogenic stem cell” or “BM stromal stem cell”. In 1991, Caplan proposed the term “mesenchymal stem cells” due to their ability to differentiate into more than one type of the cells that form connective tissue in many organs (Caplan, 1991), and this name became widely used even though it raised questions over its accuracy regarding, specially, their stemness (Bianco et al., 2008).

2.3. Definition and nomenclature

Many definitions are attributed to the acronym MSC, including multipotent stromal cells, marrow stromal cells, mesodermal stem cells, mesenchymal stromal cells, among others (Andrzejewska et al., 2019). Confusion arising from this panoply of terms and an ambiguous non-consensual definition of the MSC population, made comparisons among published studies in the 1990s and 2000s a real conundrum. This led to the proposal of new terminology and criteria by the International Society for Cellular Therapy (ISCT) (Keating, 2012).

2.3.1. Mesenchymal stem cells versus Mesenchymal stromal cells – ISCT Terminology

The lack of consensus among leaders in the field, on a capital subject, such as the basic definition of the nonhematopoietic stem/stromal cells, promoted a space of debate at the Annual Meeting of the International Society for Cellular Therapy (ISCT) in 2000. Data-based discussion concluded that the term “mesenchymal” fitted appropriately to these cells, but that compelling evidence that these cells are stem cells were lacking (Horwitz and Keating, 2000). In 2006, as confusing nomenclature persisted, the ISCT published a position paper recommending to keep MSC acronym for both, stem and stromal cells but with imperative clarification of the terminology to reduce the existing confusion and avoid misrepresentation (Dominici et al., 2006).

Even though mesenchymal stromal cells exhibit some attributes of stem cells, they do not meet the full criteria to be qualified as *bona fide* stem cells (Gomez-Salazar et al., 2020). In line with this statement, the ISCT recommended that plastic-adherent cells currently described as mesenchymal

stem cells should be termed multipotent mesenchymal stromal cells. The term “mesenchymal” was attributed because of their ontogeny and without differentiation potential connotation, and the term “stromal” because of their localization in the supportive stromal compartment of the tissues regardless of their tissue source. The denomination “stem cell” should be reserved for a subset of these mesenchymal stromal (or other) cells with demonstrable progenitor cell functionality of self-renewal and differentiation (Dominici et al., 2006).

Here, the further usage of the MSC acronym will stand for mesenchymal stromal cells, the multipotent heterogeneous population with remarkable secretory, immunomodulatory and homing properties and identified by the ISCT minimal MSC criteria which are described in the following section.

2.3.2. Minimal ISCT criteria for defining MSC

Because of increasing interest in MSC and growing clinical relevance, a need to establish a non-ambiguous and broadly accepted definition for these cells arose. The ISCT's MSC working group, proposed a set of standards to define *in vitro* expanded MSC. The following “minimal criteria” should be demonstrated before a cell could be referred to as an MSC (Dominici et al., 2006):

- *Adherence to plastic in standard culture conditions using tissue culture flasks. (Figure 23A),*
- *Specific surface antigen (Ag) expression*

By flow cytometry, $\geq 95\%$ cells must be positive for CD105 (endoglin), CD73 (5'-nucleotidase) and CD90 (Thy-1) and $\leq 2\%$ should show positive staining for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II (Human leukocyte antigen class II) (**Figure 23B**). The negative markers are hematopoietic antigens used for the exclusion of hematopoietic progenitors and endothelial cells (CD34⁺), monocytes and macrophages (CD14⁺/CD11b⁺) and B cells (CD19⁺). CD45 is expressed by all leukocytes and ensures exclusion of this heterogenic group of cells. All these populations are likely to be found in the cell preparation with MSC.

In addition to these markers, MSC can express a large number of integrin receptors (CD29, CD49a to CD49f, CD51), adhesion molecules (CD44, CD105, CD106, CD146, CD166), enzymes (CD39, CD73), growth factor receptors (CD140b, CD271, CD340, CD349), intermediate filaments (vimentin, nestin, desmin, neurofilament) and embryonic antigens (SSEA1, SSEA4), but no single molecule uniquely defines the population (Phinney and Sensébé, 2013). Characterization with these markers is facultative; they are not included in the definition of MSC and their expression can change in response to different conditions. One of the limitations in defining MSC according to surface phenotype is MSC surfaceome plasticity (see sources of MSC heterogeneity, section 2.7. below).

- *Multipotent differentiation potential*

Cells must show *in vitro* trilineage differentiation potential when exposed to dedicated culture conditions, giving osteoblasts, adipocytes and chondroblasts after the application of the adequate inductive factor(s).

The classical approach to characterize the degree of differentiation of MSC is histological staining (Dominici et al., 2006). However, several other techniques such as: RT-PCR, DNA sequencing, transcriptome analysis, Western blot or even advanced microscopy techniques have been also used to this purpose (Eggerschwiler et al., 2019).

Differentiation into osteoblasts can be induced by combination of dexamethasone, ascorbic acid and sodium β -glycero phosphate (Ciuffreda et al., 2016; Pittenger et al., 1999). These factors produce deposition of a mineralized ECM that can be detected with Alizarin Red or von Kossa staining (**Figure 23C**), and up-regulation of alkaline phosphatase activity and of secreted osteocalcin that can be measured by biochemical assays (Heino and Hentunen, 2008). Further osteogenic protein markers that can be searched are bone sialoprotein and osteopontin, or surface markers STRO-1 and alkaline phosphatase (Gronthos et al., 1999).

Adipocyte differentiation can be triggered by a combination of dimethyl sulfoxide, dexamethasone, indomethacin, insulin, isobutylmethylxanthine, among other factors (Rosen and MacDougald, 2006). By the classical approach, adipocytes are easily identified by their morphology and staining with Oil Red O (Dominici et al., 2006) (**Figure 23D**). Other read-outs include: expression of peroxisome proliferator activated receptor gamma via biochemical assays; gene or protein expression of fatty acid binding protein 4 and lipoprotein lipase, or secretion of adiponectin and leptin (Lindner et al., 2010).

Chondrogenesis is most effective when MSC are cultured in 3D models (Lindner et al., 2010). Therefore, MSC are centrifuged to form micromass pellets (Mackay et al., 1998) or embedded in hydrogels made of agarose or alginate (Erickson et al., 2009). The chondroblast differentiation protocol includes culture in media supplemented with dexamethasone, ascorbic acid, human TGF- β , insulin, transferrin, sodium selenite, and pyruvate (Mackay et al., 1998). It may additionally contain insulin growth factor (IGF) 1 and bone morphogenetic proteins (An et al., 2010). Differentiation in chondrocytes can be assessed by proteoglycans staining with Alcian blue (**Figure 23E**), toluidine blue or safranin O, as well as, immunohistochemical staining for collagen type II (Dominici et al., 2006). In

addition, detection of glycosaminoglycan content or collagen IX and versican can also be verified by biochemical assays (Lindner et al., 2010).

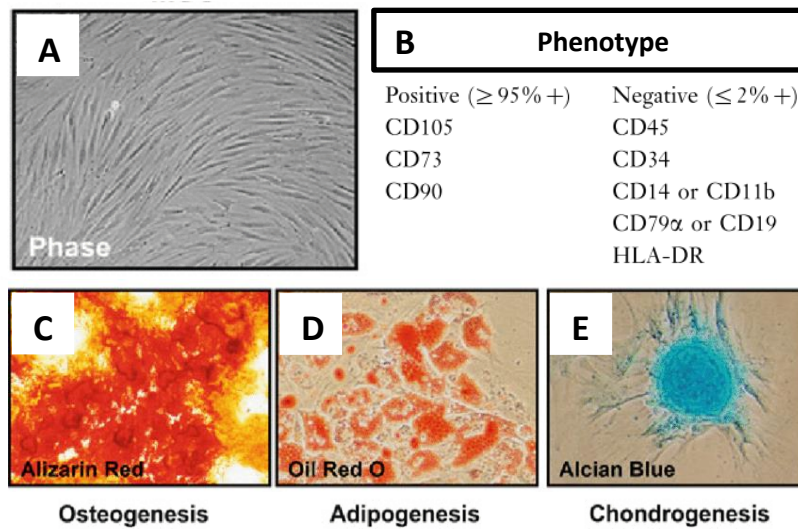


Figure 23. ISCT Minimal criteria for MSC definition

MSC are defined through their capacity to: (A) adhere on plastic, (B) express or not defined extracellular markers and their capacity to do undergo (C) osteogenesis, (D) adipogenesis and (E) chondrogenesis. Figure adapted from (Akram et al., 2013)

As well stated by the ISCT, these are “minimal” criteria used to identify cultured MSC in the laboratory, but they cannot be taken as sufficient or accepted release criteria for stocks of MSC attributed to therapeutic applications (Gomez-Salazar et al., 2020).

2.3.3. Evolution of MSC definition by ISCT

In 2013, due to the increasing interest of the MSC community in their immunomodulatory capacities and the number of studies that emerged assessing these properties, the ISCT updated the original definition to incorporate the immunoregulatory component and introduce some guideline related to the assessment of regulatory properties of human MSC (Krampera et al., 2013).

In 2019, ISCT updated their MSC definition and suggested different aspects: (1) to include the tissue origin of cells when reporting results, (2) to use stromal cell nomenclature, unless rigorous evidence for self-renewal and differentiation properties are shown, and (3) to include functional assays (RNA analyses of selected genes, flow cytometry of cell surface markers and protein analysis of MSC secretome) (Galipeau et al., 2016) to define therapeutic mechanism of action of these cells (Viswanathan et al., 2019).

Still today, despite the amendments, MSC definition may be unduly constraining because it remains not distinctive (Wright et al., 2021). And even if ISCT recognizes the impact of species difference, tissue source, and passage of cells at the time of characterization (Viswanathan et al., 2019), no specific guidelines were addressed. These elements unveil the heterogeneity of MSC and the need for reappraising and updating the MSC definition criteria.

2.4. Cell sources

2.4.1. General MSC tissue sources

MSC can be obtained from virtually all the tissues in the human body (Pittenger et al., 2019). Historically MSC were first derived from human BM (BM-MS) (Mackay et al., 1998; Pittenger et al., 1999), and were later isolated from AT (AD-MS) (Zuk et al., 2002). Tissue resident cells with characteristics of MSC were also identified and effectively isolated from dental pulp (Gronthos et al., 2000), salivary glands (Rotter et al., 2008), human organs like the gut (Lanzoni et al., 2009) and the skin (Shih et al., 2005) and even from peripheral (Fernández et al., 1997; Tondreau et al., 2005; Zvaifler et al., 2000) and menstrual blood (Meng et al., 2007).

More younger "adult" MSC sources are birth-derived tissues such as, umbilical cord blood (UCB-MS) (Erices et al., 2000; Goodwin et al., 2001; Lee et al., 2004b; Tondreau et al., 2005), Warthon's jelly (Wang et al., 2004), placenta (in 't Anker et al., 2004), among others (Diller et al., 2020). These are very interesting sources because they are rich in human MSC and of easy access, as they may be considered medical/surgical debris and *res nullius*.

The choice of the "ideal" MSC is guided by practical limitations concerning the difficulty and invasiveness of the isolation, the potential adverse effects of harvesting the cells on the donor and the cell yield, as well as differences in their biological characteristics (Berebichez-Fridman and Montero-Olvera, 2018).

The main used cell sources for clinical applications are BM, AT, and fetal or neonate tissue (specially UCB) (Jovic et al., 2022). BM and AT have been the most used sources; they are relatively easy to collect by standardized procedures especially for autologous transplantations, with AT presenting some advantages such as cell yield, less risky procedure and reduced morbidity (Phinney and Sensébé, 2013). Interestingly, over the past years the number of clinical trial using UCB-MS has increased, this source for allogeneic use is considered to be easily and painlessly obtained from donors

of a consistent young age, thus minimizing the potential effects of aging or prior health status on the MSC (Wright et al., 2021).

2.4.2. AD-MSC specific sources

AT is a complex source of MSC because:

1. There are different types of fat: visceral, omental and subcutaneous sample (Jung et al., 2015). The most common source is subcutaneous fat, as it provides larger amounts of cells during the standard surgical procedure.
2. There are different harvesting sites: subcutaneous fat can be obtained from the abdomen, chin, neck or the hip/thigh region (Jurgens et al., 2008).
3. There are several harvesting techniques, that can give “liquid” fat (*e.g.* liposuction procedures) and “solid” fat (*e.g.* resection of fat tissue, abdominoplasty).

2.4.3. AD-MSC isolation

AT can be harvested through 2 main surgical procedures: by resection, obtaining a block of fat tissue or “solid fat”, and by liposuction, either power-assisted liposuction or laser-assisted liposuction, and obtaining “liquid fat” samples (Schneider et al., 2017). Liposuction procedure presents the advantages of being safer, more well-tolerated, less invasive, and giving higher yields of viable AD-MSC, thus being preferred to abdominoplasties (Palumbo et al., 2018). In addition, harvesting methods can also impact over AD-MSC basic characteristics such as number of isolated cells, clonogenicity, or doubling time, as shown by Bajek et al. According to these studies power-assisted liposuction gave AD-MSC with higher proliferation potential and resistance to senescence when compared to laser-assisted liposuction or surgical biopsy (Bajek et al., 2017).

For the isolation of AD-MSC, the first step of the procedure differs from solid fat to liposuction. Solid fat must undergo mechanical disruption and be reduced into small pieces using a scalpel and forceps (Schneider et al., 2017). Once completed, both samples (liposuction and minced fat from resection) can be treated similarly. Minced AT are extensively washed with equal volumes of phosphate-buffered saline (PBS) and antibiotics, and then digested for 30-60 min at 37°C using collagenase, most commonly (Zuk et al., 2001) or other related enzymes (dispase, trypsin, etc) (Bourin et al., 2013). After incubation, enzyme activity is neutralized with supplemented medium and centrifuged to obtain a high-density pellet called the stromal vascular fraction (SVF).

The SVF is an heterogeneous group of cells including adipose stromal and hematopoietic stem and progenitor cells, endothelial cells, erythrocytes, lymphocytes, macrophages, fibroblasts and pericytes, among others (Schneider et al., 2017). SVF is incubated with a lysing solution to remove any contaminating red blood cells, and washed. At this point, some protocols do a facultative gradient centrifugation step and take the cells from the interface layer. Finally, collected SVF is resuspended in culture medium, filtered through a nylon mesh to remove undigested tissue fragments and cultured in plasticwares to select the plastic-adherent AD-MSC (Zuk et al., 2001). The procedure is schematized in **Figure 24**.

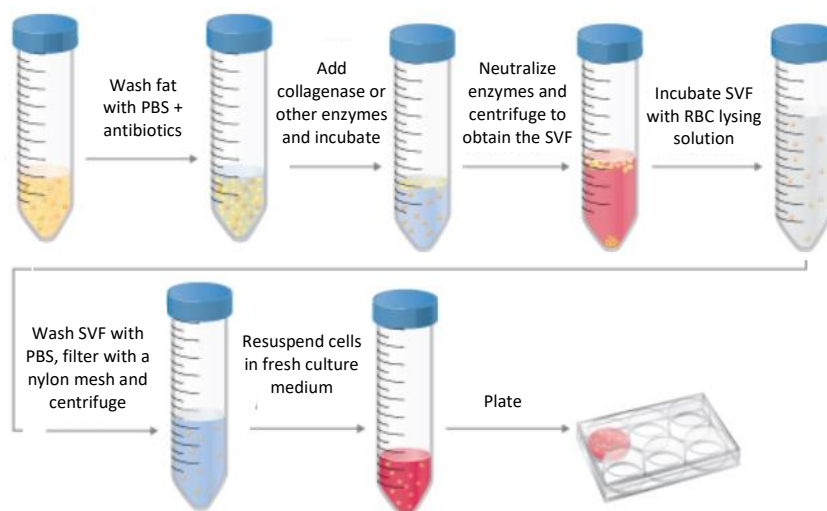


Figure 24. Simplified representation of AD-MSC isolation protocol

Adapted image from <https://www.irvinesci.com/protocol-for-mesenchymal-stem-cell-isolation>

2.5. Biological properties

MSC show tremendous potential for the treatment of many diseases, including immunological and non-immunological disorders. The therapeutic potential deployed by MSC should be considered the following aspects: (1) multilineage differentiation potential, (2) migration to the site of injury, (3) paracrine effect by secretion of soluble factors crucial for cell survival and proliferation, and (4) immune system response modulation (Wu et al., 2020).

2.5.1. Differentiation potential

As indicated above, in addition to the capacity to differentiate into osteocytes, chondrocytes, and adipocytes (Dominici et al., 2006), literature reports indicate that molecular treatment of some specific-tissue-derived MSC might also give rise to other cell types of the mesodermal lineage such as myoblasts, fibroblasts, cardiomyocytes, ligaments and tendons (Andrzejewska et al., 2019).

Furthermore, MSC are also able to differentiate *in vitro* in cells of endodermal germinal layer such as hepatocytes (Lee et al., 2004a), β - like (Timper et al., 2006) and parathyroid-like cells (Bingham et al., 2009), and of the ectodermal layer such as epithelial-like cells (Păunescu et al., 2007), neurons, astrocytes, oligodendrocytes, and Schwann cell with varying degrees of efficiency (Gervois et al., 2015). This plasticity of MSC and their self-renewal capacity make these cells interesting therapeutic targets for various diseases, including treatment and tissue regeneration. The spectrum of MSC differentiation potential is summed-up in **Figure 25**.

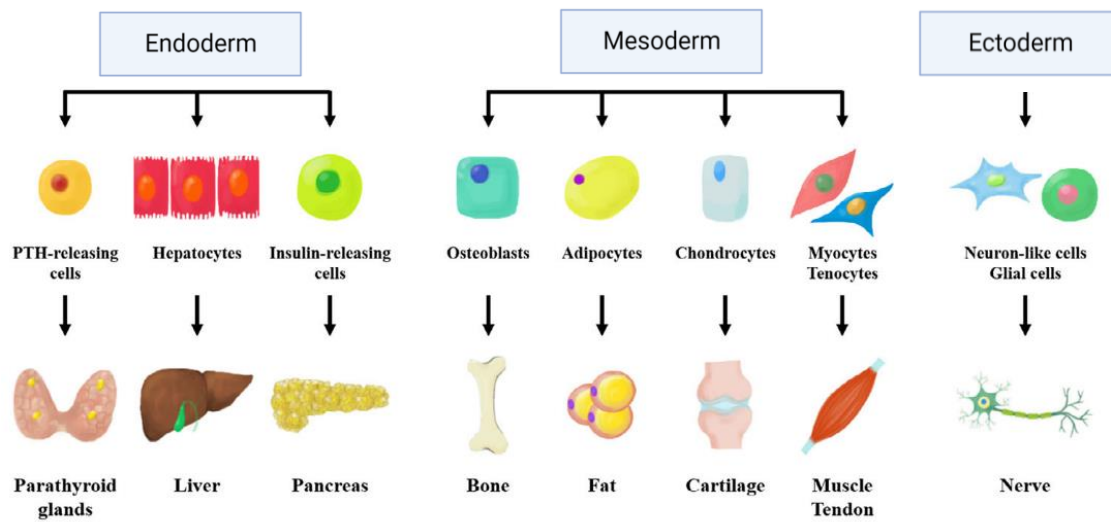


Figure 25. Schematic diagram of lineage differentiation of MSC

MSC have the potential to differentiate into endodermal (PTH/insulin-releasing cells, hepatocytes), mesodermal (bone, cartilage, fat, muscle, and tendon), and ectodermal lineages (neuron-like or glial cells). Abbreviations: PTH, parathyroid hormone; MSC, mesenchymal stromal cells. Adapted from (Oh et al., 2019)

2.5.2. Microenvironment sensing and homing

Several studies have reported the capacity of endogenous or transplanted MSC to migrate to the site of injury when tissues are damaged (Assis et al., 2010; Caplan, 2009; Chapel et al., 2003; Chen et al., 2010; Jackson et al., 2010; Kidd et al., 2009; Rustad and Gurtner, 2012), despite studies reporting the opposite (Eggenhofer et al., 2012; Fischer et al., 2009). MSC homing is the premise of their application in the regenerative medicine and the treatment of systemic diseases. Migration of the cells and release of bioactive factors that are, both, immunomodulatory and trophic, allow tissue regeneration or immunomodulation (Caplan, 2009; Ullah et al., 2019; Yuan et al., 2022).

Migration involves surface antigens and cell adhesion molecules (Chen et al., 2010) and encompasses 2 different processes according to their route of administration (Nitzsche et al., 2017). In non-systemic homing, MSC are transplanted locally at the target tissue and are then guided to the site of injury by sensing a chemokine gradient through chemokine receptors (Ullah et al., 2019), while in systemic homing, MSC are administered into the bloodstream and cells leave the blood flow and transmigrate through the endothelial barrier to reach the injury site (Nitzsche et al., 2017). This process implies 5 consecutive steps: (1) rolling, (2) activation, (3) firm adhesion, (4) crawling, and (5) transendothelial migration, similar to leukocytes' migration to inflammatory sites and is guided by homing-promoting factors released from damaged or inflamed tissues as shown in **Figure 26** (Yuan et al., 2022).

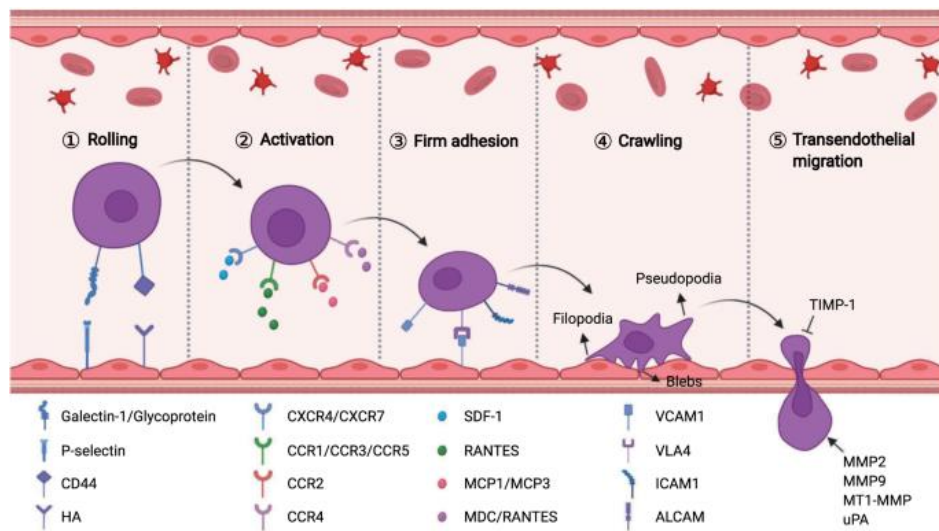


Figure 26. Schematic summarizing the molecular mechanisms of MSC systemic homing

MSC are able to migrate from the bloodstream to the site of injury/inflammation, guided by homing molecules and following five steps: (1) rolling, (2) activation, (3) firm adhesion, (4) crawling, and (5) migration. (Yuan et al., 2022)

2.5.3. Paracrine effect

MSC secrete a plethora of biologically active molecules that exert pleiotropic beneficial effects on injured or inflamed tissues. Paracrine effect, rather than cell replacement, may explain most of the MSC therapeutic benefits (Kusuma et al., 2017; Moll et al., 2020; Wright et al., 2021) since the survival and differentiation of MSC at the site of the lesion is limited (Leibacher and Henschler, 2016). Paracrine factors can be in soluble form or encapsulated in cell-secreted vesicles (Kusuma et al., 2017), **Figure 27.**

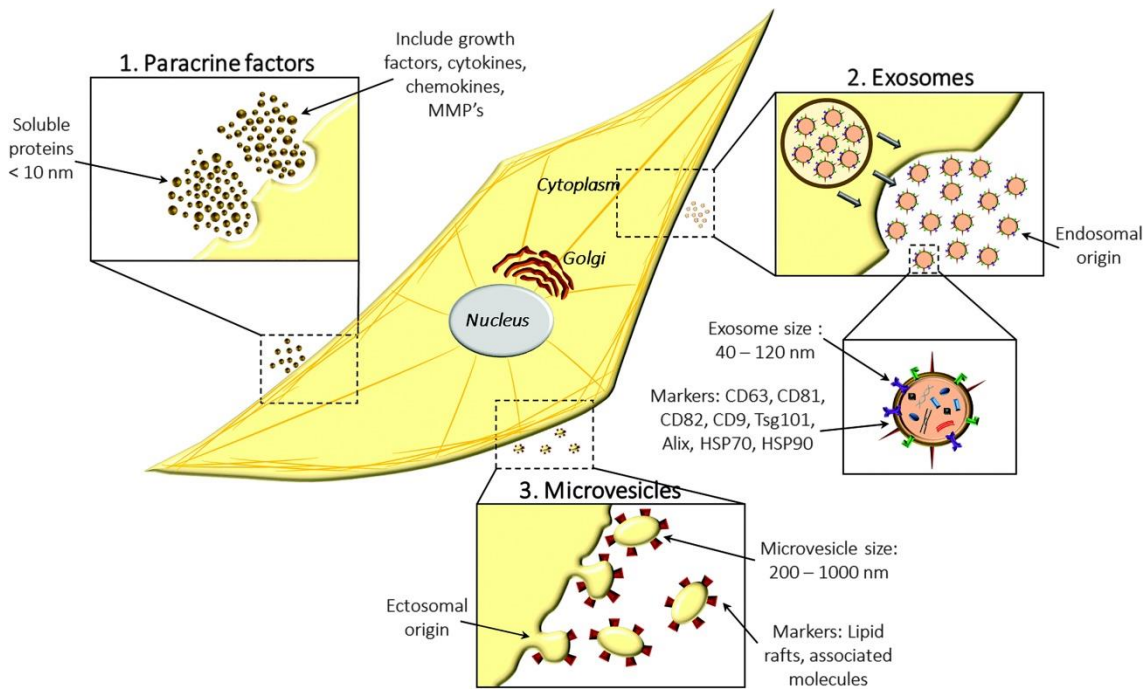


Figure 27. Schematic representation of biologically active substances released by MSC

Therapeutical effects of MSC can be exerted by: (1) paracrine factors, (2) exosomes and (3) microvesicles (Kusuma et al., 2017)

Both, secretome and sheddome respectively, constitute the conditioned medium (CM), which has been shown to recapitulate many of therapeutical effects exerted by the cells themselves when transplanted into animal models of different diseases (Linero and Chaparro, 2014).

MSC secretome is enriched in several soluble factors including: components of the ECM, proteins involved in the adhesion process, enzymes, cytokines, chemokines, immunomodulatory molecules and growth factors (Ferreira et al., 2018a). Physiologically, they play an important role in the regulation of biological functions, homeostasis, and the immune response of the body through their antimicrobial, antifibrotic, anti-apoptotic, proangiogenic, and proregenerative effects (Andrzejewska et al., 2019). A list gathering some of these molecules and the mechanism is presented on **Table 6**.

Table 6. Soluble factors involved in the different MSC paracrine effects

Fibrosis	Proliferation	Apoptosis	Chemotaxis	Angiogenesis
Ang-1	bFGF	CINC-3	CCL5	ANG
EGF	GF	bFGF-BP	CCL8	Ang-1
HGF	HGF	GH	MCP-2	CXCL9
IGF-1	IGFBP-1	HGF		MMP-1
KGF	IGFBP-2	IGF-1		MMP-2
NGF	M-CSF	OPN		TIMP-1
SDF-1	TGF α	TIMP-1		TIMP-2
TGF α		TIMP2		VEGF
VEGF		VEGF		

Abbreviations: Angiogenin (ANG), Angiopoetin-1 (Ang-1), basic fibroblast growth factor (bFGF), bFGF binding protein (bFGF-BP), chemokine (C-C motif) ligand (CCL-), chemokine (C-X-C motif) ligand (CXCL), cytokine-induced neutrophil chemoattractant 3 (CINC-3), epithelial growth factor (EGF), growth hormone (GH), hepatocyte growth factor (HGF), Insulin-like growth factor 1 (IGF-1), IGF binding protein (IGFBP), keratinocyte growth factor (KGF), metalloproteinase (MMP-), monocyte chemoattractant protein 2 (MCP-2), monocyte colony-stimulating factor (M-CSF), nerve growth factor (NGF), osteopontin (OPN), stromal cell derived factor 1 (SDF-1), tissue inhibitor of metalloproteinases (TIMP), transforming growth factor alpha (TGF α), vascular Endothelial growth factor (VEGF).

Most cell types, including MSC, produce Extracellular vesicles (EV) of different sizes (**Figure 27**) (Baharloo et al., 2020; Bayer-Wildberger et al., 2021; Harrell et al., 2020a; Kervadec et al., 2016; Li et al., 2016; Ramos et al., 2016). MSC exert some of their therapeutic effects and paracrine signaling through extracellular vesicles (EV) (Kusuma et al., 2017), which include exosomes (40–120 nm) formed in the multivesicular endosomes, microvesicles (200–1,000 nm) formed from plasma membrane budding, and apoptotic bodies released by dying cells (Ferreira et al., 2018a). The EV contain and deliver several categories of bio-active molecules (cytokines, growth factors, lipids, RNA, DNA, miRs...). They express surface makers either in a general fashion (CD9, CD63, CD81), or specific of the producing cell type (CD44, CD90, CD73 for the MSC), or specific of biological capacities (HLA-DR, CD273...). EV have pleiotropic effects *in vivo* in healthy and pathological conditions. In several studies, the EV display the same biological capacities as their producers (Kervadec et al., 2016; Kim et al., 2018b; Lima Correa et al., 2021; Phinney and Pittenger, 2017) while avoiding the issues of cell distribution and immune rejection raised by cell transplantation.

These advantages are due to their small size allowing systemic delivery, and to a low expression of histocompatibility markers. EV produced by MSC mimic their immunomodulatory capacities on T, B, NK and DC (Aiello et al., 2017; Chen et al., 2019a; Crain et al., 2019; Mardpour et al.,

2019) and MSC are one of the best source for the large scale production of EV (Baharloo et al., 2020; Pachler et al., 2017). There are several ways to isolate and purify EV (Witwer et al., 2019) based on different physico-chemical properties, however it remains challenging to obtain highly purified and characterized preparations. Recommendations have been emitted by the International Society of Extracellular vesicles to prepare and characterize homogeneously the EV in view of clinical uses (Théry et al., 2018). The production and standardization of EV will reach maturity in medical industry and may emerge as an attractive substitute for cells.

2.5.4. Immunomodulation

MSC have the ability to regulate the function of most of the effector cells in the immune response (Andrzejewska et al., 2019) by direct contact or through soluble factors, exosomes and microvesicles, or a synergy of these tools. The immune system plays an integral role in regulating tissue repair and regeneration and is capital in chronic inflammatory diseases and autoimmunity, therefore, MSC are key players and deserve in-depth attention. The principal mechanisms involved in MSC immunomodulation are, thus, especially described in the section below.

2.6. *Mechanisms involved in immunomodulation*

MSC possess immunomodulatory potential that operate as a result of the direct action of MSC themselves on the different cellular actors of the immune system, or through the action of MSC-imprinted cells (monocytes, macrophages, Tregs, etc).

Immunomodulation can be traduced as either activation or suppression of the immune system, and MSC can be very plastic and display both pro- and anti-inflammatory functions (Le Blanc and Davies, 2015). Because of the increasing interest of MSC application in the treatment of immune-mediated and inflammatory disorders, here, we will mainly focus on MSC actions that explain the beneficial outcomes obtained in these pathological contexts. However, it should be remembered that the cellular microenvironment and inflammatory milieu determine MSC phenotype and their local or systemic effects on the immune system.

MSC grown *in vitro* have the ability to interact and regulate the function of the majority of the effector cells involved in the processes of primary and acquired immune response. Interestingly, some of their effects can be exerted independently of their metabolic status (Song et al., 2020; Weiss and Dahlke, 2019), as studies evidenced that apoptotic, metabolically inactivated, or even fragmented MSC possess immunomodulatory capacities (Chang et al., 2012; Gonçalves et al., 2017; Luk et al., 2016; de

Witte et al., 2018). The effect of MSC and their involved mechanism on the most important immune cell types are summarized in **Figure 28** and described in details below.

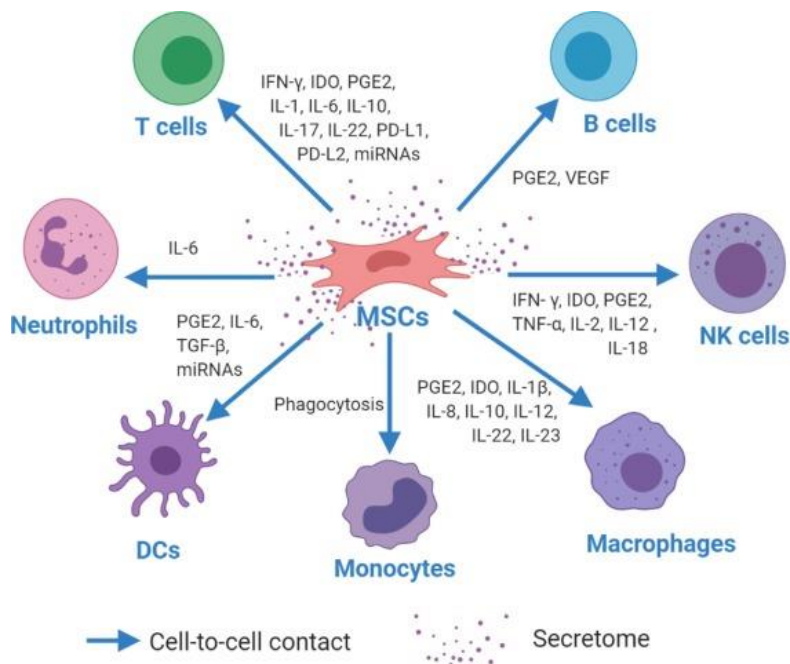


Figure 28. Representation of immunomodulatory interactions between MSC and immune cells

MSC exert immunomodulatory functions mainly via interactions with immune cells, such as T cells, B cells, natural killer (NK) cells, macrophages, monocytes, dendritic cells (DC), and neutrophils through, both, cell-to-cell contacts (blue arrows) and paracrine activity (shown by secretome). Abbreviations: ICAM-1, intercellular adhesion molecule-1; IDO, indoleamine-pyrrole 2,3-dioxygenase; IFN, interferon; IL, interleukin; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2; PGE2, prostaglandin E2; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor- α ; VEGF, vascular endothelial growth factor. (Song et al., 2020)

2.6.1. Actions over innate immunity

The innate immunity plays a crucial role in activation of the adaptive immune reaction but also in clearing pathogens targeted by the adaptive immune response. MSC exert immunomodulatory effects on soluble and cellular components of the innate immune system, leading to inhibition or resolution of inflammatory responses and stimulating the regenerative process (de Castro et al., 2019; Hass et al., 2011; Zhao et al., 2016).

2.6.1.1. Effects on the complement

The complement cascade's primary roles are fighting infection and clearing out immune complexes and damaged cells to maintain immune homeostasis. However, it is implicated in the rejection of transplanted allografts, tissue damage in AID, as well as, rapid clearance of systemically

circulating MSC after infusion by instant-blood-mediated-inflammatory-response (Le Blanc and Davies, 2015).

MSC express complement inhibitors CD46 (membrane cofactor protein), CD55 (decay accelerating factor (DAF)), most predominantly CD59 (membrane inhibitor of reactive lysis), and secrete Factor H allowing to partially inhibit activation of the complement system and also protecting them from complement induced death (Tu et al., 2010). The intervention of these molecules on the complement cascade is shown in **Figure 29**

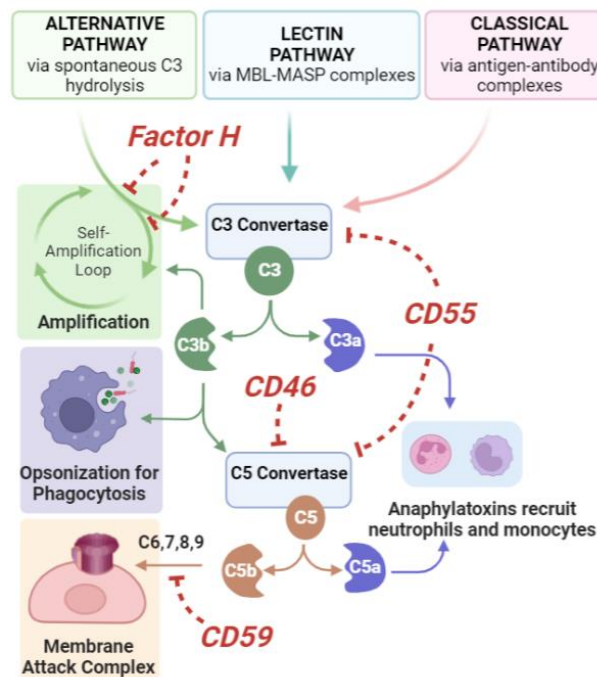


Figure 29. Complement cascade regulation by MSC

The classical, lectin, and alternative pathways converge to convert C3 to C3 convertase, an enzyme capable of initiating a cascade that results in cell membrane pore formation, known as the membrane attack complex (MAC), and subsequent cell lysis. MSC are able to modulate the cascade by expressing plasma membrane or soluble complement regulatory proteins. CD59, blocks MAC complex formation; CD46, acts as a cofactor to help inactivate C3b and reduce C5 convertase formation; and CD55, inhibits the cleavage of C3 and C5 by blocking the formation of C3 and C5 convertases and accelerating their decay. The secreted Factor H acts also as a cofactor in C3b inactivation, C3b is a component of C3 convertase in the alternative pathway, thus, inhibiting its formation. Created with Biorender. *Abbreviations:* MBL: mannose-binding lectin; MASPs: MBL-associated serine proteases; PAMP: pathogen-associated molecular pattern; Fb: factor B.

2.6.1.2. Monocytes / Macrophages

Monocytes are circulating mononuclear phagocytic cells that patrol the body and orchestrate an immune response in times of infection and inflammation. When recruited to tissues, they are capable of differentiating into macrophages and DC. On their side, macrophages are generally

considered terminally differentiated cells specialized in engulfing and eliminating foreign organisms and apoptotic cells.

MSC-monocytes interaction *in vitro* has been shown to lead to suppression of the differentiation of monocytes to DCs, this was dependent on MSC-to-monocytes ratio, and mediated in a certain degree by cell-to-cell contact but mostly by soluble factors (Deng et al., 2016; Jiang et al., 2005; Nauta et al., 2006; Spaggiari et al., 2009). Prostaglandin E2 (PGE2) was shown to play a crucial role in this process and may be helped by IL-6 and monocyte colony stimulating factor (M-CSF) (Spaggiari et al., 2009). MSC promote the polarization of monocytes/macrophages toward an anti-inflammatory/immune-regulatory (M2) phenotype and increase their phagocytic activity. This effect is mediated through cell–cell contact and soluble factors, such as, IL-6 and hepatocyte growth factor (HGF) (Deng et al., 2016), TNF- α –stimulated gene/protein (TSG)-6 (Ko et al., 2016), (PGE2) (Manferdini et al., 2017; Vasandan et al., 2016) in human MSC, and interleukin 1 Receptor Antagonist (IL1-RA) and TGF- β in murine MSC (Liu et al., 2019; Luz-Crawford et al., 2016). Anti-inflammatory monocytes secrete high levels of IL-10 and have decreased levels of IL-12p70, TNF- α , and IL-17 expression

When MSC are administered *in vivo*, they briefly reside in the lungs and are rapidly up-taken by monocytes/macrophages in a process called efferocytosis, which can be distinguished from phagocytosis because the former is immunologically silent (Yin and Heit, 2021). MSC uptake induce phenotypical and functional changes in phagocytes that are able to migrate to remote body sites mediating and transferring the immunomodulatory effects of MSC (Ko et al., 2016; Vasandan et al., 2016; de Witte et al., 2018). A simplified representation of MSC effects on these cells is shown in **Figure 30**.

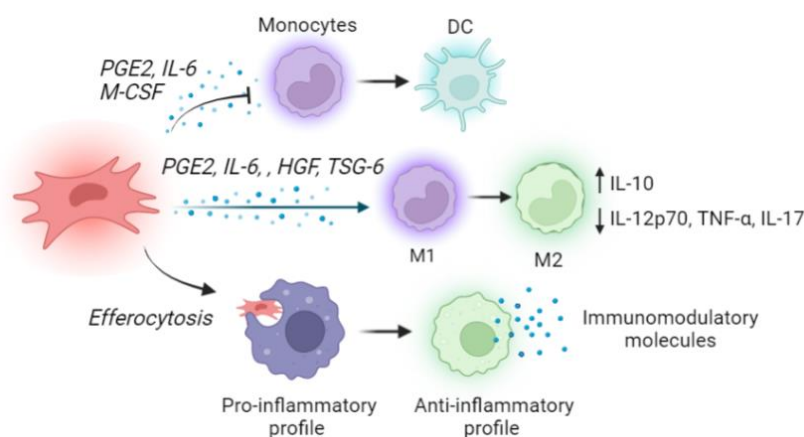


Figure 30. Immunomodulatory effects of MSC on monocytes and macrophages

MSC inhibit differentiation of monocytes towards dendritic cells (DC) and immunomodulate monocytes and macrophages, from pro-inflammatory towards anti-inflammatory profiles, through secretion of soluble factors (small blue molecules), cell-to-cell contact (arrows), or efferocytosis. Created with Biorender

Phagocytic cells are key in MSC-mediated immunomodulation, as their depletion abrogates the ability of MSC to suppress T cell proliferation *in vitro* and their immunomodulatory effect *in vivo* in transplantation models (Ko et al., 2016; Weiss and Dahlke, 2019).

2.6.1.3. Natural killer (NK) cell

NK cells are involved in the elimination of virally infected or tumorigenic cells. They exert cytotoxic effects over their target cells through the release of perforins and granzymes, and with no need of any priming or prior activation by APC (de Castro et al., 2019).

MSC are strong inhibitors of NK cells proliferation and function. Spaggiari et al. have first shown that the IL-2 induced proliferation of NK cells was strongly inhibited by the presence of MSC (Spaggiari et al., 2006). Coculture of NK and MSC induced, also, down-regulation of the activating receptors on the surface of NK cells that correlated with impaired NK cytotoxic activity and cytokine production (Patel et al., 2010; Spaggiari et al., 2009). Both inhibition of NK-cell proliferation and of the cytotoxic activity were mediated by synergistic effects of indoleamine 2,3-Dioxygenase (IDO) expression and production of PGE2 by MSC. NK cell-mediated cytotoxicity can also be suppressed by MSC HLA-G5 secretion (Hass et al., 2011; Selmani et al., 2008), **Figure 31**.

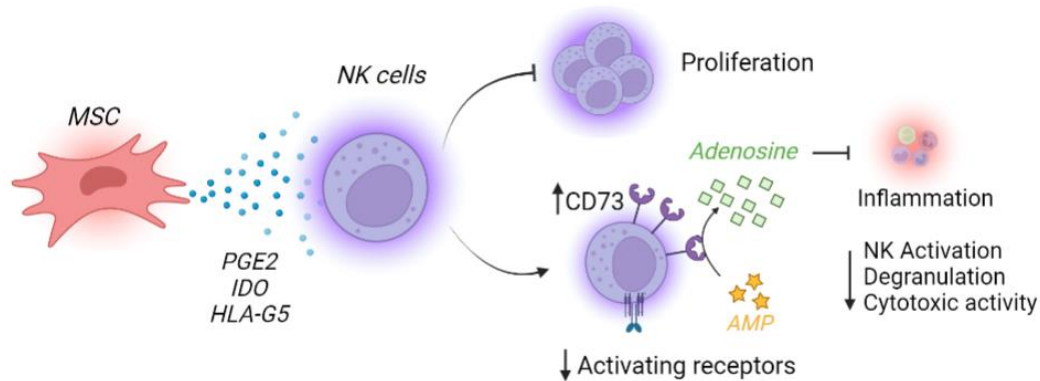


Figure 31. Immunomodulatory effects of MSC on NK cells

Didactic representation of MSC immunomodulatory action on NK cells including: inhibition of the proliferation, and impaired NK activation, cytotoxic activity and degranulation. MSC main paracrine molecules mediating these effects are IDO, PGE2 and HLA-G5. Created with Biorender. Abbreviation: AMP, adenosine monophosphate

Finally, another group demonstrated that MSC–NK interactions stimulate expression of the endonuclease CD73 on the surface of NK cells (Chatterjee et al., 2014). This enzyme converts adenosine monophosphate to adenosine, a molecule that suppress inflammation by regulating various immune cells. Up-regulation of CD73 was concomitant to higher enzyme activity and adenosine accumulation.

In presence of adenosine analogs, NK produced less cytokines and expressed reduced levels of degranulation, demonstrating that CD73⁺ NK cells can regulate NK cell activation in an autocrine or paracrine manner, thus preventing NK degranulation (Chatterjee et al., 2014).

2.6.1.4. Dendritic Cells (DC)

DC play a major role in the uptake, transport, and presentation of antigens with the capacity to stimulate naive T lymphocytes, thus linking the innate and adaptive immune systems (de Castro et al., 2019). The ability of Ds to initiate an immune response depends on their maturation, which allows transition from antigen-processing (immature DC) to APC (mature DC) (Jiang et al., 2005).

In the presence of MSC, monocytes-derived as well as CD34⁺-derived DC generation is impaired (Deng et al., 2016; Jiang et al., 2005; Nauta et al., 2006; Spaggiari et al., 2009). Human and murine MSC were also shown to retain DC in their immature state by blunting the expression of class II HLA and costimulatory molecules, the secretion of pro-inflammatory cytokines (IL-6 and IL-12p70) and their migratory capacities (English et al., 2008; Jung et al., 2007; Reis et al., 2018a; Spaggiari et al., 2009; Wang et al., 2008b), which are important for antigen presentation, leading to reduced support to T cell proliferation (Ramasamy et al., 2007). MSC effect on DC maturation is mediated by increasing the expression of miR-23b and the inhibition of the NF-κB pathway (Wu et al., 2017a), through regulation of STAT1 and STAT6 (Dong et al., 2018), as well as through the release of TSG-6 (Liu et al., 2014), **Figure 32.**

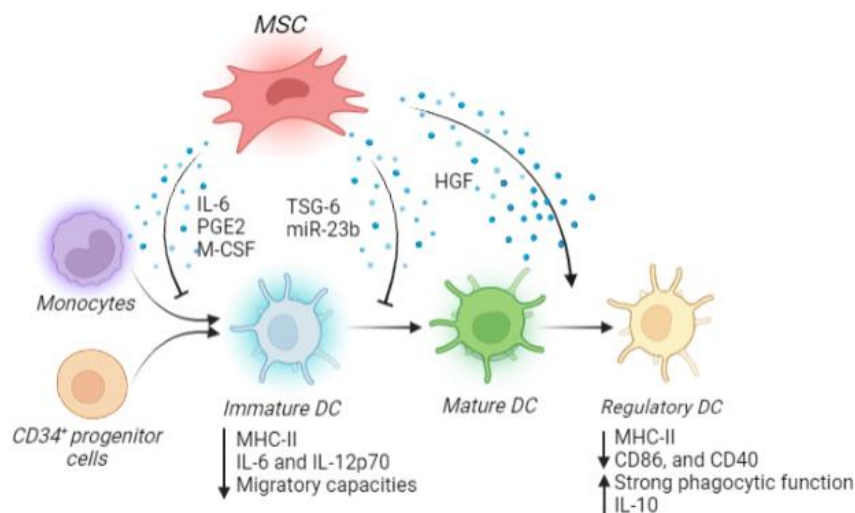


Figure 32. Immunomodulatory effects of MSC on dendritic cells

MSC are able to inhibit the differentiation of precursor cells (monocytes and CD34⁺ progenitor cells) into DC. They are also able to impair maturation of immature DC by reducing the expression of major histocompatibility complex (MHC) type II, the secretion of proinflammatory cytokines, and their migratory capacities. On the other hand, mature DC are usually induced towards a regulatory profile with strong phagocytic function and IL-10 production. Created with Biorender.

In peripheral blood, DC can originate from myeloid lineage (mDC) or plasmacytoid lineage (pDC), each with a different phenotypic and functional profile. MSC have different effects on both cells subsets, they reduce the secretion of TNF- α in mDC, and increase the secretion of IL-10 in pDC (Aggarwal and Pittenger, 2005) leading to a state of immune tolerance by inducing the formation of regulatory DC (DCregs). Others groups confirmed MSC induction of DCreg, characterized by low expression of MHCII, CD86, and CD40 molecules, strong phagocytic function, and the ability to inhibit T cell proliferation, mediated by HGF secretion, notch signalling, among other mechanisms (Cahill et al., 2015; Ge et al., 2010; Zhang et al., 2009), which evade apoptosis, have enhanced phagocytosis capacities and inhibit T cell activation and proliferation (Li et al., 2022).

2.6.1.5. Neutrophils

Neutrophils are the most abundant innate immune cells in circulation, they accumulate rapidly at the site of injury in response to pathogen-associated molecular pattern stimuli and act against invading pathogens by producing neutrophil extracellular traps (NET) and releasing antimicrobial substances such as reactive oxygen species (ROS) (de Castro et al., 2019). MSC and neutrophils interaction can lead to different outcomes depending on the context. In infectious microenvironments, MSC presence can increase neutrophils viability thanks to release of soluble factors (Raffaghello et al., 2008) and production of EV (Mahmoudi et al., 2019), and enhance neutrophils' phagocytic activity to clear infection (Brandau et al., 2014; Hall et al., 2013; Mahmoudi et al., 2019). However, in contexts where these neutrophils' mechanisms become overactivated, MSC efficiently dampen the secretion of ROS, peroxidases and proteases and inhibit NET formation, contributing to homeostasis and niche protection (Jiang et al., 2016).

2.6.2. Actions over adaptive immunity

2.6.2.1. T cells

It has been demonstrated that MSC interact tightly with T cells. High concentrations of MSC have been shown to inhibit the proliferation of activated T lymphocytes (CD4⁺ and CD8⁺ T cell subsets) in a dose-dependent and non-antigen specific manner, while supporting their survival in a quiescent state (Benvenuto et al., 2007; Gieseke et al., 2010; Glennie et al., 2005; Nicola et al., 2002). However, when MSC are present in low concentrations, immuno-supportive effects on T cell proliferation mediated by soluble factors such as IL-6 and MCP-1 have also been described (Marinescu et al., 2021; Najjar et al., 2009; Zhou et al., 2013).

Regarding T cell subsets, MSC have been shown to directly inhibit the differentiation of nascent CD4⁺ T cells to Tfh in experimental autoimmune models (Jang et al., 2016; Liu et al., 2015; Park et al., 2015). As Tfh cells provide help for antigen-specific B cells and their frequency correlates with production of auto-Ab, suppressing Tfh cells alleviates AID. MSC are capable of reducing the level of pro-inflammatory cytokines synthesized by T lymphocytes, such as TNF- α , IFN- γ and IL-17, and of increasing synthesis of anti-inflammatory cytokines, like IL-4, inducing a shift from a proinflammatory Th1 and Th17 profile to an anti-inflammatory Th2 state in T cells *in vitro* and *in vivo* (Bai et al., 2009; Fiorina et al., 2009; Ge et al., 2010; Li et al., 2022; Park et al., 2015). In the same line, MSC interaction can also lead to Th1 and Th17 apoptosis (Sakai et al., 2011; Wang et al., 2008a) and induce, expand and support the survival of Treg *in vitro*, and *in vivo* (Akiyama et al., 2012; Ge et al., 2010; Negi and Griffin, 2020; Selmani et al., 2008).

Several mechanisms are implicated in the suppression of activated T cells and their reduced secretion of pro-inflammatory cytokines (TNF- α and IFN- γ) (Andrzejewska et al., 2019). These mechanisms include: (1) cell–cell interactions through Fas/Fas ligand (Akiyama et al., 2012; Zhao et al., 2012), PD-L1 and PD-L2 (Guan et al., 2018; Sheng et al., 2008a; Wang et al., 2013b), CTLA-4 (Gaber et al., 2018) and adhesion molecules (Ren et al., 2010a). Some of these molecules can also have immunosuppressive capacities over T cells when secreted (Davies et al., 2017; Gieseke et al., 2010); (2) secretion of paracrine factors such as kallikrein and the metabolites produced by IDO-1 (Aggarwal and Pittenger, 2005; Ge et al., 2010; Meisel et al., 2004a; Plumas et al., 2005), HLA-G5 (Selmani et al., 2008), PGE2 (Aggarwal and Pittenger, 2005; Kim et al., 2018a), HGF, TGF- β (Glennie et al., 2005; Nicola et al., 2002), galectin-9 (Gieseke et al., 2013), NO (in rodents), IL 10, HO-1 and others (de Castro et al., 2019); (3) secretion of microvesicles that interact directly with T cells and induce cell cycle arrest, as well as, modulation of T cell subsets (Lee et al., 2020; Shen et al., 2021); and (4) indirect modulations orchestrated as collateral effects of MSC over DC, monocytes and macrophages. These mechanisms are schematized in **Figure 33**.

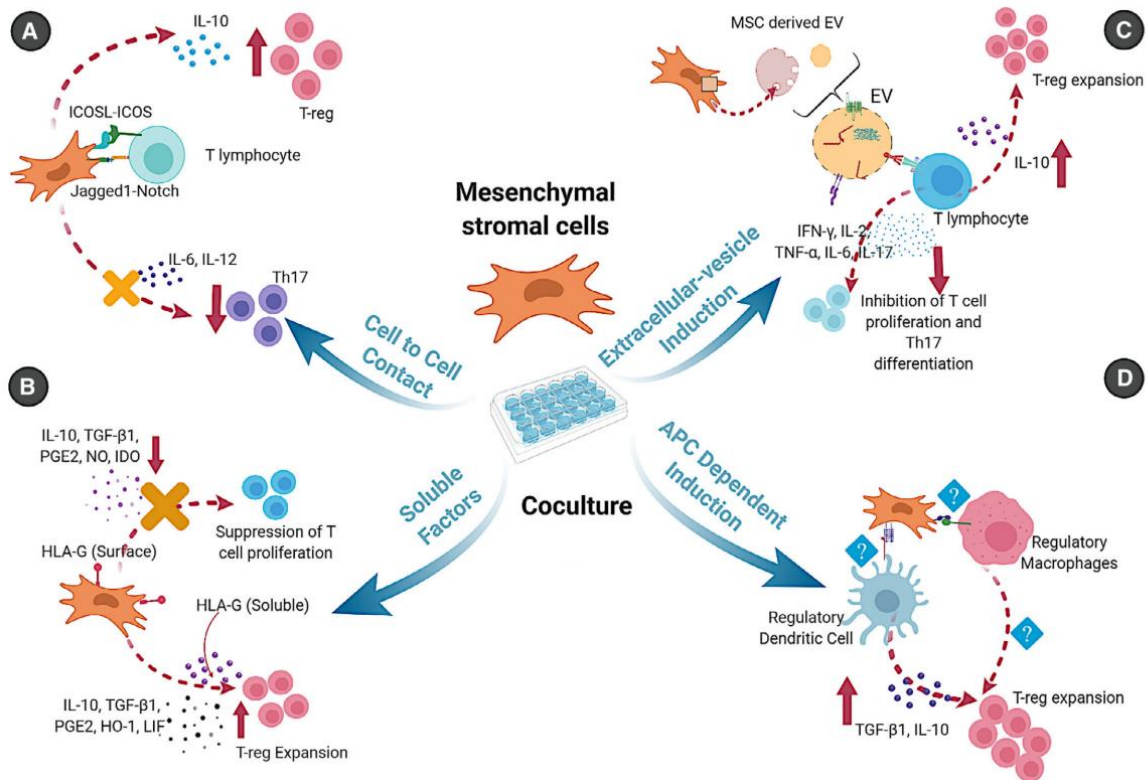


Figure 33. Schematic representation of the different mechanisms used by mesenchymal stromal cells (MSC) to modulate T cells subsets

MSC can interact with T cells through: (A) Cell-to-cell contact: MSC and T lymphocytes express complementary extracellular molecules on their surfaces, which allow their straight contact and consequent modulation. (B) Secretion of soluble factors: MSC secrete many soluble factors such as transforming growth factor beta 1 (TGF-β1), prostaglandin E2 (PGE2), heme oxygenase-1 (HO-1), nitric oxide (NO), indoleamine 2,3-dioxygenase (IDO), human leukocyte antigen G5 (HLA-G5) and leukemia inhibitory factor (LIF) that induce T-reg expansion while suppressing other T cell proliferation. (C) Extracellular vesicle induction: MSC-derived extracellular vesicles carrying specific RNAs, proteins and other bio-molecules induce polarization of CD4⁺ T cells towards T-reg by increasing production of IL-10 while decreasing IL-17, IL-2, TNF-α, IFN-γ, and IL-6. (D) Antigen presenting cell-dependent induction: MSC effects on antigen presenting cells (dendritic cells, monocytes, macrophages) induce regulatory phenotypes that promote Treg through IL-10 and TGF-β1 and others. (Negi and Griffin, 2020)

2.6.2.2. B cells

B lymphocytes are responsible of specific Ab production to fight and eliminate pathogens but they are also responsible for auto-Ab production in AID. MSC can act on B lymphocytes directly and indirectly, through mechanisms similar to those already described for T cells.

(1) Under immunological quiescent conditions, MSC have been shown to increase the survival of B-cells and to keep B cells in a non-proliferating state, thanks to cell-cell contact. (2) When B cells are activated by a cocktail of factors or in presence of T cells, MSC are able to decrease the differentiation of B cells into plasmablasts and their migration capacities, to limit the synthesis of Ig

like IgM, IgG, and IgA classes, to reduce their proliferation through T cells inhibition and to induce IL-10-producing B cells, known as regulatory B cells (Corcione et al., 2006; Franquesa et al., 2015; Glennie et al., 2005; Luk et al., 2017). Breg cells induced by MSC have been described with different phenotypes in literature and induction was shown to be partially mediated by cell-to-cell contact via PD-1 and PD-L1, extracellular vesicles and soluble factors such as IDO, PGE2, BAFF, stromal cell-derived factor-1 α (SDF-1 α) and others; these data are reviewed by Liu and colleagues (Liu et al., 2020a). **Figure 34** recapitulates these Breg subsets and the mechanisms involved in their induction.

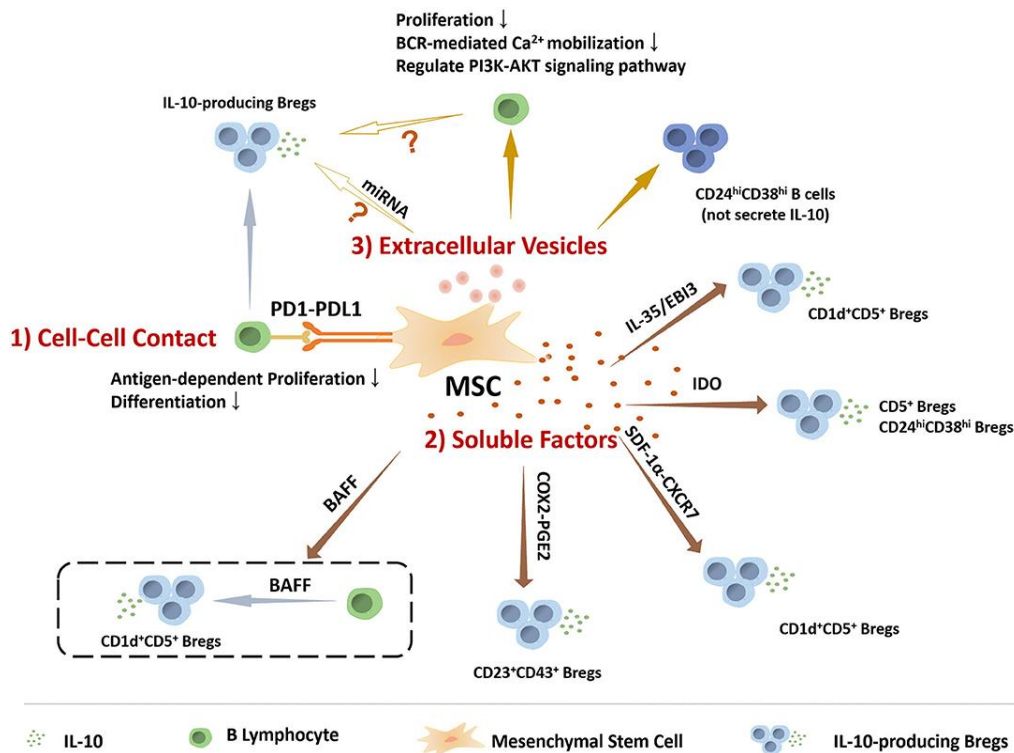


Figure 34. The role of MSC in regulating the IL-10 producing regulatory B cells

MSC perform functions on modulating IL-10 producing regulatory B cells via many manners, including (1) Cell-to-cell contact: MSC play roles in B cells via PD1-PDL1 pathway to inhibit antigen-dependent proliferation and differentiation, and induce Breg. (2) Soluble factors: IL-10-producing Breg subsets, including CD5⁺ Breg, CD24^{high}CD38^{high} Breg, CD1d⁺CD5⁺ Breg, and CD23⁺CD43⁺ Breg, are mediated by MSC-secreting soluble factors. (3) Extracellular Vesicles: MSC-EV could inhibit B cell proliferation and BCR-mediated Ca²⁺ mobilization, regulate PI3K-AKT signaling pathway in B cells that is critical for Breg cell development, and induce CD24^{high}CD38^{high} B cell subpopulation, a classic phenotype of Breg, but without IL-10 production. (Liu et al., 2020a)

Similarly, *in vivo* studies showed that MSC were able to induce improvement in experimental autoimmune models by decreasing the frequency of plasma cells and suppressing B cells activation, proliferation and differentiation (Che et al., 2014; Dang et al., 2020) or also inducing Breg cells (Park et al., 2015).

To sum up this chapter, Figure 35 summarize MSC immunomodulation mechanisms. Additionally, the most important molecules, their mechanism of actions and effect on target cells are presented on Table 7.

Table 7. Soluble and membrane-bound molecules involved in MSC immunomodulatory effects

Molecule	Mechanism of action	Effect on target cells
<i>Soluble molecules</i>		
HGF	Interaction with its receptor Met or hepatocyte growth factor receptor	Induces IL-10 expression in monocytes, inhibits Th1 and DC activities, and promotes IL-10 positive Treg cells
HLA-G5	Interaction with the receptors immunoglobulin-like transcript (ILT) 2 and 4	Inhibits the cytotoxic function of CD8 ⁺ T and NK cells, cytokine production of Th1 and Th17 cells, and induces Treg generation
IDO	Tryptophan depletion in the local microenvironment with production of kynurenine catabolites	Inhibits T cells and B cells proliferation, induces an anti-inflammatory profile in monocytes and macrophages
IL-1ra	Interaction with IL-1R and inhibition of IL-1 α /IL-1 β -signaling	Induces M2 polarization in macrophages, promotes Treg, attenuates antigen-presenting properties of DC, inhibits Th1 and Th17 cells
IL-6	Signaling through IL-6R. Activated intracellular pathways are under study and may vary between targets	Inhibits DC maturation and apoptosis of neutrophils. induces D _{Creg} , M2 macrophages and secretion of other anti-inflammatory molecules (IL-1Ra and IL-10)
PGE2	Interaction with E type prostanoid or EP receptors 2 and 4, induction of AMPc and activation of protein kinase A and phosphatidylinositol-3 kinase pathways	Inhibits T cells proliferation and impairs their differentiation into Th1 and Th17 subsets. Induces M2 profile in monocytes and macrophages. Inhibits DC maturation and induces D _{Creg} . Impairs NK cells function
TGF- β	Interaction with TGF receptor type I and II and activation of SMAD transcription factors	Inhibits DC maturation and T cell activation, and impairs Th1 and Th2 differentiation. Promotes Treg and Breg
<i>Molecules involved in cell-to-cell contact</i>		
ICAM-1	Interaction with LFA-1, cell adhesion	Inhibits T cell proliferation and differentiation
PD-L1 / PD-L2 (also soluble)	Interaction with PD-1 at the T cell surface and transduction of an inhibitory signaling	Inhibits lymphocyte proliferation, reduces IL-2 secretion, suppresses CD4 ⁺ T-cell activation, and induces T-cell death
VCAM-1	Interaction with VLA-4, cell adhesion	Inhibits T cell proliferation and differentiation

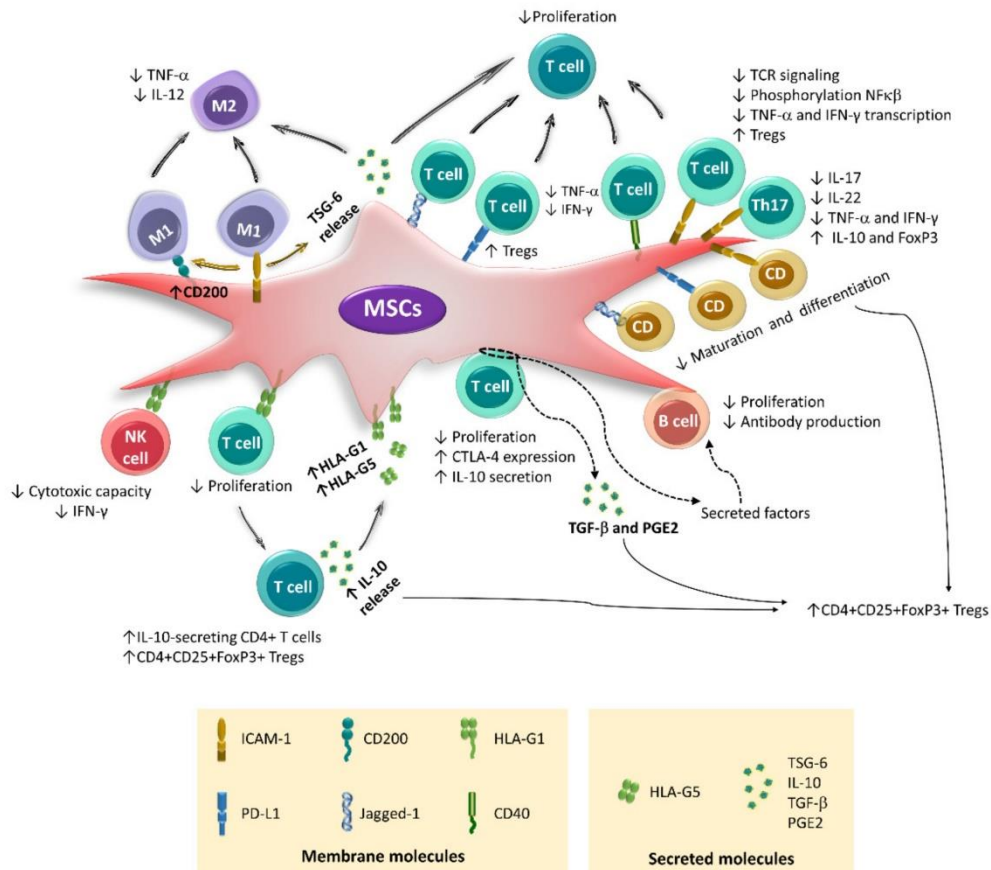


Figure 35. Immunoregulatory mechanisms mediated by MSC

The main membrane molecules involved in the immunoregulation exerted by MSC are shown. Cell–cell interactions, in addition to affecting the proliferation, differentiation, and effector function of immune cells, also increase the immunoregulatory capacity of MSC. The contact of M1 macrophages with MSC through ICAM-1 induces an M2 phenotype in macrophages, while in MSC, the expression of CD200 and TSG-6 is increased (brown arrows), which also favors the differentiation of M2 macrophages. Conversely, ICAM-1, PD-L1, and jagged-1 decrease the secretion of cytokines and proliferation of activated T lymphocytes, as well as the maturation and differentiation of DC. Furthermore, CD40 affects the proliferation of T cells, while HLA-G1 induces Treg differentiation. HLA-G1 is also involved in the decrease in effector function of NK cells. Conversely, direct contact of MSC with T lymphocytes (circle with dotted lines) induces changes in T lymphocytes and stimulates the secretion of TGFβ and PGE2, as well as factors that affect the function of B lymphocytes (dotted lines). However, it is unknown exactly which molecules are involved in this interaction. (López-García and Castro-Manrreza, 2021)

2.7. Sources of MSC heterogeneity

The ISCT definition of MSC applies to cells from all species and tissues, yet the recently exposed guidelines were established based on human BM-MSC (Wright et al., 2021). Applying these guidelines to MSC from many tissue sources and species imply that all MSC are phenotypically and functionally similar. However, several studies report that species, tissue sources, donor intrinsic characteristics, isolation method, culture conditions, and several other aspects induce MSC differences. These differences are exhibited in MSC surface marker expression, their culture requirements, their longevity in culture, their transcriptome, their response to stimulation, and their growth rate. The most important factors inducing cells heterogeneity are described below.

2.7.1. Species

Human MSC share common features with MSC derived from other animals but have also different characteristics. Differences include: culture conditions with regard to attachment factors, media formulation and also culture survival, senescence and immortalization frequencies (Phinney and Sensébé, 2013; Wright et al., 2020).

Discrepancies have also been reported in surface marker expression, with absence of some expected positive markers and positivity for human negative markers (Chamberlain et al., 2007; Lee et al., 2014; Ranera et al., 2011; Wright et al., 2020). In terms of differentiation potential, not always the trilineage capacity is confirmed in all animal species and time required for differentiation may also vary (Wright et al., 2021). In addition, mechanisms involved in biological properties such as immunosuppression are species-dependent; indeed, human express extremely high levels of IDO and very low levels of inducible NO synthase (iNOS), whereas mouse MSC expressed abundant iNOS and very little IDO (Ren et al., 2009).

This work will mainly focus on human derived MSC characteristics, properties and mechanisms of action.

2.7.2. Cell sources

Differences have been described among MSC of various tissue origin in regard to the genetic, epigenetic, phenotypic, cytokine profile and biological properties (Billing et al., 2016; Chen et al., 2015; Hass et al., 2011; Ho et al., 2018). This suggests that MSC heterogeneity reflects the diversity of their natural niches (Kolf et al., 2007) and this biological imprint may be retained by cultured cells (Costa et al., 2021). Studies showing differences in MSC from different tissue sources of the same donor add

evidence to natural niche influence (Mohamed-Ahmed et al., 2018). As our work is centered on the use of MSC coming from AT for clinical applications, descriptions of characteristics and comparison of biological properties will be focused mainly on AD-MSC regarding other common sources such as BM-, UCB-, PB-MSC, etc.

- *Proliferation capacity and senescence*

AD-MSC exhibit greater proliferative capacity than BM-MSC (Jin et al., 2013; Kern et al., 2006; Li et al., 2015a; Mohamed-Ahmed et al., 2018), but less than UCB-MSC (Eiró et al., 2014; Kern et al., 2006) and human uterine cervical stem cells (Eiró et al., 2014). Life span reduction and senescent profile at high passages, arrive earlier in BM-MSC than in AD-MSC; however, UCB-MSC seem to have better resistance to senescence than the 2 former sources (Jin et al., 2013; Kern et al., 2006). Of note, BM-MSC are rarer than AD-MSC, then, it is necessary to expand them much more than AD-MSC to reach expected and useful quantities for therapeutic applications. This in turn may accelerate their senescence.

- *Differentiation potential*

BM-MSC and AD-MSC were able to confirm their tri-lineage differentiation potential, while a lack of differentiation of UCB-MSC towards adipocytes has been reported (Costa et al., 2021; Heo et al., 2016; Kern et al., 2006). AD-MSC present decreased osteogenic potential and increased adipogenic potential compared to BM-MSC. (Danišovič et al., 2009; Li et al., 2015b; Xu et al., 2017). In addition AD-MSC possess also significantly greater potential for angiogenesis and vasculogenesis (Costa et al., 2021; Strioga et al., 2012; Terunuma et al., 2019), and higher immunomodulatory capacities when compared to BM-MSC (Li et al., 2015a; Melief et al., 2013; Ménard et al., 2020; Strioga et al., 2012).

- *Phenotype*

Because of the low specificity of the ISCT marker panel, expanded stromal cells from many tissues meet the minimal criteria for MSC identity. However, the tissue from which MSC are obtained play a crucial role on MSC phenotype (Wilson et al., 2019).

A list of the most recurrently expressed MSC extracellular markers reported in literature (mainly reviews) is presented in **Table 8** (Baer et al., 2012; Cho et al., 2017; Chong et al., 2012; Donnenberg et al., 2015; Flanagan et al., 2021; Hass et al., 2011; Reis et al., 2018b; Rojewski et al., 2008; Samsonraj et al., 2017; Tondreau et al., 2005). Nevertheless, it is important to clarify that discrepancies are easily found between authors, for some markers, due to differences between clones of Ab, to variations in culture conditions, duration and cell density (Strioga et al., 2012).

Table 8. Extracellular markers expressed by in vitro expanded MSC derived from different tissues

Source	Positive Markers	Negative Markers	References
Adipose Tissue	CD9, CD10, CD13, CD26, CD29, CD36, CD44, CD46, CD47, CD49a, CD49d, CD51, CD54, CD55, CD58, CD59, CD61, CD63, CD73 (SH3), CD81, CD90, CD95, CD98, CD99, CD105 (SH2), CD106, CD140a, CD146, CD147, CD151, CD164, CD165, CD166, CD227, CD282, CD340, CD305, HLA I, STRO-1,	CD1a, CD3, CD11b, CD14, CD19, CD31, CD34, CD45, CD79 α , CD133, CD144, HLA-DR, MIC A/B, SSEA-1, SSEA-4	(Cho et al., 2017) (Lv et al., 2014) (Hass et al., 2011) (Rojewski et al., 2008) (Baer et al., 2012) (Donnenberg, 2015)
Bone Marrow	CD9, CD13, CD29, CD31, CD44, CD49a to CD49e, CD51, CD54, CD58, CD59, CD61, CD62L, CD71, CD73 (SH3), CD90, CD102, CD104, CD105 (SH2), CD120a, CD120b, CD140a, CD140b, CD146, CD166, CD200, CD221, CD271, CD279, CD340, CD349, SSEA-4, STRO-1,	CD1a, CD3, CD4, CD5, CD11a, CD11b, CD14, CD18, CD19, CD25, CD31, CD34, CD38, CD45, CD62E, CD62P, CD133, CD178, HLA-DR	(Cho et al., 2017) (Lv et al., 2014) (Hass et al., 2011) (Rojewski et al., 2008) (Samsonraj, 2017) (Reis et al., 2018b)
Peripheral Blood	CD29, CD44, CD54, CD90, CD105 (SH2), CD133, CD166	CD1a, CD3, CD14, CD31, CD34, CD45, CD271	(Chong et al., 2012) (Hass et al., 2011), (Rojewski et al., 2008) (Tondreau et al., 2005)
Umbilical Cord Blood	CD13, CD29, CD44, CD73, CD90, CD95, CD105, CD133	CD14, CD20, CD34, CD38, CD45, CD271	(Flanagan et al., 2021) (Hass et al., 2011) (Rojewski et al., 2008)

Some principal differences described in literature for AD-MSC include: moderately or strongly expression of CD36, CD49d (integrin α 4) and CD54 (intercellular adhesion molecule-1, ICAM-1), and reduced CD106 (vascular cell adhesion molecule-1, VCAM-1) and CD200 (OX-2 membrane glycoprotein), the opposite being described for BM-MSC. In addition, low expression of CD49f (integrin α 6) and podocalyxin-like protein 1 were reported in AD-MSC, while the expression of these surface markers is relatively high in BM-MSC (Bourin et al., 2013; Strioga et al., 2012).

- *Transcriptomic and proteomic profiles*

Several studies compared MSC transcriptomic profiles (Al-Nbaheen et al., 2013; Barrett et al., 2019; Cho et al., 2017; Noël et al., 2008; Zhang et al., 2022), identifying differentially expressed genes and potential signatures as well as enriched pathways in MSC from one source when compared to another. In the same line, analysis of the factors released by MSC of different sources showed source

imprint (Kehl et al., 2019; Shin et al., 2021) and may explain, for example, differences in biological properties (Li et al., 2015a; Melief et al., 2013; Strioga et al., 2012)

2.7.3. Donor variability

Donor variation is well recognized as a fundamental source of variability in MSC populations. Factors such as age, sex and health status of MSC donors may introduce heterogeneity in MSC characteristics. With age, MSC diminish their yield, as well as, their regenerative and immunosuppressive capacities (Mattiucci et al., 2018; Rojewski et al., 2008; Siegel et al., 2013). As examples, studies showed an inverted correlation between donor age and osteogenic potential in human female AD-MSC (Zhu et al., 2009) and regarding health status, MSC from SLE were reported to show early signs of senescence and impaired immunomodulatory capacities (Che et al., 2014; Li et al., 2022)

Furthermore, donor variations impact the clinical and commercial development of MSC-based therapies, especially autologous ones, with respect to defining the characteristics critical for required clinical effects. (Wilson et al., 2019)

2.7.4. Culture conditions

The phenotype and biological features of MSC could be dynamically altered by culture itself. As an example, AD-MSC express CD34 when freshly isolated, although the expression of CD34 gradually declines with successive passages (Strioga et al., 2012), so, negativity of CD34 expression in AD-MSC may be considered as a bias induced by culture (Busser et al., 2015). Similar case was reported for BM-MSC and the acquisition of CD44 expression with culture (Qian et al., 2012).

A study assessing the variability of MSC treated by 5 different centers with different culture strategies showed that manufacturing conditions contributed more to MSC variability than the biological source of material. The factors inducing the highest differences are basal medium formulations (Xu et al., 2017; Yang et al., 2018), medium supplementation (fetal bovine serum (FBS) *versus* PL) (Bieback et al., 2019; Menard et al., 2013; Reis et al., 2018b), initial seeding densities, the number of passages (Kozłowska et al., 2019; Merimi et al., 2021a), and the length of time MSC are maintained in culture and frozen (Stroncek et al., 2020).

2.8. Therapeutic applications

Mesenchymal stromal cells (MSC) are intensively investigated in clinical trials due to their wide-ranging physiological effects including the maintenance of tissue homeostasis and regeneration, as well as the immunomodulatory activities. In the last years, their indications have expanded from regenerative medicine, to graft-versus-host disease (GVHD), autoimmune and inflammatory diseases, myocardial infarction, and acute respiratory distress syndrome. MSC have even been tested for COVID-19, 65 MSC-based clinical trials were registered in 2020 in this indication (Levy et al., 2020). Till last year, 1014 MSC-based clinical trials (CT) have been registered in US National Institutes of Health's Clinical Trials platform (clinicaltrials.gov) database (1995-2021) (Jovic et al., 2022). An overview of these CT and their details, is shown in **Figure 36**. Over 300 clinical trials of MSC therapies have been completed and concluded to MSC tolerability and safety profile, as well as, promising therapeutic benefits in some clinical settings (Zhou et al., 2021). Some trials led to regulatory approvals of MSC in a few countries, as shown in **Table 9**.

Table 9. MSC products with regulatory approval

Name	MSC type	Administration	Indication	Country of approval (Year)	Company
Queencell	Autologous AD-MSC (SVF)	Subcutaneous injection	Subcutaneous tissue defects	South Korea (2010)	Anterogen Co. Ltd.
Cellgram-AMI	Autologous BM-MSC	Intracoronary	Acute Myocardial infarction	South Korea (2011)	Pharmicell Co. Ltd.
Cartistem	UC-MSC	Implantation in lesion site	Knee articular cartilage defects	South Korea (2012)	Medipost Co. Ltd.
Cupistem	Autologous AD-MSC	Parenteral	Crohn Disease fistulas	South Korea (2012)	Anterogen Co. Ltd.
Prochymal	Allogeneic BM-MSC	Intravenous infusion	Graft versus host disease	Canada (2012) New Zealand (2012)	Osiris Therap. Inc Mesoblast Ltd.
Neurona-ta-R	Autologous BM-MSC	Intrathecal injection	Amyotrophic lateral sclerosis	South Korea (2014)	Corestem Inc.
Stempeucel	Allogeneic BM-MSC	Intramuscular	Critical limb ischemia	India (2016)	Stempeutics Research PVT
Temcell HS Inj	Allogeneic BM-MSC	Intravenous infusion	Graft versus host disease	Japan (2015)	JCR Pharmaceutical
Stemirac	Autologous BM-MSC	Intravenous infusion	Spinal cord injury	Japan (2018)	Nipro Corporation
Alofisel	Allogeneic AD-MSC	Local Injection (fistulas)	Complex perianal fistulas in Crohn Disease	Europe (2018)	TiGenix NV/Takeda

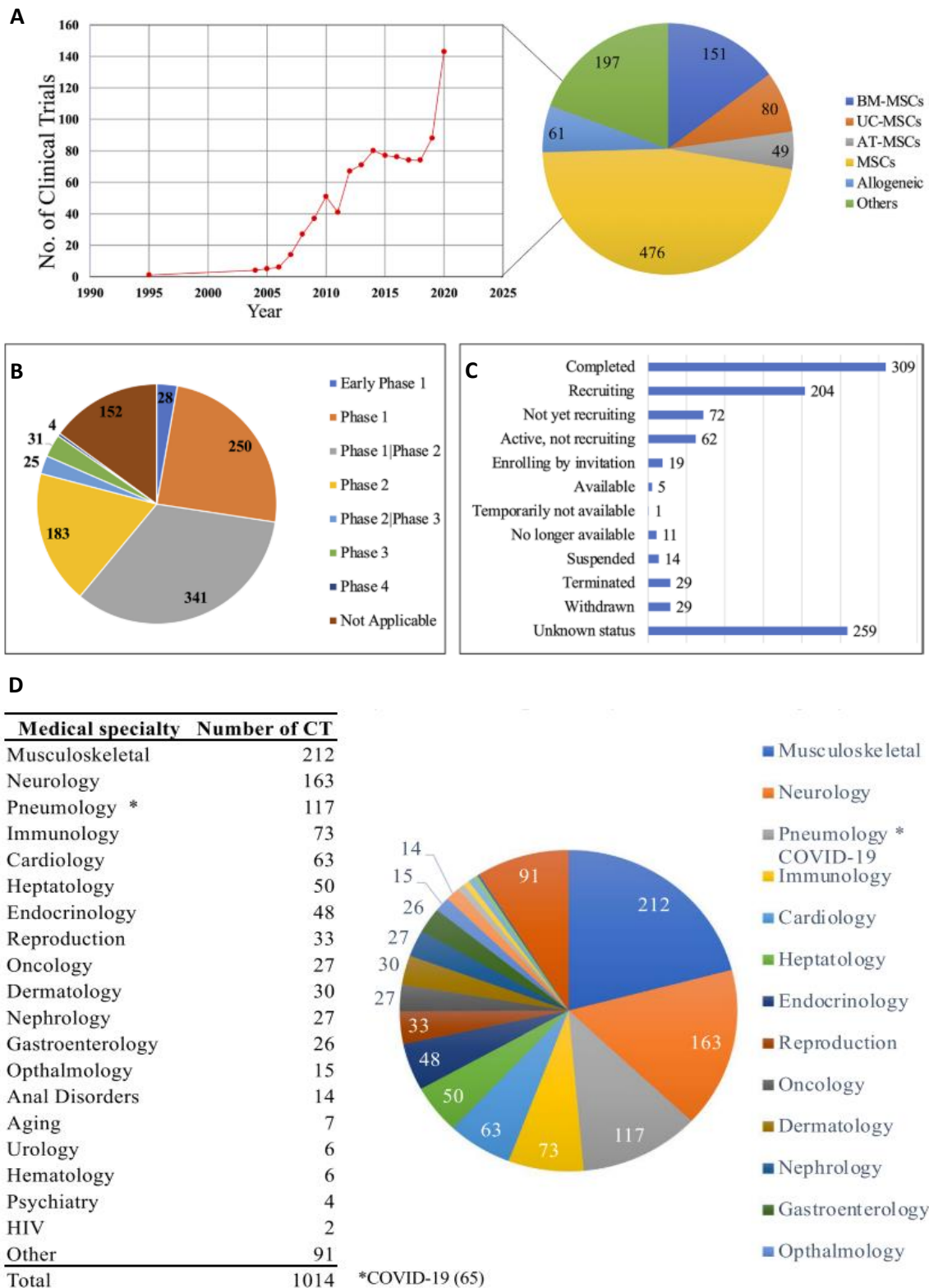


Figure 36. Overview of global trends in MSC-based cell therapy

(A) Line plot yearly registered mesenchymal stem cells-based clinical trials at ClinicalTrials.gov since the first use in 1995 up to 2020. And pie chart of number of clinical trials based on main sources used: BM-MSCs, UC-MSCs, AT-MSCs. (B) Pie chart distribution of clinical trials according to investigation phases. (C) Bar blot of clinical trials according to the study status. (D) Overview of the types of diseases targeted by these registered clinical trials. (Jovic et al., 2022).

2.8.1. Regenerative medicine

Several properties have made MSC appealing in the field of regenerative medicine (Hu et al., 2018), such as their capacity to migrate, engraft, and functionally influence the repair process within the site of injury and damage (Shojaei et al., 2019; Wang et al., 2017c). MSC play a tissue engineering role, to enhance tissue regeneration through growth factors and scaffolds; for example, to generate tissue-engineered skin or cartilage, which have been assessed in clinical trials.

Accumulation of MSC adjacent to the damaged tissue following their administration can be seen after “systemic” or “local” transplantation. The inflamed environment of injured or damaged tissues mobilizes MSC to the target site under the action of platelet-derived growth factors, SDF-1, CCL5, CCL21 and other chemokines (Hocking, 2015).

Once in place, MSC-mediated tissue regeneration involved hypothetically 2 mechanisms: (1) cell replacement and (2) cell “empowerment”, as schematized in **Figure 37** (Wang et al., 2014b). The first mechanism is directly exerted by recruited MSC, *in situ*, they differentiate into functional cells to replace the damaged ones (Wang et al., 2014b). However, the cell replacement therapeutic mechanism is discussed (Merimi et al., 2021b).

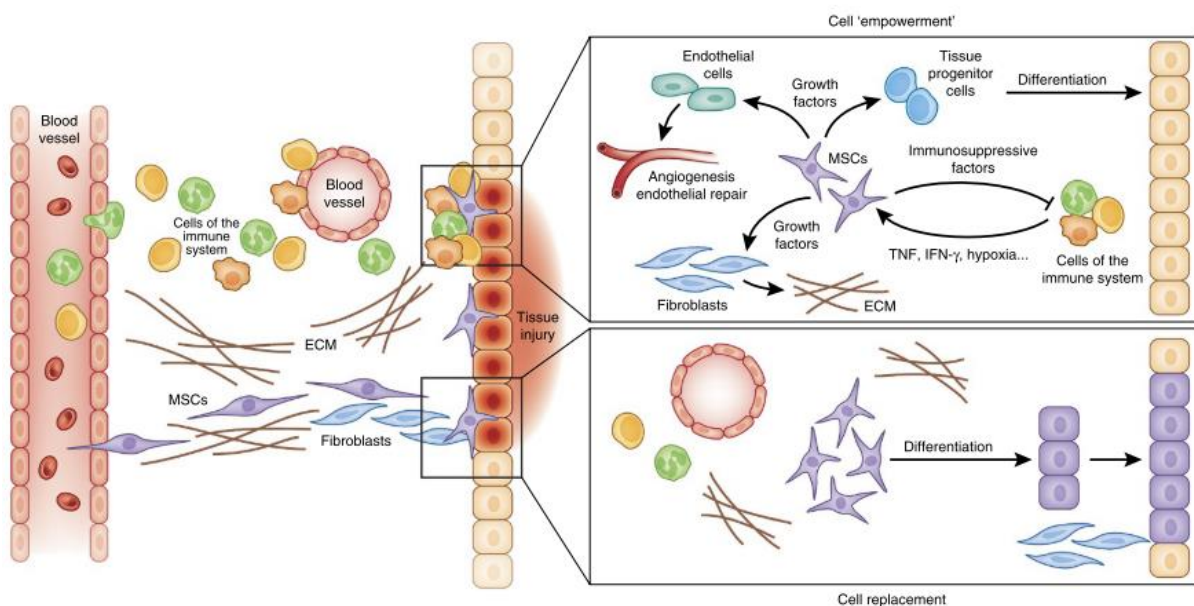


Figure 37. Mechanisms of MSC-based therapy for tissue regeneration

MSC collaborates in tissue regeneration by mobilization to tissue injury, differentiation into functional cells and replacement of damaged cells (cell replacement mechanisms) and through the secretion of paracrine factors that prepare the microenvironment and stimulate fibroblast, endothelial and progenitor cells to differentiate (cell “empowerment”). (Wang et al., 2014b)

On the other hand, in response to inflammatory cytokines, MSC can participate to tissue regeneration indirectly, by preparing the microenvironment. These is done by: (1) modulating the progression of inflammation and helping in dead tissue removal, (2) increasing vascularisation and nutrient supply to ease migration and proliferation of regenerative cell types, (3) stimulating endothelial cells, fibroblasts and tissue progenitor cells, *in situ*, to repair damage (Hu et al., 2018; Merimi et al., 2021b; Wang et al., 2014b). MSC effectively participate in the tissue repair process through their immunomodulatory, trophic, antibacterial, antifibrotic, and proangiogenic functions (Huayllani et al., 2020).

MSC constitute the most commonly used adult stem cells in regenerative medicine (Squillaro et al., 2016). Their applications include: bone reconstruction, cartilage repair and regeneration of tissues associated with the musculoskeletal system. Among them, osteoarthritis (OA) is the most widespread musculoskeletal disorder in adults, it leads to cartilage damage associated with bone changes and synovial inflammation, causing pain and disability. MSC-based therapies are relevant for chronic and degenerative disorders, such as OA, where no curative treatments are available (Ruiz et al., 2015). A phase I CT, conducted in France and Germany, tested different doses of intra-articular autologous AD-MSCTransplantation in 18 patients with symptomatic and severe knee OA. Results showed safety profile and significant improvements in pain levels and function in low MSC-doses treated patients (Pers et al., 2016). A placebo-controlled double CT-blind phase IIb study to assess clinical and structural efficacy is currently ongoing.

They are also very attractive for their antifibrotic potential in several organs (Usunier et al., 2014). Indeed, preclinical and clinical trials have shown MSC ability to improve outcomes in fibrosis induced by radiotherapy (Chapel et al., 2013), autoimmune pathologies (Voswinkel et al., 2013) and other etiological agents. There are no current FDA approved MSC-based product, but the regulatory authority of Republic of Korea MFDS in 2012 approved the use of Cartistem, a composite of culture-expanded allogeneic hUCB-MSCTand hyaluronic acid hydrogel, for the treatment of traumatic or degenerative osteoarthritis (Lim et al., 2021). Currently, there are more than 200 clinical trials testing MSC efficacy on musculoskeletal diseases (Jovic et al., 2022).

2.8.2. Hematological Pathologies and GVHD

MSC have the capacity to support hematopoiesis and potentially enhance marrow recovery following chemotherapy or radiotherapy. Thus, they represent good candidates to facilitate the engraftment of HSCs used as therapy for several hematological pathologies, and reduce GVHD severity (Squillaro et al., 2016). GVHD is an immunological disorder, characterized by the attack of donor's cells against healthy recipient tissues and organs. It is a severe complication of HSCT and may manifest as acute (aGVHD) or chronic (cGVHD) forms, both with poor overall survival (Zhao et al., 2019). MSC exert multifactorial effects, including: paracrine activity involving secretion of proteins/peptides and hormones; transfer of mitochondria by way of tunneling nanotubes or microvesicles; and transfer of exosomes or microvesicles containing RNA and other molecules calming GVHD (Kelly and Rasko, 2021), **Figure 38**.

The first MSC transplantation in GVHD context was pioneered by Le Blanc et al. in 2004, in a case of a young boy presenting steroid resistant grade IV aGVHD of the gut and liver. The

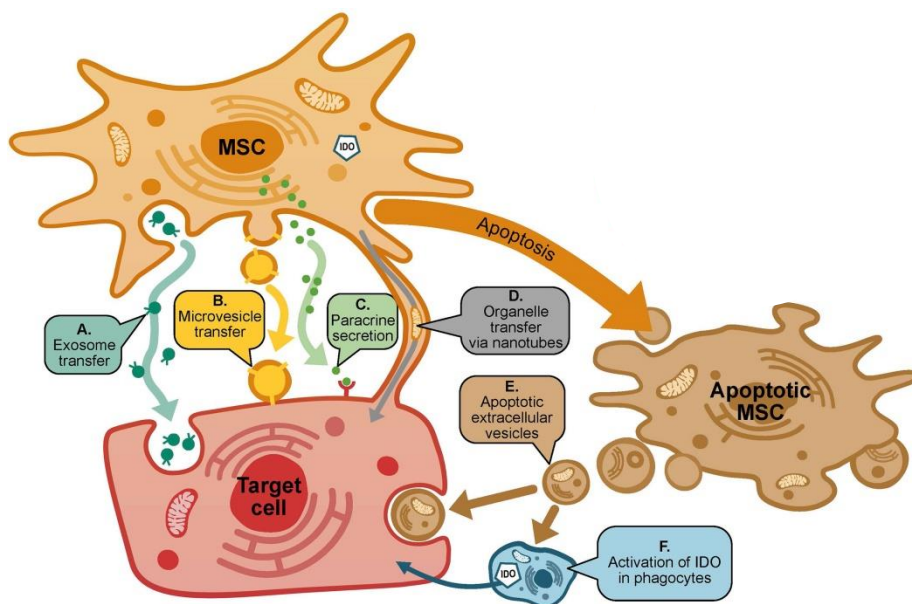


Figure 38. Mechanisms of action of MSC in GVHD

MSC may exert many effects on target cells via diverse potentially-overlapping mechanisms. Target cells include (i) donor and host immune cells, including T cells, B cells, NK cells, monocytes and dendritic cells; and (ii) host cells susceptible to damage by GVHD, e.g. cells of the skin, gastrointestinal tract and liver. Potential mechanisms through which MSC may act include (A, B): transfer of exosomes or microvesicles containing RNA and other molecules; (C) paracrine activity including secretion of proteins (including IDO), peptides and hormones; (D) transfer of organelles via tunneling nanotubes; (E, F) MSC apoptosis results in the release of apoptotic extracellular vesicles that act on target cells, as well as induction of IDO production in recipient phagocytes.

transplantation reported very good results, remarkable clinical response and no complications (Le Blanc et al., 2004).

Since then, multiple studies have been performed to explore the possible benefits of MSC in GVHD. After encouraging phase II results, Osiris Therapeutics sponsored a phase III trial of allogeneic BM-MSC from random donors for the treatment of steroid-refractory GVHD (NCT00366145). The study misfired to induce durable clinical response in adult patients, but it suggested responsiveness in pediatric patients (Kebriaei et al., 2020). The Osiris-backed BM-MSC product, Remstemcel-L, has been approved in Canada, New Zealand (Prochymal®, currently Ryoncil®), and Japan (Temcell®) for restricted use in children with GVHD (Cyranoski, 2012).

But discrepancies in results are evident in literature regarding MSC's efficacy for GVHD management (Hashmi et al., 2016; Zhao et al., 2019). A first systematic review and meta-analysis of uncontrolled studies with single-arm design showed that MSC treatment had a positive effect on 6-month survival in patients with steroid-refractory aGVHD (Hashmi et al., 2016). However, a systematic review gathering information of 10 controlled randomized clinical trials performed before 2018, demonstrated substantial improvements in terms of complete response and overall survival for cGVHD after MSC-treatment, but no major improvements in terms of engraftment, incidence of aGVHD, relapse, death, death due to relapse, or death due to infection (Zhao et al., 2019).

In pediatric patients, last results of a multicenter phase III, single-arm study have provided evidence of the safety, tolerability, and efficacy of Remstemcel-L as first-line therapy after initial steroid failure (Kurtzberg et al., 2020a, 2020b), however the FDA recommended to provide a complementary controlled randomized trial; while real-world data of Temcell® used in Japan demonstrated limited effects (Murata et al., 2021).

Features that may explain poor MSC-based therapy outcomes in these trials, are cell fitness, viability, immune compatibility, doses and lack of recognition of potential responders among patients (Galipeau et al., 2021). Considering that MSC are typically transfused in patients after several expansions cycles in cultures and within a few hours after thawing, one can hypothesize that allogeneic, likely senescent human MSC directly retrieved from cryostorage, with its associated effect on viability, functionality, and *in vivo* persistence, are less optimal than metabolically fit materials (Galipeau and Sensébé, 2018a)

Alternative sources may be the answer for better results, placenta-derived decidual stromal cells seem to hold promise of better response rates compared to BM-MSC for severe acute GVHD (Ringden et al., 2018).

Other on-going studies in hematology field include the use of MSC for non-malignant red blood cells disorders, hemoglobinopathies, myelodysplastic syndromes, severe aplastic anemia, among others (Rodríguez-Fuentes et al., 2021)

2.8.3. Cardiovascular disorders

MSC transplantation therapy has been studied intensively for the treatment of cardiovascular diseases, especially myocardial infarction. MSC can protect the myocardium thanks to their immunoregulatory ability, as well as their anti-apoptotic, anti-fibrotic and pro-angiogenic properties. These features impact on the surrounding environment favoring differentiation of myocardial cells around infarcted areas, leading to cardiovascular repair (Guo et al., 2020). Promising results have been found for diseases such as dilated cardiomyopathy and ischemic or non-ischemic heart failure; however, CT are still in their early stages, evaluating safety and efficacy of the transplanted MSC (Rodríguez-Fuentes et al., 2021). Potential risks of undesirable differentiations of BM-MSC into osteogenic or chondrogenic structures within the heart tissue were identified early (Breitbach et al., 2007). This prompted some teams to prime their cells using dedicated cocktails promoting cardiogenic commitment (Bartunek et al., 2013). While the phases I and II suggested tolerance, feasibility, and efficacy, long-term clinical outcomes of placebo-controlled studies suggested benefit only in an ischaemic heart failure subpopulation defined by advanced left ventricular enlargement (Bartunek et al., 2020).

2.8.4. Neurological diseases

The anti-apoptotic, paracrine, and multidirectional ability of MSC to differentiate has driven their current evaluation in translational research and CT for the treatment of neurological disorders including stroke, traumatic brain injuries, spinal cord injury, Alzheimer's disease, amyotrophic lateral sclerosis and many others (Andrzejewska et al., 2021). More than 160 clinical trials using MSC or MSC-derived EV as treatment for neurological diseases were registered in the ClinicalTrials.gov database, the majority targeting Alzheimer Disease (Jovic et al., 2022).

2.8.5. AID and inflammatory disorders

AID are chronic and systemic disorders, characterized by over-activation of immune cells and a sustained immune response to self-antigens, leading to the damage and dysfunction of multiple organs. As for the MG, current treatments include immunosuppressive agents, corticosteroids and non-steroidal drugs, but clinical outcomes are still limited and patients suffer from various adverse

effects imparted by these therapies. MSC transplantation from different sources has become a promising therapeutic approach for AID due to their anti-inflammatory and immunomodulation properties (Yang et al., 2021). More than a hundred CT have been registered in the clinicaltrials.gov database regarding AID (Wang et al., 2018a). For some AID, MSC efficacy has only been tested in experimental models.

2.8.5.1. Systemic lupus erythematosus

SLE is a chronic AID characterized by the activation of autoreactive T and B lymphocytes leading to the production of a variety of auto-Ab that recognize mainly, but not only, nuclear acids and their binding proteins as autoantigens. The formation of immune complexes, persistent tissue inflammation and high levels of pro-inflammatory cytokines, result in multiple organ injuries, as seen in brain, joints, blood vessels, kidneys, and skin. (Mok and Lau, 2003). The conventional treatment of SLE relies primarily on high doses of corticosteroids, cyclophosphamide, and other immunosuppressive and biological agents. Although these drugs have markedly improved outcomes in SLE patients, their use is associated with severe side effects (Squillaro et al., 2016).

Preclinical studies have shown that MSC therapy can improve the signs and symptoms of SLE by promoting the proliferation of Th2, Treg and Breg cells while inhibiting the differentiation CD4⁺ T cells into Th1, Th17, and Tfh cells or from B cells into plasma cells, thus reducing Ab production. The synergy of immunomodulation effects restores a normal Th1/Th2 and Treg/Tfh ratio, correcting the over-activation of the immune system in patients with SLE (Li et al., 2021a). MSC-based clinical trials suggested that MSC transplantation is safe and tolerable in SLE patients (Wang et al., 2017b) and induced improvement of the clinical symptoms in active and refractory patients (Sun et al., 2010; Wang et al., 2013a, 2014a). MSC treatment reduced SLE disease activity index, the levels of proteinuria, auto-Ab and complement components and restored immune cell subsets balance (Sun et al., 2010). However, repeated infusion every 6 months may be necessary to avoid disease relapse for some patients (Wang et al., 2014a), raising the question of the immunogenicity and rejection of these cells. Allogeneic MSC are recommended for SLE treatment, as clinical studies with autologous MSC revealed abnormal profile and impaired capacities. Patients BM-MSCs are not immunosuppressive and do not ameliorate disease symptoms (Carrion et al., 2010; Cheng et al., 2019; Sun et al., 2007). Eleven CT are currently registered in clinicaltrials.gov (to June, 2022), and use UC-MSCs and BM-MSCs as therapeutic candidates. The majority of clinical trials are in Phase I/II and aim to assess safety and efficacy of MSC in patients.

2.8.5.2. Multiple Sclerosis

MS is an autoimmune-mediated disease of the central nervous system, initiated by autoreactive immune cells (mostly T cells) and targeting components of the brain or spinal cord, *i.e.* the oligodendrocytes, the astrocytes, or the neurons. The pathologic hallmarks include neuroinflammation, demyelination, axonal loss, gliosis and neurodegeneration. Therapies of MS disorders mainly focus on diminishing inflammation, but they remain relatively disappointing. Therefore, cell-based therapy using MSC that manifest immunomodulatory properties are promising in MS treatment (Yang et al., 2021).

Preclinical studies of MSC's effect on MS have been done, mostly, using the experimental autoimmune encephalomyelitis (EAE) animal model. Several authors have shown that MSC transplantation improves the course of EAE mice when administered at early stages. MSC deeply modulate the immune response, reduce inflammation and microglial activation, promote neuroprotection, and foster remyelination and endogenous neurogenesis through the secretion of neurotrophic factors and anti-inflammatory cytokines (Bai et al., 2009; Gerdoni et al., 2007; Kassis et al., 2008; Zappia et al., 2005).

In MS patients, trials involved a limited number of subjects, with active or progressive disease, treated mostly with autologous, BM-MSC administered either by lumbar puncture (intrathecally) or IV using variable doses. Allogeneic sources have also been proposed in some studies, by treating patients with UC-MSC (Li et al., 2014a; Riordan et al., 2018) or placenta MSC (Lublin et al., 2014). Overall, MSC treatment showed a favorable safety profile and potential benefic effects, nevertheless, conclusions regarding efficacy were limited due to restricted number of enrolled patients. A first large phase I/II clinical trial aiming at evaluating the safety of a single IV infusion of autologous BM-MSC compared to placebo and the efficacy of these cells at clinical and imagery levels (Uccelli et al., 2019) should soon report its results.

2.8.5.3. Crohn's Disease

Crohn's disease (CD) is a chronic inflammatory disease of the gastrointestinal tract and has unknown etiology. Perianal fistulas are a common complication of CD that severely impair patients' quality of life and cause substantial morbidity. It is not an AID per se, but shares some similar features, like pro-inflammatory milieu, local uncontrolled immune response and chronically inflamed tissue that impair fistula healing (De La Portilla et al., 2013). Additionally, a high percentage of patients present Ab that can be classified as auto-Ab to intestinal and non-intestinal self-constituents, or/and anti-microbial Ab which target microorganisms, including bacteria, yeasts and fungi (Mitsuyama et al.,

2016). Regular treatments include steroids to suppress the immune system's abnormal inflammatory response that is causing the symptoms, diet and nutrition adaptation and in an important proportion of patients surgery is necessary.

The therapeutic benefit of human MSC has been reported in a number of experimental models of inflammatory diseases including inflammatory bowel disease (Gonzalez-Rey et al., 2009; González et al., 2009). In patients, BM-MSC from CD subjects were shown to be functionally analogous to those of healthy individuals (Chinnadurai et al., 2015), while another group reported dampened *in vitro* immunosuppressive properties of mesenteric and subcutaneous AD-MSC in patients compared to controls (Serena et al., 2017). Despite the differences, autologous AD-MSC and BM-MSC have shown promising results from a safety and efficacy perspective (Ciccocioppo et al., 2011; Duijvestein et al., 2010; Garcia-Olmo et al., 2009). Allogeneic AD-MSC injections were also shown to be effective (De La Portilla et al., 2013).

A phase III trial evidenced that a single intralesional injection of allogeneic AD-MSC (Cx601) was a safe and effective treatment for complex perianal fistulas in CD. A higher remission rate was observed in MSC-treated patients, when compared to placebo-treated counterparts, at 6 and 12 months post-injection (Panés et al., 2016, 2018). Clinical remission after treatment may be sustained for up to 2 years in these patients, as shown by García-Olmo et al. (Garcia-Olmo et al., 2022). However, conclusions are limited by the low number of patients that entered the study extension. These favorable results allowed a marketing approval for Cupistem and Alofisel (Cx601) to treat complex perianal fistulas in adult patients with non-active/mildly active luminal CD. The former product is based on autologous AD-MSC and was approved by the Korean Ministry of Food and Drug Safety (MFDS) in 2012, while the latter is an allogeneic expanded AD-MSC approved for its use in Europe since 2018.

2.8.5.4. Rheumatoid arthritis

RA is a systemic AID principally affecting synovial joints. Disease hallmarks include invasive synovium inflammation, presence of auto-Ab targeting citrullinated or carbamylated peptides, and progressive cartilage and bone destruction. Current conventional treatments include steroid drugs, anti-rheumatic drugs and new biotherapies targeting cells or effectors. However, many patients still do not receive desired clinical outcomes from current therapies and MSC have been considered as a potential therapeutic approach for RA due to their immunomodulatory properties (Sarsenova et al., 2021).

Evaluation of MSC efficacy in RA animal models, was done using mainly a collagen-induced arthritis (CIA) model in mice. Tested human MSC were mostly obtained from UC, but the use of cells

coming from BM or AT was also reported. Overall results show that cells injection lead to amelioration of the severity of CIA, relief of systemic symptoms, reduction of joint inflammation, synovial cellularity and bone destruction through MSC regulation on immune cells and inflammatory cytokines involved in the course of RA (Sarsenova et al., 2021; Zhang et al., 2019).

In patients, few trials have been performed. Early clinical studies indicate that both autologous (Ghoryani et al., 2020; Shadmanfar et al., 2018) and allogeneic (Álvaro-Gracia et al., 2017; Park et al., 2018a; Wang et al., 2013c, 2019) MSC transplantation are safe and effective for treatment of refractory RA patients. No serious adverse effects have been reported during these studies and the patients treated by MSC showed a moderate reduction in serum inflammatory markers, symptomatic amelioration and significant disease remission (Hwang et al., 2021). More studies, especially multicenter ones, are required to examine the long-term safety of MSC injections and their respective clinical applications (Hwang et al., 2021).

2.8.5.5. Systemic Sclerosis

Systemic sclerosis (SSc) is a rare AID, whose pathophysiology is still not fully elucidated. It is characterized by extensive tissue fibrosis with increased matrix deposition in skin and internal organs, microvascular alterations and activation of the immune system with auto-Ab against various cellular antigens. There is no curative treatment to date and only symptomatic treatments are commonly proposed to patients to alleviate pain and improve function (Farge et al., 2021; Peltzer et al., 2018).

Preclinical studies have shown beneficial effect of MSC-based treatment in different animal models of local or systemic sclerosis, and are reviewed by Rozier et al. (Rozier et al., 2018). Regarding clinical studies, phase I CT evaluated the safety of autologous SVF containing MSC in patients with SSc with hand disability. Study results outlined the safety profile and suggested potential efficacy (Granel et al., 2015; Park et al., 2020b) and the effect persisted at least 22-30 months after injection (Daumas et al., 2017). However, two randomized double blind, placebo-controlled trials of this therapeutic agent failed to confirm previous results, showing only efficacy trends in SVF-treated patients (Khanna et al., 2022) or improvements that were not superior when compared to placebo group (Daumas et al., 2022). To sum up, these first results need to be taken with caution in terms of efficacy and larger CT would be required.

The question that remains is whether autologous or allogeneic AD-MSC or BM-MSC should be used in the clinics, since studies are ambiguous about phenotypic or functional profiles of MSC from SSc patients compared to healthy counterparts (Griffin et al., 2017; Larghero et al., 2008; Scuderi et

al., 2013). Currently, CT trials using autologous SVF or allogeneic MSC sources such as UC, Wharton's jelly, and BM are ongoing.

2.8.5.6. Other autoimmune diseases

MSC have also shown encouraging results in animal models of type I Diabetes mellitus (Lee et al., 2006; Madec et al., 2009; Yang et al., 2021), psoriasis (Chen et al., 2019b; Imai et al., 2019; Lee et al., 2016), primary Sjögren's syndrome (Chen et al., 2018; Chihaby et al., 2021; Tian et al., 2020), and Myasthenia Gravis (Ben-Ami et al., 2011; Sudres et al., 2017). Meta-analysis of controlled clinical trials in type I diabetes are still debating the positive outcomes of MSC transplantation and randomized-controlled trials with larger number of patients are still needed to properly conclude on the efficacy (de Klerk and Hebrok, 2021). A few studies have been reported regarding psoriasis and primary Sjögren's syndrome (Chen et al., 2016; Xu et al., 2012a), showing positive outcomes but limited conclusive power due to the small size of studied population. The encouraging results of preclinical studies in MG constitute the background of this current project, reason why they will be explained more extensively in a dedicated section.

2.8.6. Risks in MSC treatment.

One of the main concerns in MSC-based therapy is MSC malignant transformation during *in vitro* expansion or following infusion, leading to tumor formation. Also, as a collateral effect of the immunosuppressive and trophic effects exerted by MSC (Barkholt et al., 2013) one may be scared of the development of already existing malignant cells in the host. To discard the possibility of MSC products containing cells with abnormalities it was suggested that karyotyping analysis should be performed (Barkholt et al., 2013). However, conversely to murine MSC, humans MSC are minimally susceptible to oncogenic transformation *in vivo*, and long-term culture was shown not to induce chromosomal alteration. Continued passaging leads to loss of already existing aneuploidy and any culture-induced aneuploidy leads to senescence without evidence of transformation, negating the risk of cancer formation (Stultz et al., 2016; Tarte et al., 2010).

A potential undesired effect of AD-MSC is their potential evolution toward fibrosis. Kim and colleagues reported a case in which IV infusion of autologous AD-MSC, in a patient with chronic kidney disease, resulted in severe interstitial fibrosis and inflammatory cell infiltration, which could suggest nephrotoxicity of the applied MSC (Kim et al., 2017). Another potential complication of MSC injection is thrombus formation around the cells through a coagulation mechanism, also known as instant-blood-mediated-inflammatory-response which is responsible of significant loss of transplanted cells,

but which can also cause pulmonary embolism due to cell trapping (Tatsumi et al., 2013; Wu et al., 2017b). However, it should be noted that MSC were used in several hundreds of clinical trials, and that the incidence of adverse events has been extremely rare, if any. Current data from clinical trials tend to ensure the safety profile of MSC use (Musiał-Wysocka et al., 2019) but do not completely preclude the set-up of more long-term safety studies.

We cannot status yet whether autologous or allogenic cells would be preferred in applications dedicated to MG. Autologous cells would not be rejected by the patients. However, at variance with more classical regenerative medicine applications of MSC, *e.g.* bone or cartilage repair, it is not expected that the therapeutic MSC need to integrate for a long time in the patient body. It is also challenging to prepare autologous cells, case by case, on a patient basis. The preparation needs the extraction of cells from AT or BM, under local or general anesthesia, which are especially risky in MG patients and may not be accepted by Health Ethics Committees until the efficacy has been strongly established. Finally, in some AID (RA, SLE, SSc), the MSC from patients are not as efficient as allogenic cells from healthy young donors. On the other hand, allogenic cells can be produced and characterized in large amounts, but their injection could trigger an immune response and their rejection. Indeed, allogenic MSC were rejected upon transplantation in mice (Eliopoulos et al., 2005) and a significant proportion of patients developed specific allo-Ab after infusion of allogenic MSC (Sanabria-de la Torre et al., 2021). This medical dilemma is not resolved yet (Ankrum et al., 2014a). Of note, many of the ongoing clinical trials in AID use allogenic cells.

2.9. MSC priming

Some authors claim that MSC are not inherently immunosuppressive and thus, need to be activated in order to display full capacities. This theory is supported indirectly by results demonstrating that priming, influences MSC properties *in vitro* and *in vivo* (Hu and Li, 2018) and are necessary to activate the immunomodulatory activity (Ferreira et al., 2018b; Saparov et al., 2016). On the other hand, as exposed above, cell cultures harbour initial heterogeneity because of several factors affecting their gene expression, phenotype, secretome and biological properties. Priming appears then to potentially “correct” such variations permitting the use of more uniform therapeutic products with enhanced immunosuppressive potential, which may lead to higher clinical benefits in patients (Guerrouahen et al., 2019). This enthusiasm may be tempered by the fact that, up to now, cells used in clinical trials were not primed before use. However, one may consider that they are placed in inflammatory contexts *in vivo*, and that this may be sufficient for triggering their activation. Also, some

conditions of culture may be sufficient to trigger activation. The future use of primed cells prepared in this intent (Mebarki et al., 2021) in forthcoming trials should allow fixing this controversy.

Cell priming consists in preparing cells for some specific function, by empowering one or more biological capacities (effects) and adapting their physiology to better face the challenge (disease),

Figure 39.

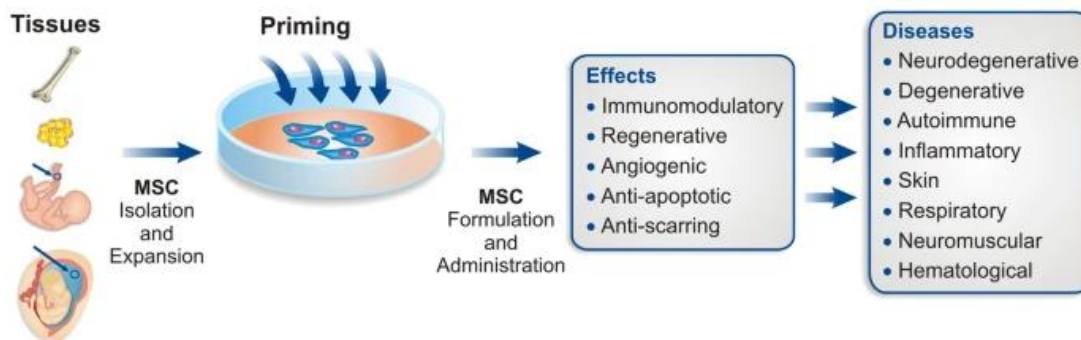


Figure 39. Overview of the production of primed MSC for the treatment of different types of diseases

MSC obtained from different sources are expanded in culture and primed to boost their biological properties (listed here under the title of “Effects”), making them better candidates to treat different diseases (Noronha et al., 2019)

Several priming approaches have been developed to improve MSC function, survival, and therapeutic efficacy (Noronha et al., 2019) and can be ordered into 5 classical categories: (a) MSC priming with hypoxia, (b) with inflammatory cytokines or mediators, (c) with pharmacological drugs and chemical/biological agents, (d) using dedicated 3D culture conditions and (e) by cellular conditioning. We will now focus more precisely on these strategies. The term “priming” will be used to refer to single molecules used in a pharmacological way, and the term “conditioning” will refer to approaches leading to sometimes complex and as yet unknown modifications of cell properties.

2.9.1. Hypoxia licensing

Standard cell culture practice generally uses normoxic oxygen tension of atmospheric pressure (21% O₂), while physiologic oxygen availability in the tissues is generally much lower (0.7–7% O₂). MSC expansion in environments with high levels of oxygen may lead to cellular oxidative stress and DNA associated lesions, as well as to early senescence (Bétous et al., 2017; Estrada et al., 2012; Kwon et al., 2017; Tsai et al., 2011). Hypoxia priming has been used to reproduce the *in vivo* MSC niche conditions, aiming to improve the therapeutic efficacy of MSC from different species and/or tissues (Noronha et al., 2019).

Used as a conditioning strategy, hypoxia has been shown to improve MSC cell survival by downregulating apoptosis related proteins such as p21 and their related pathways E2A/p21 (Tsai et al., 2011), enhancing MSC pro-survival markers (Chacko et al., 2010) and enhancing MSC release of vascular endothelial growth factor (VEGF) and platelet growth factor (Chacko et al., 2010; Ohnishi et al., 2007) which also boosts MSC related angiogenesis. Hypoxic conditioning of MSC has been shown to greatly alter cell metabolism during expansion, enhancing glycolysis (Wobma et al., 2018), reducing ROS production and telomeric shortening rates (Estrada et al., 2012; Lavrentieva et al., 2010; Wobma et al., 2018). It also favors MSC migration through induction of the CXCL12–CXCR4 axis (Han et al., 2022) and increases the stemness of cells by up-regulation of related genes such as OCT4, KLF-4, Nanog and Nestin. Regarding immunomodulation capacities, hypoxic pretreatment enhances the immunomodulatory effect of MSC by stimulating the production of anti-inflammatory cytokines, and exosomes biogenesis and secretion (Gupta et al., 2022; Xu et al., 2016). The hypoxic stress is controlled by the stabilisation and activation of the Hypoxia-Inducible Factor (HIF)-1 α subunit of the HIF-1 transcription factor (Gupta et al., 2022; Martinez et al., 2017; Tsai et al., 2011), **Figure 40**.

After hypoxia, MSC elicit improved inhibitory effect on PBMCs proliferation and function, through increased IL-10 secretion and FasL expression (Jiang et al., 2015), through the production of T-cell inhibitory lactate levels due to MSC faster glucose consumption (Wobma et al., 2018) or even through up-regulation of ICAM-1 on MSC surface (Han et al., 2022) that was shown to limit T cell activation (Zheng et al., 2021). Hypoxia has also been shown to markedly upregulate expression of IDO which is critical in immunoregulation by MSC through induction of T cell anergy and generation of Treg (Roemeling-Van Rhijn et al., 2013). Although hypoxia enabled MSC to produce several growth factors and chemokines more efficiently, it accelerates proteasome-mediated degradation of COX2 and decreases PGE2 in MSC (Sareen et al., 2020).

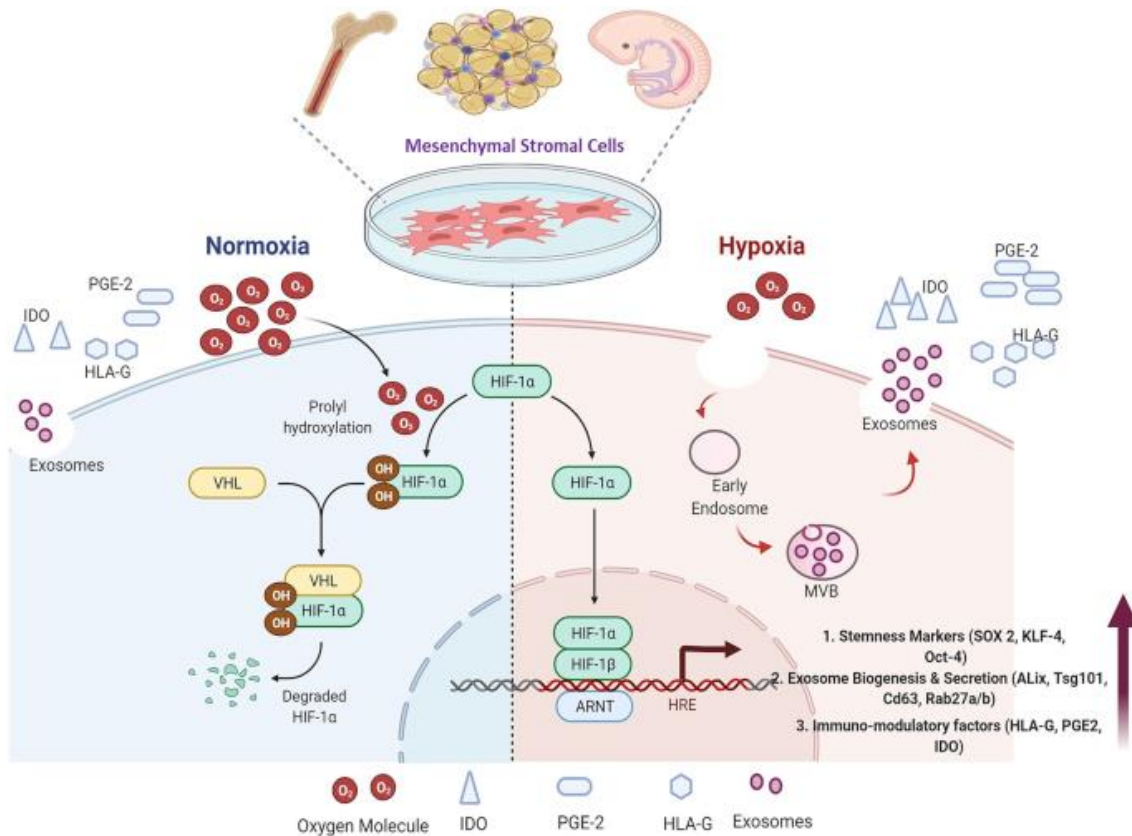


Figure 40. MSC changes induced hypoxia conditions and orchestrated by HIF-1

Under normoxic conditions, HIF-1 α is recognized by VHL, a component of an E3 ubiquitin ligase complex. This interaction thereby promotes the rapid degradation of HIF-1 α . Under hypoxia, prolyl hydroxylation is suppressed; HIF is stabilized and translocated to the nucleus as heterodimer with HIF-1 β or its analog ARNT. Binding to Hypoxia Response Element enhances: MSC stemness, exosomes biogenesis and secretion, and immunomodulatory factors production by MSC. *Abbreviations*; hypoxic response factor HIF-, von Hippel-Lindau tumor-suppressor protein (VHL), aryl hydrocarbon receptor nuclear translocator (ARNT), hypoxia response element (HRE) (Gupta et al., 2022).

2.9.2. Cytokines

Paracrine signaling is an essential component of MSC therapy, especially regarding the immunomodulatory functions of MSC. The profiles of MSC can be modulated during *in vitro* manufacturing to enhance specific functional attributes and adapt them to respond better to a given pathological context (Srinivasan et al., 2022). Proinflammatory cytokines are able to influence the expression of immunomodulatory molecules and cell-adhesion proteins by MSC, and are therefore used to enhance immunoregulatory properties, as shown in **Figure 41**.

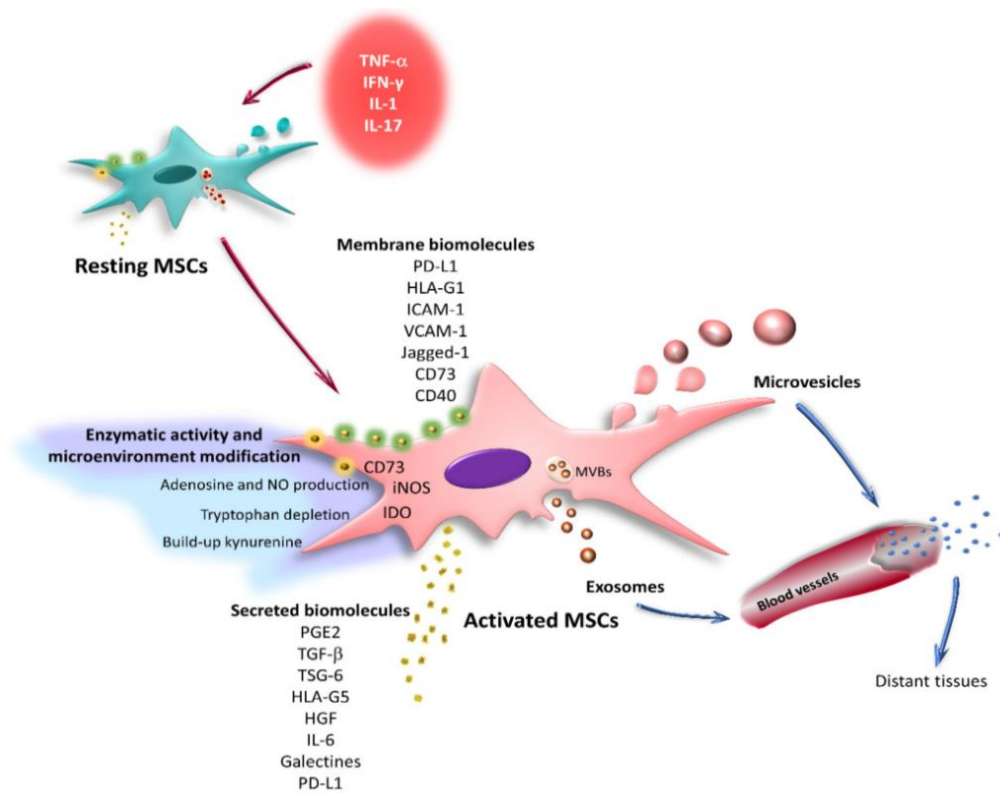


Figure 41. Immunoregulatory mechanisms of primed MSC

MSC are generally in a resting state with low expression of the molecules involved in their immunoregulatory function. However, when exposed to proinflammatory cytokines such as TNF- α , IFN- γ , IL-1, and IL-17, these cells become activated. This event enhances the expression of immunoregulatory molecules in MSC, which can be secreted or remain attached to the cell membrane, and exert their function through paracrine effect or cell-cell contact, respectively. In addition, resting and activated MSC release extracellular vesicles (exosomes and microvesicles), which are capable of traveling through body fluids and reaching distant sites, where they establish contact with immune cells. (López-García and Castro-Manrreza, 2021)

The panoply of molecules that can be used is large, and they may act solely or in combination. The effects of the most commonly used cytokines to activate MSC, are presented in the sections below.

2.9.2.1. IFN- γ priming

IFN- γ is one of the best-known activators of MSC immunomodulatory properties. Studies suggesting the role of IFN- γ in activating the immunosuppressive capacity of MSC started in the early 2000s (Krampera et al., 2006; Meisel et al., 2004a) and since, it became largely studied. Upon IFN- γ exposition, MSC upregulate several anti-inflammatory and immunomodulatory factors, including IDO (Meisel et al., 2004a), HLA-G5 (Wang et al., 2016), CCL2 (Rafei et al., 2009), PGE2, HGF, TGF β and PD-L1 (Ryan et al., 2007; Sheng et al., 2008b). Additionally, expression of chemokine ligands (CXCL9,

CXCL10, and CXCL11), and adhesion proteins (VCAM-1 and ICAM-1) were also reported as enhanced (Wang et al., 2016).

IFN- γ -treated MSC (γ MSC) are able to modulate innate as well as adaptive immune responses. They were shown to suppress the proliferation of CD4⁺ and CD8⁺ T lymphocytes and NK cells, in a contact-dependent manner (Chinnadurai et al., 2014; Sheng et al., 2008b), but also through the secretion of soluble factors, notably IDO (Boyt et al., 2020; François et al., 2012a; Mebarki et al., 2021a; Meisel et al., 2004a; Ryan et al., 2007). Enhanced expression of IDO is dependent on the dose and duration of IFN- γ stimulus (Boyt et al., 2020). γ MSC were also capable to significantly inhibit Th1 cytokine (IFN- γ , TNF- α , and IL-2) production and T cell degranulation through B7H1 and B7DC/PD1 pathways (Chinnadurai et al., 2014); and to increase the number of Treg cells and inhibit Th17 cells when cocultured with PBMC (Wang et al., 2016).

Noone and colleagues demonstrated that IFN- γ -primed MSC suppressed NK activation more efficiently and were more resistant to NK cytotoxicity than non-primed MSC. These effects were in part mediated by boosted synthesis of IDO and PGE-2. Additionally, IFN- γ pretreated MSC increased expression of nonclassical MHC ligands for the NK inhibitory receptor CD94/NKG2A and reduced expression of UL16 binding protein (ULBP1–3), ligands of the activator receptor NKG2D (Noone et al., 2013), thus evading NK-dependent destruction. More interestingly, IFN- γ -primed MSC were shown to preserve their immunosuppressive properties despite cryopreservation (Chinnadurai et al., 2016) and IFN- γ -priming was proposed to restore these properties in senescent cells (Chinnadurai et al., 2017).

The efficacy of γ MSC has been investigated in several pre-clinical models. However, results have shown inconsistent efficacy. For example, in mouse models, allogeneic murine γ MSC protected against lethality from acute radiation syndrome but failed to alleviate acute GVHD (Chinnadurai et al., 2021). In contrast, Polchert and colleagues reported that MSC pre-treated with IFN- γ could suppress GVHD more efficiently and increased mice survival using fewer cells, when compared with non-activated MSC (Polchert et al., 2008). In experimental colitis, human γ MSC showed enhanced migration potential to inflammatory sites and clinical improvements in mice, through significant reduction of mucosal damage and inflammatory responses (Duijvestein et al., 2011). On the contrary, Burand and coworkers have shown that infusion of thawed IFN- γ -primed human MSC failed to ameliorate a murine model of ischemia/reperfusion retinal injury (Burand et al., 2017).

Differences in clinical outcomes may be influenced by MSC origin, but also by priming protocols, as there is no common agreement on how much IFN- γ is necessary or for how long the priming should be done in order to get optimal results. Recent studies have shown that dose and

duration of IFN- γ should be tailored to the individual donor to maximize immunomodulation properties, as donor-to-donor variability plays an important role when discussing the efficacy of MSC (Boyt et al., 2020).

Noteworthy, IFN- γ may also lead to other unintended changes in phenotype and secretion profile of MSC causing conflicting cellular responses. For example, sustained exposure to IFN- γ led to inhibition of STAT3 activity and impaired MSC proliferation and differentiation (Vigo et al., 2017), and was able to induce senescence-like features in murine MSC (Yang et al., 2017), which are undesired outcomes for MSC production.

IFN- γ activation may also induce changes in cell size and granularity, and It was reported that morphological changes would predict the immunosuppressive capacities of IFN- γ treated MSC cultures (Klinker et al., 2017). Long-term expansions upon treatment should be assessed to evaluate the potential toxicity. Priming with IFN- γ , was also consistently shown to upregulate the expression of class I and class II HLA molecules, making MSC more immunogenic and more prone to rapid clearance, *in vivo*, following administration. This was evidenced in studies reporting a heightened *in vitro* immune response and *in vivo* donor-specific sensitization in response to primed allogeneic MSC (Cho et al., 2008; Schu et al., 2012). Stimulation of cells with IFN- γ activates also numerous parallel pathways that are not necessarily related and do not contribute to MSC immunomodulation properties, through different mediators as shown in **Figure 42**.

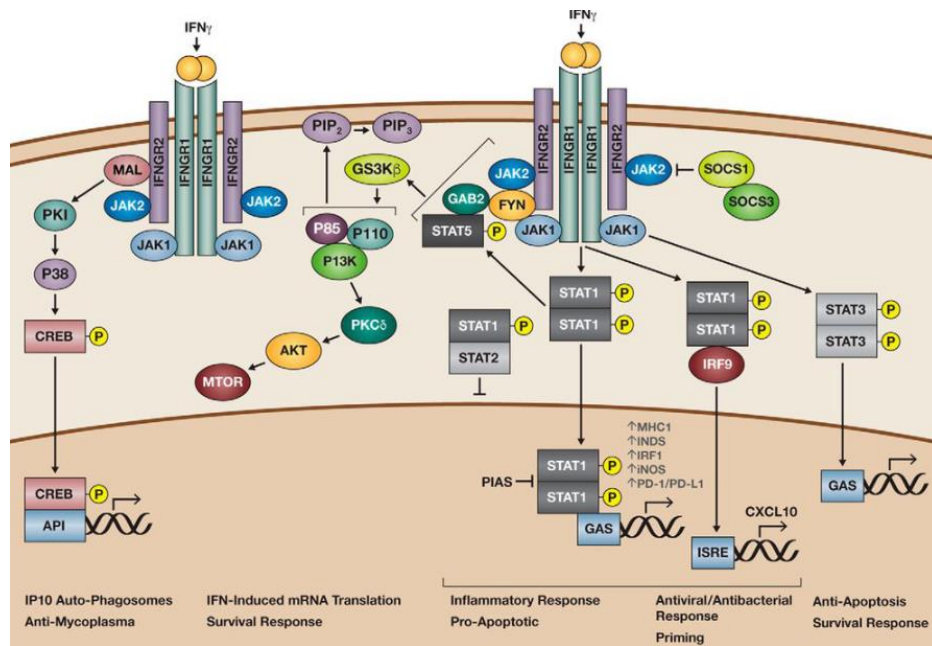


Figure 42. Overview of the signaling pathways elicited by IFN- γ

As a dimer, IFN- γ (orange) binds the receptors IFNGR, which is composed of the IFNGR1 and IFNGR2 subunits, and the kinases Jak1/Jak2. Phosphorylation of Jak1 and JAK2 results in the phosphorylation of STAT1 (center) which translocates to the nucleus and binds to GAS, inducing genes associated with inflammatory response such as HLA-A, NOS-2, IRF1, PD-1, and PD-L1. Gas-induced genes can also be modulated through STAT3 signaling (right). Other responses that are triggered by IFN- γ are antiviral and antibacterial responses through STAT-1/IRF9 signaling. Recruitment of adaptor proteins associated with IFNGR2 such as MAL and Fyn results in non-canonical STAT1 signaling. MAL acts via MAPK p38 phosphorylation, which induces up-regulated expression of the chemokine IP-10, anti-mycoplasma proteins, and formation of autophagosomes (left). GSK3 β activation and Fyn elicit pSTAT5 recruitment to activate PI3K to regulate cell membrane permeabilization. Alternatively, IFN- γ activation of GSK3 β via PKC δ activates AKT/mTOR regulation of survival responses (Green et al., 2017)

2.9.2.2. TNF- α priming

Since TNF- α is the first cytokine released by activated T lymphocytes, it has been proposed to provide the initial stimulus for MSC priming when in contact with PBMC (López-García and Castro-Manrreza, 2021). TNF- α priming promotes increased MSC secretion of immunoregulatory factors such as PGE2, IL-10, IL-6, NO, IGF-1 and HGF (English et al., 2007; Jin et al., 2016; Nogueira-Pedro et al., 2021; Prasanna et al., 2010), of chemokines (e.g., CCL2, CCL13, CCL17, CCL19, CCL22, CCL23 and CXCL2) (Jin et al., 2016) and increased surface expression of ICAM-1 (Montesinos et al., 2020) and VCAM-1 (Lu et al., 2016). Literature also reported enhanced exosomes production by TNF- α stimulated MSC, with high levels of CD73 exosomal expression. Authors have evidenced that TNF- α -induced changes are mediated through the nuclear factor (NF)- κ B signaling (Ting et al., 2021; Zhong et al., 2017).

Compared to IFN- γ priming, TNF- α treatment exerts a milder up-regulation of some of the cited molecules (Prasanna et al., 2010) and does not affect the expression of PD-L1 on the surface nor the

secretion of IDO (Davies et al., 2017; English et al., 2007; Prasanna et al., 2010). Most importantly, it does not impact the expression of HLA II molecules, thus impacting less on cells immunogenicity (López-García and Castro-Manreza, 2021).

As an alternative, some studies have proposed the use of both molecules for priming MSC and have shown that TNF- α and IFN- γ exert a powerful synergistic effect on the expression of immunoregulatory molecules in MSC from different sources (English et al., 2007; Jin et al., 2016; Montesinos et al., 2020; Ren et al., 2010b; Zhong et al., 2017; Zimmermann and Mcdevitt, 2014). In addition to an up-regulation of the already cited immunomodulatory molecules, priming with both TNF- α and IFN- γ was shown to increase factor H production by MSC, which is an important inhibitor of complement activation (Tu et al., 2010). *In vitro* studies of IFN- γ and TNF- α primed MSC have demonstrated increased suppressive activity towards activated T cells compared to untreated MSC (Cuerquis et al., 2014). The commitment of monocyte differentiation into IL-10-secreting M2 macrophages (CD14⁺CD206⁺) and the induction of CD4⁺IL-10⁺ and CD8⁺IL-10⁺ Treg subpopulations (François et al., 2012a) , amplify the immunosuppressive properties of primed MSC. The effects were shown to be mediated through IDO activity and PD-L2 expression by MSC after priming (François et al., 2012a).

TNF- α and IFN- γ treatment of MSC can also impact the content of EV. Exposure of BM-MSK to TNF- α and IFN- γ induces the release of exosomes enriched in ICAM-1, COX-2 and PGE2, which have a greater capacity to interact with monocytes and T lymphocytes, and an enhanced ability to mitigate inflammation (Harting et al., 2018). MSC-primed exosomes, associated with higher IFN- γ , IL-10 and IDO contents, were also able to decrease effectively T lymphocyte proliferation, through the induction of Treg (CD25⁺FOXP3⁺) (Zhang et al., 2018).

In vivo, MSC primed with IFN- γ and TNF- α were capable of inhibiting delayed-type hypersensitivity responses (Szabó et al., 2015). The effect of IFN- γ and TNF- α alone or in combination over MSC paracrine profile is shown in **Figure 43**.

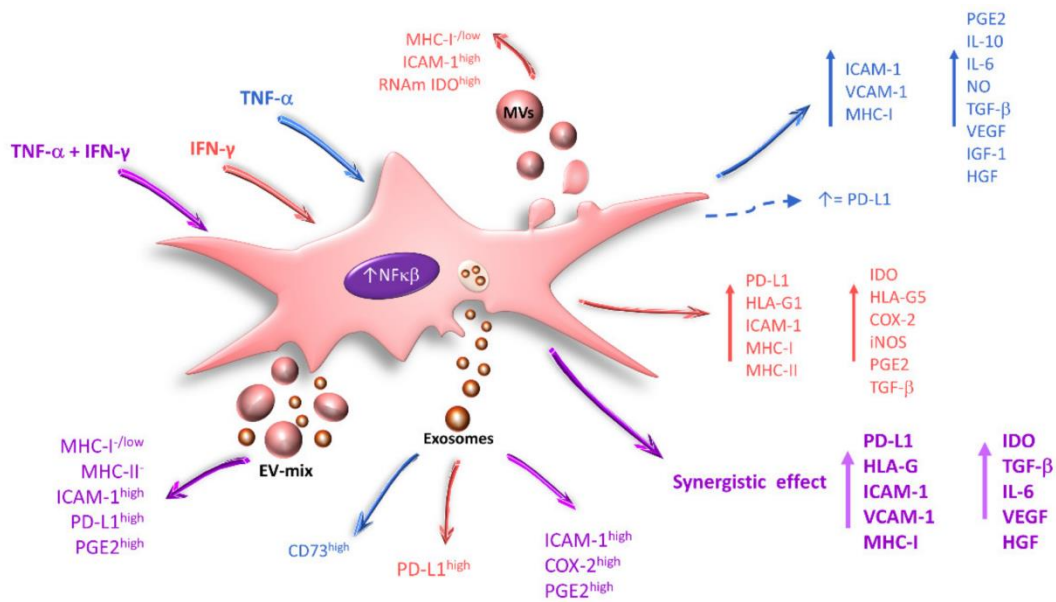


Figure 43. Effect of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ on the expression of immunoregulatory molecules by MSC

Changes in the expression of immunoregulatory molecules on MSC or in exosomes, multivesicular bodies, and extracellular vesicles-mix released by them, upon MSC stimulation with $\text{TNF-}\alpha$ (blue), $\text{IFN-}\gamma$ (red), or $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ (purple) are indicated. (López-García and Castro-Manrreza, 2021)

2.9.2.3. Interleukin-1

Interleukin-1 α/β (IL-1 α/β) are important inflammatory factors, both need IL-1R1 for signal transduction, and this receptor is abundantly expressed in human MSC (Fan et al., 2012). Groh and colleagues first showed that when coculturing MSC with PBMC, monocytes could activate MSC through IL-1 β secretion and enhance MSC capacities to inhibit activated T cell proliferation through the secretion of $\text{TGF-}\beta$ 1 (Groh et al., 2005). IL-1 β priming of human BM-MSC involves up-regulation of genes related to $\text{NF-}\kappa\text{B}$ pathway activation and implied in biological processes such as cell survival, migration and adhesion, chemokine production, angiogenesis, and modulation of the immune response. Furthermore, IL-1 β enhanced recruitment of neutrophils, monocytes, lymphocytes, and eosinophils by MSC (Carrero et al., 2012). Conditioned media produced by MSC primed with IL-1 showed immunomodulatory properties, decreasing the secretion of inflammatory mediators, such as IL-6 and $\text{TNF-}\alpha$, and increasing production of IL-10 by LPS-activated cells (Redondo-Castro et al., 2017).

In vivo, IL-1 β -primed MSC have been shown to attenuate the development of colitis in a murine experimental model. Primed cells were able to polarize peritoneal M2 macrophages, increase frequencies of Treg and Th2 cells, and decrease percentage of Th1 and Th17 cells in the spleen and mesenteric lymph nodes of infused mice when compared with mice receiving resting MSC (Fan et al., 2012).

2.9.2.4. Interleukin-17

IL-17A, another pro-inflammatory cytokine, was recently reported as an alternative approach for MSC priming. IL-17 increased proliferation of human and murine BM-MSC in a dose-dependent manner (Huang et al., 2009, 2006; Mojsilović et al., 2015). In human MSC, this effect was mediated by the generation of ROS upon activation of the NF- κ B activator 1 (ACT-1) and TNF receptor-associated factor 6 (TRAF-6). IL-17 priming was also able to induce greater migration, motility, and osteogenesis in MSC (Huang et al., 2009) and it impaired adipogenesis (Shin et al., 2009). However, results in the literature are not unanimous regarding the last point.

Human MSC, primed with IL-17, increased the expression of metalloproteinases (MMP) 1, MMP13, and CXCL6, and other genes associated with chemotaxis response and did not increase expression of class II MHC and co-stimulatory molecules. This signature may be involved in T cell recruitment and immunosuppression, while maintaining their hypoimmunogenic phenotype (Sivanathan et al., 2017). Functional studies *in vitro* showed that IL-17-primed MSC were able to suppress T cells proliferation, to induce strong down-regulation of CD25 expression in activated CD4, to inhibit Th1 cells cytokine production (IFN- γ , TNF- α , and IL-2) and to induce Treg cells (Sivanathan et al., 2015); these effects are mediated by IL-6 secretion by IL-17 primed MSC. By the way, IL-6 therefore participates to immunomodulation.

2.9.2.5. Combinatory strategy

In the physiological context, after MSC infusion, the cells encounter different stimuli, the balance of which dictates the MSC activation towards dedicated phenotype and function (López-García and Castro-Manreza, 2021). In addition to the simple combination of IFN- γ and TNF- α presented above, several teams have analyzed the effect of combined cytokines on the biology of MSC, and studies have determined that MSC treated with mixtures of proinflammatory cytokines have a greater immunoregulatory capacity (Gómez-Ferrer et al., 2021; Horie et al., 2020), and are able to overcome donor-specific variations in MSC immunomodulatory potency (Andrews et al., 2022).

The combination of TNF- α , IL-1 β , and IFN- γ , also referred as “cytomix”, is used to drastically enhance the efficacy of MSC. *In vitro*, MSC primed with this cocktail overexpressed cell adhesion molecules (VCAM-1 and ICAM-1) (Ren et al., 2010b) and molecules related to immunosuppression (IDO, COX2 and PD-L1). EV derived from primed-MSC were produced in greater number and had also enhanced immunosuppression capacities over CD4⁺ and CD8⁺ activated T-cell proliferation (Gómez-Ferrer et al., 2021).

When used *in vivo*, infusion of such EV improved the integrity of the tissues which contained higher proportion of M2 cells, and decreased infiltration of CD45⁺ cells and M1 macrophages in a delayed-type hypersensitivity mouse model (Gómez-Ferrer et al., 2021). MSC primed with this mixture have also shown positive outcomes in an animal model of ventilator-induced lung injury (VILI) by restoring oxygenation, reducing inflammatory cytokines and neutrophils accumulation in the bronchoalveolar lavage, improving lung function and promoting injury resolution (Horie et al., 2020).

Other alternative cocktails are available: (1) MCS pretreated with the mix of IL-17 with IFN- γ and TNF- α alleviated Concavalin A-induced liver injury in an iNOS-dependent manner (Han et al., 2014). (2) Human MSC treated with IL-1 β , IL-6, and IL-23 cocktail showed higher levels of TGF- β and lower levels of IL-4 than untreated MSC, and enhanced immunosuppressive capacities. (3) The combination of IFN- γ , TNF- α and IL-6 enhanced the immunosuppressive capacity of MSC on T cells through IDO production and up-regulation of HLA-G, TGF- β 1 and COX-2 (Crop et al., 2010a).

These results demonstrate that the type of inflammatory stimulus affects MSC gene, phenotype and secretome profiles, and their functional immunomodulating responses.

2.9.3. Pharmacological drugs and small molecules

A series of pharmacological drugs and small molecules have been used to prime MSC and enhance their therapeutic capacities. Their effects vary greatly, but most stimuli improve MSC survival and modulate their differentiation potential, regenerative capacities, homing, mobilization, engraftment and/or immunomodulatory properties to variable extents (Noronha et al., 2019). The effects of molecules influencing the immunomodulatory aspect are described below and summarized in **Figure 44**.

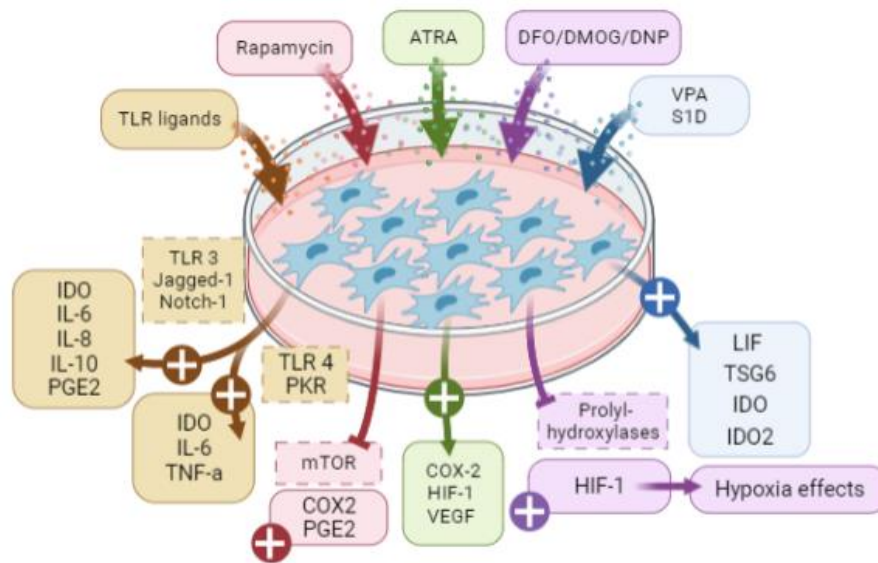


Figure 44. Schematic representation of the effect of drugs and biological/chemical molecules on MSC immunomodulation molecules

Treatment of MSC with TLR ligands, rapamycin, all-trans Retinoid Acid (ATRA), dimethyloxalyglycine, desferrioxanine, valproic acid, 2,4-dinitrophenol (DNP), sphingosine-1-phosphate modulates the gene expression or secretion of immunomodulatory molecules as shown in solid boxes. Dashed-contoured boxes show proteins or pathways that mediate and are important in triggering the effect. Created with Biorender

Epigenetic regulatory molecules such as DNA-demethylating agent, 5-azacytidine (5-Aza) and the histone deacetylase inhibitor valproic acid modulate differently MSC proliferation, migration and immune capacities, although they had little influence on cell phenotype and the multipotency capacities. Valproic acid-primed UC-MSC exhibited enhanced cells activation, migration and expression of Leukemia Inhibitory Factor (LIF), TSG-6, IDO1 and IDO2; 5-Aza significantly improved migration, but severely inhibited MSC's anti-inflammatory response (Lim et al., 2017). Combination of valproic acid and the bioactive lipid sphingosine-1-phosphate also improved the anti-inflammatory activity of MSC.

Hypoxia-mimetic reagents can potentially enhance immunomodulation capacities of MSC by cellular processes dependent of HIF-1 activation (Noronha et al., 2019). This category includes: inhibitors of prolyl-hydroxylases (desferrioxamine and dimethyloxalyglycine), 2,4-dinitrophenol, the volatile anesthetic isoflurane and hypoxia-inducible factors (HIF-1, HIF-1 α , HIF-1 β) (Alagesan et al., 2022).

All-trans retinoic acid upregulates the expression of genes involved in MSC survival, migration and angiogenesis (COX-2, HIF-1, CXCR4, CCR2, VEGF, Ang-2 and Ang-4), some of which have also immune implication, and boost immunomodulation capacities through increased PGE2 production in dose-dependent manner (Pourjafar et al., 2017). Human BM-MSC primed with all-trans retinoic acid and cocultured with activated PBMCs from ankylosing spondylitis patients secreted high levels of IL-6,

reduced CD4⁺ T cell proliferation and spondylitis-related pathogenic cytokines TNF- α , IL-17 α and IFN- γ (Li et al., 2015c).

IS such as rapamycin, everolimus, FK506 or cyclosporine, and glucocorticoids such as budesonide and dexamethasone, increase the immunosuppressive potency of MSC (Girdlestone et al., 2015; Kim et al., 2015; Wang et al., 2017a). IS-treated MSC were shown to better suppress T-cell proliferation *in vitro* and authors related the effect to either MSC capacity to shuttle some drugs to the target cells (Girdlestone et al., 2015) or to mTOR signaling inhibition leading to COX-2 and PGE2 up-regulation (Kim et al., 2015; Wang et al., 2017a). *In vivo*, rapamycin-treated MSC were tested in 2 different pre-clinical models of aGVHD and were shown to significantly inhibit the onset of disease in one model (Girdlestone et al., 2015) and to ameliorate clinical scores and decrease Th17 cell subset in the other (Kim et al., 2015). Other IS like azathioprine or mycophenolate mofetil did not modify suppressive activity of MSC (Girdlestone et al., 2015). The enhanced immunosuppression observed with glucocorticoids was explained by up-regulation of IDO expression and activity, and mediated through glucocorticoid receptor and FOXO3 (Ankrum et al., 2014b).

Biological molecules may also modulate the MSC capacities. Conventional pathogen-associated molecular patterns (PAMPs) that activate the immune system through TLR signaling were reported in literature as MSC priming molecules. Pre-treatment of MSC with poly(I:C), a TLR3 ligand, resulted in enhanced immunosuppressive function by upregulating IDO and other anti-inflammatory molecules. *In vivo*, poly(I:C)-treated MSC were able to modulate Treg and Th1/Th17 activities (Qiu et al., 2017), and improved clinical and pathological manifestations of mice in experimental models of colitis (Qiu et al., 2017) and sepsis (Zhao et al., 2014). MSC primed with LPS, a TLR4 ligand, have provided heterogeneous results (Zeuner et al., 2015). While brief priming periods with LPS induced higher MSC secretion of pro-inflammatory cytokines (Waterman et al., 2010), a day-lasting priming strategy was shown to increase MSC's IDO1 production and enhance significantly their immunosuppressive effects on T-cells *in vitro* (Opitz et al., 2009). *In vivo*, exosomes secreted by LPS-treated MSC show better regulatory properties than those obtained from non-treated MSC, favoring M2 macrophage polarization and inducing resolution of chronic inflammation in diabetic mice model (Ti et al., 2015).

2.9.4. 3D Culture

MSC culture in a 3-dimensional (3D) environment is another type of conditioning that aims to more closely mimic the physiological conditions of cell niches. In contrast with conventional 2D monolayer cultures, a spheroid 3D culture comprises several cells layers: the most external zone

contains cells with active metabolism and proliferation, the intermediate quiescent zone harbours cells with minimum metabolic activity, and the inner zone, where cells are exposed to much lower levels of oxygen and nutrients, contains hypoxic cells (Foglietta et al., 2020).

MSC grown in spheroids have shown greater angiogenic, anti-inflammatory, anti-fibrotic, and regenerative effects with improved cell survival and stemness profile (Cesarz and Tamama, 2016; Cheng et al., 2013; Noronha et al., 2019; Potapova et al., 2007). 3D MSC-conditioning was shown to upregulate TSG-6 expression (Bartosh et al., 2010), PGE₂, TGF- β 1 and HGF levels (Bartosh et al., 2010; Park et al., 2014; Ylöstalo et al., 2012; Zimmermann and Mcdevitt, 2014) and VEGF (Potapova et al., 2007), the latter mediated by activation of the ERK/AKT signaling pathway (Lee et al., 2012).

In vitro, conditioned medium produced by MSC spheroids impairs the production of TNF- α , IL-6, IL-12p40, IL-23, and CXCL2 by activated macrophages and increases the production of anti-inflammatory cytokines IL-1ra and IL-10, as compared to the effect of conditioned medium produced by monolayer-MSC cultures. Increased levels of TSG-6 and COX-2, lead to high PGE-2 production and conversion of proinflammatory M1 macrophages into anti-inflammatory M2 macrophages (Ylöstalo et al., 2012). *In vivo*, MSC spheroids have been shown to decrease neutrophil activity and the secretion of proinflammatory molecules, such as TNF- α , IL-1 β , CXCL2 and PGE₂ in mouse models of peritonitis (Bartosh et al., 2010) and of hepatic ischemia-reperfusion injury (Sun et al., 2018).

2.9.5. Cellular conditioning

As priming aim is to prepare MSC for some specific function, and adapting their physiology to better face the challenge in the *in situ* context (proinflammatory milieu, hypoxia, etc), some authors have also tested to culture MSC in direct or indirect contact with PBMC (Chinnadurai et al., 2018; Crop et al., 2010b, 2010a), which are cells that MSC will encounter when administered to patients. Stimulation of MSC with PBMC allows to study the effect of a range of inflammatory cytokines that are associated with immune responses.

Only one study analyzed the effect of co-culturing non-stimulated PBMCs with MSC for relatively long periods (7 days). Results showed that after direct or transwell coculture systems, MSC significantly increased the expression of IL-8, IL-6, TNF- α , VEGF- α , and basic Fibroblast Growth Factor (bFGF). In addition, the anti-inflammatory factor IDO was highly increased, while no IL-2, IFN- γ , and IL-10 expression were detectable (Crop et al., 2010b). When PBMC activated by a mixed lymphocyte reaction (MLR) were used to condition MSC, changes were identified in the morphology, gene expression, and function of MSC. MLR induced growth of MSC in a star-shaped clustered distribution instead of a typical monolayer distribution and MSC presented higher proliferation rate when

compared to control condition. Regarding gene expression, MSC cultured with PBMC activated by MLR presented increased levels of COX-2, which may result in increased production of anti-inflammatory PGE2, and could explain the improved capacity to inhibit the proliferation of mitogen- or alloantigen-stimulated lymphocytes. IL-6, IL-8, IL-1b and IL-33 expression were also increased in these cells, while only moderate HLA expression was reported (Crop et al., 2010a).

Chinnadurai and colleagues studied the effect of PBMC activated with Staphylococcal enterotoxin B (SEB), and instead of healthy controls they studied GVHD and Crohn disease's MSC. Through restricted secretome analysis and selective genomics, they described effector pathways of MSC that are regulated by activated PBMCs (Chinnadurai et al., 2018). Results showed that expressions of CXCL9 and CXCL10 were upregulated by MSC upon interaction with PBMCs, and were correlated with T cell suppression. In addition, they showed that VEGF, HGF, and CCL2 are constitutively secreted by MSC and are not modulated by SEB-activated PBMC, and that VEGF and CCL2 could predict T cell suppression by MSC. Finally, this study proposed potential interesting MSC potency readouts.

3. Cellular therapy for Myasthenia Gravis

A few studies have analyzed the use of MSC for the treatment of MG. Most of them were carried out in EAMG rodent models (Kong et al., 2009a, 2009b; Sudres et al., 2017; Yu et al., 2010), and only one was conducted in humans (Gabr and Abo Elkheir, 2016). Sources of MSC used in the studies included BM, AT and dental pulp.

The first preclinical study was published by Kong et colleagues in 2009 using EAMG rats immunized with a synthetic peptide mimicking the rat AChR α subunit. *In vitro*, the authors showed that rat BM-MSC were able to inhibit the proliferation of AChR-specific T and B cells obtained from immunized animals. Treatment also reduce the production of Ig, decreased the Th1 and Th17 subsets, as well as the secretion of their hallmark cytokines (IFN- γ , IL-6, IL-17), and increased the Th2 and Treg subsets (Kong et al., 2009a, 2009b). These effects were mediated, at least partially, by the secretion of TGF- β (Kong et al., 2009a) and IDO (Kong et al., 2009b). *In vivo*, IV infusions of syngeneic BM-MSC improved significantly the clinical status of animals and induced important weight gain. They modulated Th subsets as observed *in vitro* and reduced IL-6 levels in serum. The authors concluded that MSC injection can ameliorate the severity of EAMG by interfering with autoreactive cell's proliferation and by normalizing Th subsets. Benefits were associated to production of TGF- β and IDO. In this MG context, the use of MSC appears to represent a promising therapeutic strategy.

However, as these results were obtained using rat MSC, questions remained regarding human MSC (hMSC) efficacy in MG treatment. Yu and colleagues were the first group to test hMSC cells in an EAMG mouse model. C57BL6/J mice were immunized with a peptide analogue to the *Torpedo marmorata* AChR α subunit, and were treated twice with 1×10^6 foetal human BM-MSC. Similar to the results shown by Kong et al., hMSC could definitely inhibit the proliferation of AchR-specific lymphocytes, *in vitro* and *in vivo*. Soluble factors and cell–cell contact may be involved in the inhibiting process (Yu et al., 2010). The authors also evidenced decreased auto-Ab levels in mice sera, which may explain the improved clinical symptoms. Additionally, Ulusoy et al. demonstrated that benefits of hMSC could be extended to other EAMG mice models, such as the one induced using the extracellular domain of human MuSK protein (Ulusoy et al., 2015). In this model, IV administration of dental pulp MSC reduced lymph node cell proliferation in response to MuSK stimulation, circulating levels of anti-MuSK IgG and their deposit at the NMJ. It improved clinical symptoms of MuSK-related EAMG, presumably through suppression of CD11b+ cells. Altogether, these findings suggest that xenogeneic MSC (hMSC) were as efficient as rat MSC in mitigating the clinical signs of mice EAMG.

Despite the encouraging results, the conditions in which the cells were tested were limited. The EAMG model is obtained using a vaccination-mimicking strategy, and is well-suited for the study of auto-Ab action. However, the characteristic MG immune dysregulations and thymus abnormalities are missing. The development of humanized mouse model (Schönbeck et al., 1992; Spuler et al., 1996; Sudres et al., 2017) would give answers about MSC impact on other MG pathophysiological features. Indeed, Sudres and collaborators developed a humanized MG mouse model called NSG-MG (described in detail in the section 1.8.3) and tested the effect of AD-MSC conditioned with PBMC (cMSC) or not conditioned (resting MSC, rMSC) (Sudres et al., 2017) injected intravenously, as compared to the injection of vehicle alone. cMSC infusion reduced anti-AChR Abs in the serum of NSG-MG mice and the proliferation of human cells in peripheral organs, and increased AChR expression at the NMJ. MSC treatment reduced and delayed the occurrence of MG symptoms and improved clinical status. cMSC effects were stronger compared to rMSC ones. The authors proposed potential mechanism to explain the ameliorations : (1) the down-regulation of genes involved in inflammation and autoimmunity (TNF- α), in germinal center formation and maintenance (CD40, CD40L, PD-L1) and in B cell activation (BAFF), within the grafted thymic fragments; (2) the up-regulation of DAF (decay-accelerating factor, CD55), a regulator of the complement that limits MAC damage and modulates T cell activation in the thymus and lymphoid organs. These mechanisms are illustrated in **Figure 45**.

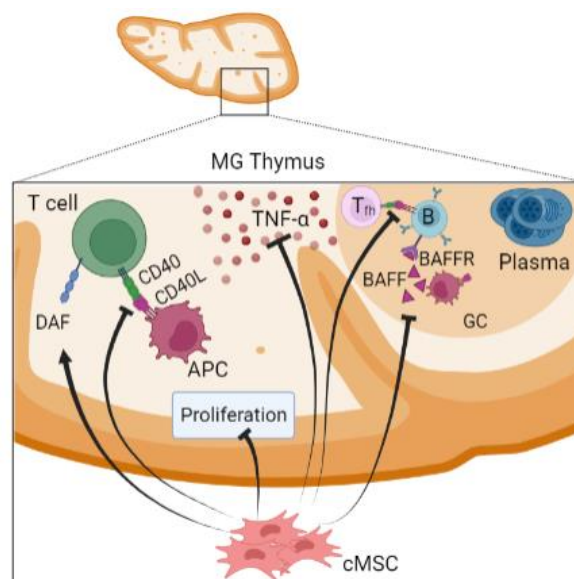


Figure 45. Schematic diagram summarizing the immunosuppressive effects of cMSC in the NSG-MG model

The inhibition of TNF ligand (i.e., TNF- α , BAFF, and CD40L) mRNA expression in the human thymus may occur in T cells or antigen-presenting cells (APCs). In addition to anti-inflammatory effects, conditioned mesenchymal stem cells (cMSC) modulate accessory molecules. The mRNA expression of the costimulatory molecule CD40 is reduced and the mRNA expression of the coinhibitory molecule CD55 is augmented in comparison with the untreated group. In addition, the inhibition of the CD40-CD40L interaction in B cells may explain the observed reduction of AChR-specific Abs in the serum of treated mice. In the thymus cellular proliferation is decreased. TFH: T follicular helper cell; B, B cell; GC, germinal center. Adapted from (Sudres et al., 2017).with Biorender.

In clinical practice, only one study investigated the benefits of treating patients with MSC in the pure context of MG. The study was performed in Cairo, Egypt and reported only as an oral presentation at the 22nd Annual ISCT Meeting (2016) (Gabr and Abo Elkheir, 2016). The trial enrolled 7 refractory MG patients; each of them received two IV doses of 200×10^6 cells at 3 months of interval and were followed monthly for one year. Improvements in MG condition were tracked using clinical examination and quantitative MG score assessment. The authors reported significant improvement of the MG score starting from first month after injection. The improvement was maintained for the whole follow up period in 5 out of the 7 patients.

Additionally, there were 2 reported cases of MG patients presenting with concomitant diseases, and that benefited from MSC treatment for the latter condition. One MG patient was diagnosed with amyotrophic lateral sclerosis and frontotemporal dementia and received, in a compassionate context, autologous BM-MSC. Transplantation in this patient improved both cognitive and motor disabilities (Petrou et al., 2014). The other case is more recent, since a MG patient received 4 doses of 500.000 MSC/kg of body weight, every other day, for treating a COVID-19 cytokine storm. The authors reported reduction in the cytokine levels and significant improvement after the treatment, and suggested that MSC may be helpful for COVID-19 treatment in autoimmunity context (Olano et al., 2022).

Thesis objectives

MG is an autoimmune disorder mediated by auto-Ab responsible of a breakdown in the communication between the nerve and the muscle, leading to fluctuating fatigable weakness. While the muscle is considered as the target organ, the thymus is implicated in the outset of most AChR⁺ MG cases. The pathogenesis of MG involves the hyperactivity of various innate and adaptive immune cells, activation of complement system, deficiency of immunomodulation mechanisms and impairment of immune homeostasis.

MG treatment combines symptomatic drug therapy, immunosuppressants, thymectomy and supportive therapy. However, there are still important unmet needs for the MG patient population. Notably, 10% of patients are treatment intolerant or refractory, and 80% of patients fail to achieve complete remission despite regular treatment uptake (Mantegazza and Antozzi, 2018), while suffering severe side-effects. Thus, the development of more efficient and less toxic therapeutic options are of interest.

MSC possess broad immunoregulatory capabilities, modulating both adaptive and innate immunity by direct cell-cell contact, secretion of potent molecules, and production of extracellular vesicles. These cells and potentially interact with all the cellular and molecular immune actors. By targeting simultaneously several pathogenic mechanisms, conditioned MSC (cMSC) therapy has an excellent chance to induce beneficial long-term effects in auto-immune indications such as MG, and to become a promising therapeutic approach. Indeed, preclinical studies performed in MG mouse models, using murine and human MSC, have shown encouraging results improving mouse clinical symptoms and alleviating disease course. Our team previously demonstrated that human AD-MSc grown in FBS and transplanted either in resting state (rMSC) or after a conditioning step with PBMC (cMSC) were able to improve the biological and clinical outcomes in our NSG-MG model. Results evidenced that cMSC were much more efficient than rMSC as therapy for MG, implying that the coculture step with PBMC enhanced immunomodulatory capacities in MSC. Interestingly, the conditioning of MSC using classical and unmanipulated, non-activated PBMC is poorly known.

To move towards a clinical perspective, we should consider some general principles for the use of future cells as therapeutic products: (1) production of cells should avoid the use of animal products, considered as potential biohazards ; (2) the cells maybe easily characterized, at all steps of their production, and before the administration, and these markers may provide cut-offs for validation; (3) the biopotency of the cells may be ascertained, at least before their administration; (4) whenever

possible, mechanisms of action may be proposed; (5) whenever possible, the sources of variability should be discarded or substituted by more standardized tools.

The first immediate consequence of these requisites is that research-grade (RG-) MSC grown in FBS should be replaced by clinical grade (CG-) MSC grown in a safer growth substrate, such as human PL. Then, because phenotype and biological features of MSC can be dynamically altered by culture conditions including medium supplementation, the immunomodulation capacities of CG-cMSC should be validated *in vitro* and in the NSG-MG model. The second consequence is less immediate but may consider the replacement of the PBMC used for conditioning, which harbour biological variability, by a standardized tool.

This doctoral project aims first to characterize the phenotype, to assess the functional capacities and to propose mechanisms of action of CG MSC conditioned by PBMC. We will therefore propose signatures. Secondly, the secretome of cells and cocultures will be explored to propose molecular cocktail for replacing the use of PBMC. To achieve the general objective, the following tasks were performed:

(1) We characterized and compared RG-MSC and CG-MSC profiles and identified some changes induced by FBS replacement by PL. The phenotype of both cell types was screened, in resting state, for the expression of large number of extracellular markers, using flow cytometry and correlation of their general phenotypic profile was studied.

We then (2) explored the effects and mechanisms deployed by the interactions between PBMC and MSC, as compared to the effect of IFN- γ , used as a control at cellular and molecular levels. Therefore, (2A) we assessed the reciprocal interactions between MSC and PBMC *in vitro*, as reflected via modifications in MSC gene expression levels. Through RNAseq study we analyzed the overall (new) immunomodulatory pathways induced by PBMC coculture step and searched for deregulated genes classified as membrane-associated proteins as candidate markers for characterizing MSC phenotypic profile. We confirmed genes signatures by qPCR. (2B) We assessed the modifications of phenotypical extracellular markers expression in MSC using flow cytometry and analyzed subpopulation changes in both MSC and PBMC upon interaction and activation using mass cytometry (CyTOF). (2C) We analyzed and compared the secretome of PBMC and MSC, either alone, in coculture, and after conditioning using a proteomic approach (Proseek Multiplex, by Olink) to identify the main proteins responsible for conditioning effect.

(3) We assessed the functional efficacies of cMSC and their supernatant, *in vitro*, through their capacity to inhibit the proliferation of activated T cells and to induce Treg.

(4) We evaluated their efficacy, *in vivo*, using our NSG-MG animal model.

Setting up multi-approaches analysis using various strategies, we obtained complementary validations of the phenotypical, immunological, and mechanical traits, allowing to propose phenotypic, genic, proteic, functional signatures and new potency markers. This will help the comprehension of the mechanisms of action, and to design cut-offs, readouts and criteria for production and quality control of MSC, simply conditioned by PBMC.

II. RESULTS

Conditioning of mesenchymal stromal cells by peripheral blood mononuclear cells triggers original mechanisms of immunomodulation

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Abstract

Mesenchymal Stromal Cells (MSC) are multipotent progenitors presenting immunomodulatory capacities that can be enhanced by a co-culture step with peripheral blood mononucleated cells (PBMC), making them attractive tools for the treatment of autoimmune diseases. MSC derived from adipose tissue were conditioned by PBMC (cMSC), left untreated (rMSC), or activated by IFN- γ (γ MSC) and were characterized and compared for their gene expression profiles, phenotypes and functional capacities *in vitro* and *in vivo*. Gene expression study by RNAseq showed differential expression of 244 genes between rMSC and cMSC, while 2089 and 3614 genes were differentially expressed when comparing γ MSC with rMSC and cMSC, respectively. Validation analysis suggested that the immunomodulation mechanisms of cMSC involved *CCL11*, *DPP4*, *ICAM1*, *IL6*, *PCD1LG2*, *PTGS2*, *TNIP1*, *TNIP3* and *ZC3H12A*, while γ MSC involved *CCL8*, *CXCL9*, *CXCL10*, *CXCL11*, *CD74*, *IDO*, *TGF- β* . RNAseq also suggested phenotypical markers distinguishing cells of different conditions. Flow cytometry analysis showed that in cMSC, the expression of CD26, CD54, CD273 and CD318 was increased, CD55 decreased, and HLA molecules were not modulated. In contrast, major changes in MSC phenotype were induced by IFN- γ activation (increased CD54, CD73, HLA molecules, CD317, reduced CD59). Single cell clustering by mass cytometry suggested cMSC and rMSC proximity and underlined the phenotypic alterations induced by IFN- γ . The secretome analysis provided 22 candidate molecules involved in conditioning by PBMC, and 40 molecules involved in cMSC immunomodulatory mechanisms. *In vitro*, cMSC supernatants were the only ones able to reduce proliferation of activated T cells by at least 50%. Finally, cMSC were challenged in a humanized MG mouse model, and cMSC-treated mice presented MG scores lowered by 50% compared to untreated mice from 2 weeks post-injection. This work unveiled treatment-dependent phenotypic and transcriptomic signatures and shed light on molecular actors involved in cMSC immunomodulation capacities upon cellular conditioning.

Introduction

Mesenchymal Stem/Stromal Cells (MSC) are non-hematopoietic, multipotent progenitor cells that can be isolated from various somatic human adult tissues. Their immunoregulatory capabilities affect both adaptive and innate immunity through cell-cell contact, soluble mediators and the production of nano-sized vehicles acting as intercellular messengers (Andreeva et al., 2017; Baharloo et al., 2020; Ben-Ami et al., 2011; Ménard and Tarte, 2013). They can reduce B and NK cell responses, act on monocytes and macrophages polarization and inhibit dendritic cells maturation (Andrzejewska et al., 2019; Benvenuto et al., 2015; Negi and Griffin, 2020). MSC also broadly suppress T-cell activation and proliferation *in vitro* via soluble mediators and reduce the ability of antigen-presenting cells to exercise their role and to produce pro-inflammatory cytokines (Djouad et al., 2007; Hof-nahor et al., 2012; Melief et al., 2013), therefore exerting indirect immunosuppressive effects on B and T lymphocytes.

Efficient MSC can be prepared in large numbers from adult adipose tissue and benefit from international consensus regarding the production and validation for clinical trials (Galipeau et al., 2016; de Wolf et al., 2017). Animal additives (especially fetal calf or bovine serum) are replaced by platelet lysate (PL), which influences their growth, some phenotypic and functional characteristics and their level of activation (Loisel et al., 2017; Menard et al., 2013; Reis et al., 2018b; Viau et al., 2019) but reduces the inter-donor variabilities (Guess et al., 2017; Menard et al., 2013; Reis et al., 2018b). MSC-based clinical trials targeting autoimmune conditions (systemic lupus erythematosus, MS, rheumatoid arthritis, Sjögren syndrome) (Leyendecker et al., 2018; Squillaro et al., 2016) provided mixed results, until the deep revisiting of concepts regarding cell preparations, indications, and patient stratifications, and promising clinical results were achieved recently (Daumas et al., 2022; Galipeau and Sensébé, 2018b; Panés et al., 2018). Effective application of MSC to temper auto-immune reactions may require potentiated and immediate immunosuppressive effects.

To become strongly immunosuppressive, MSC can be conditioned by peripheral blood mononuclear cells (PBMC) or primed by factors such as IFN- γ , TNF- α or interleukins (Ben-Ami et al., 2011; Buyl et al., 2020; Chinnadurai et al., 2018; Guan et al., 2017a; Guess et al., 2017; Kim et al., 2018a; De Witte et al., 2017). These molecular licensing approaches usually require supra-physiological doses of agents and do not induce the full mechanistic spectrum of immunomodulation by MSC since they use only restricted specific paths, while the conditioning by naïve, healthy PBMC seems a more physiological modality whose mechanism is largely unknown.

Autoimmune diseases (AID) are important causes of morbidity and mortality in occidental countries (Wang et al., 2015b). Among them, *Myasthenia Gravis* (MG) is mediated by pathogenic antibodies (Ab)

targeting the neuromuscular endplate (mainly the acetylcholine receptors, AChR), and is associated with severe defects in immune regulation, chronic cell activation, and inflammation (Berrih-Aknin and Le Panse, 2014; Hughes et al., 2004; Vilquin et al., 2019). The thymus is frequently involved in the pathophysiology (Truffault et al., 2017b). Not only regulatory T cells are deficient (Balandina et al., 2005), but effector cells are resistant to suppression (Ben-Ami et al., 2014; Gradolatto et al., 2014). T cells from MG patients exhibit a Th1/Th17/Tfh signature involving several pro-inflammatory cytokines, with TNF- α and IL-17 playing a key role in this process (Gradolatto et al., 2014). MG leads to abnormal fatigability and can be life-threatening. Despite symptomatic treatments with acetylcholinesterase inhibitors, immunosuppressants and new biotherapies, there is still no curative treatment for this invalidating disease and the life-long medications, especially glucocorticoids, have serious deleterious side-effects affecting several tissues and organs (Gilhus et al., 2011).

The classical animal models of MG are based on active immunization with exogenous AChR, or passive transfer of Ab (Tuzun et al., 2015). Beneficial effects of MSC were shown using syngeneic MSC in rats (Kong et al., 2009a) or human MSC in mouse (Yu et al., 2010). We developed a humanized model, called NSG-MG, based on grafting pieces of MG thymus in immunodeficient mice (NOD-SCID-Gamma, NSG). These thymic fragments including germinal centers, B cells producing anti-AChR Ab, and the pathological inflammatory environment, induced most animals to develop Ab and clinical symptoms (Sudres et al., 2017). Using MSC grown in fetal bovine serum (FBS, RG MSC) we previously compared the effect of human resting MSC (rMSC) with cMSC in NSG-MG mice. The results showed that cMSC are superior to rMSC for their therapeutic effects, associating a decreased incidence of MG, a decreased level of anti-AChR Ab, and restoration of the AChR expression at the diaphragm (Sudres et al., 2017).

The conditioning of MSC using non-activated PBMC is poorly known. To elucidate the mechanisms and to consider future clinical translations, clinical grade (CG) MSC should be used and phenotypic and functional changes of the cells upon conditioning need to be identified, permitting to propose markers for their potency and their validation. We have therefore explored the mechanisms deployed in this context, as compared to the effect of IFN- γ used as a control. We first (a) validated the use of CG MSC, and assessed (b) the reciprocal interactions between MSC and PBMC *in vitro* as reflected via modifications in MSC gene expression levels; (c) the modifications of phenotypical markers in MSC and the modulation of PBMC subsets upon interaction and activation; (d) the differential secretomes produced by cells alone, in coculture and following conditioning; (e) the functional efficacy of MSC *in vitro* (f) and their efficacy *in vivo* using the NSG-MG model. We set up a multi-approach analysis of the interactions between MSC and PBMC and their consequences using complementary strategies: classical and mass cytometry, RNAseq, proximity extension assay, and preclinical evaluations. We

obtained a multi-functional validation of the phenotypical, immunological, and mechanical traits, allowing the proposal of new potency markers for CG MSC simply conditioned by PBMC.

Materials & Methods

Human samples

Mesenchymal Stromal Cells

Human adipose-derived MSC were isolated from lipoaspirates from patients undergoing cosmetic liposuction, *as res nullius*, according to the French Laws and regulations.

Research Grade cells (RG1 to RG4) were obtained from 4 different donors (Dr. Danièle Noël, Montpellier, France) (Maumus et al., 2013; Rozier et al., 2021). All individuals gave written consent and approval was obtained from the French Ministry of Higher Education and Research (DC-2009-1052). Adipose tissue was digested with collagenase type II for 1 h at 37°C and centrifuged (300 g). The stromal vascular fraction was collected and cells were filtered successively through 100 µm, 70 µm and 40 µm cell strainers (BD-Biosciences, Le Pont de Claix, France). Cells were seeded at the initial density of 4000 cell/cm² in αMEM medium (Gibco Laboratories) containing 10% FBS (PAA Laboratories, Mureaux, France), 100 µg/mL penicillin/streptomycin (Gibco, Thermo-Fisher, Les Ulis, France), 2 mmol/mL glutamine (Gibco) and 1 ng/mL basic fibroblast growth factor (bFGF) (Biotechne, R&D Systems, Lille, France) and cultured at 37°C in 5% CO₂. Upon reaching confluence, cells were trypsinized, characterized by their phenotype and trilineage differentiation potential and frozen in αMEM medium supplemented with 20% FBS and 10% DMSO (Sigma Aldrich, Saint Louis, USA). They were used at passage 1-3.

Clinical grade (CG) adipose tissue-derived MSC were collected from donors undergoing liposuction (Percy Military Hospital, Pr. Christophe Martinaud). Donors of M1, M2, M3, M4 and M5 were women aged of 42, 57, 39, 49 and 42 years, respectively. Adipose tissues were washed 3 times with PBS (Biological Industries, Kibbutz Beit-Haemek, Israel) and digested in type I Collagenase (Sigma Aldrich, Lyon, France) for 1 h at 37°C then centrifuged (200g). The stromal vascular fractions were washed with αMEM (Biological Industries) supplemented with 10% human albumin (LFB, Les Ulis, France). Isolated mononuclear cells were seeded between 10 000 and 20 000 cells/cm² and cultured at 37°C in 5% CO₂ in a medium containing αMEM, 2UI/mL Sodium Heparin (Sanofi, Paris, France) and 5% human platelet lysate (hPL) obtained from the French army blood transfusion center (Clamart, France) as previously described (Doucet et al., 2005). When reaching 80-90% of confluence, MSC were trypsinized

(Trypzean®, Sigma-Aldrich) and frozen in α MEM supplemented with 10% human albumin and 10% DMSO.

Peripheral Blood Mononuclear Cells

PBMCs used for MSC conditioning and CFSE assay were obtained from venous blood from healthy volunteer donors (EFS, Rungis, France) using the Lymphoprep density gradient centrifugation protocol (Axis-Shield, Oslo, Norway), suspended in FBS (HyClone, Utah, USA) supplemented with 10% DMSO and stored frozen at -150°C. Donors of P1, P2, P3, P4, P5 were 35, 48, 64, 20, 51 years old respectively.

MG thymus

MG thymic fragments were provided by medical collaborators with the authorization of French Ethical Committee (CCP Ile de France Paris 7, authorization ID RCB 2010-A00250-39). They were obtained from patients undergoing thymectomy at Montsouris or Cochin Hospital (Paris, France) who gave written informed consent for the use of *res nullius* samples obtained in the course of surgeries and were anonymized. Two MG female thymuses (16 and 21 years old), with no thymoma, were included.

Cell Cultures

MSC thawing, culture and expansion

Frozen MSC were thawed and washed in α MEM, seeded at a density of 4,000 to 5,000 cells/cm² in non-coated plastic vessels (Corning, Boulogne-Billancourt, France) and grown in α MEM supplemented with 100 μ g/mL penicillin/streptomycin (Gibco) and either 10% FBS (Hyclone) and 1ng/mL of bFGF for RG-MSC, or 5% PL (MacoPharma, Tourcoing, France) and 2U/mL of heparin (Sigma Aldrich) for CG MSC. Adherent cells were cultured with regular change of medium and passaged once using 0.05% Trypsin/EDTA (Gibco). They were harvested at the end of passage 1 (80% confluence) and frozen at a concentration of 1×10^6 cells/mL in α MEM medium supplemented with 20% FBS and 10% DMSO.

MSC conditioning or priming, production of supernatant and conditioned media

In vitro, MSC conditioning consisted of a 3-day coculture with allogeneic PBMCs and priming consisted in 2 days activation with IFN- γ . Briefly, passage 2 CG MSCs were thawed and seeded at density of 4000 cells/cm² for 4 days, then plated into 6-well plates at 4000 cells/cm² in growth medium. Four days later, as MSC reached a density between 30 000 and 40 000 cells/cm², the wells were equipped with Transwell membrane cell culture inserts (1 μ m pore size, Becton Dickinson, Rungis, France) and thawed allogeneic PBMCs (2,5:1 PBMC to MSC ratio) were added to produce conditioned MSC (cMSC) or

medium alone to produce resting MSC (rMSC). In parallel, 6-well plates of MSC grown as above were incubated with IFN- γ for 48h (500 UI/ml, Biotechne, R&D Systems) to produce γ MSC. These concentrations and incubation lapses have been previously determined experimentally.

Upon treatment, MSC and PBMC cells were harvested. The coculture supernatant was collected, centrifuged (645g, 10 min) and frozen. The PBMC were counted and frozen. The rMSC, cMSC and γ MSC were harvested by trypsination, counted and splitted for uses in different experimental set-ups: one fraction was frozen for future mass cytometry analyses and another one for RNA extraction; one fraction was used for immediate phenotypical analysis by flow cytometry; and one fraction was seeded at 50,000 cells/cm² for production of rMSC, cMSC and γ MSC supernatants for 3 days, at which time this medium was centrifuged (645g, 10min) and kept frozen. Four MSC cultures were combined with 5 PBMC donors.

Flow cytometry phenotyping

Resting or conditioned cells were washed with PBS, and incubated with monoclonal fluorochrome-conjugated Ab (30 min at 4°C). Control isotypes were used for all types of Ig, and for all fluorochromes. At least 50.000 cells were acquired using a BD FACS Canto II cytometer (BD Biosciences) or a CytoFlex S (Beckman Coulter, Villepinte, France). Data was analyzed with FlowJo software version 10.7.1 (Tree star, Olten, Switzerland). The geometric mean fluorescence intensity (MFI) for each marker was compared among conditions and to the corresponding isotype control. A first set of phenotyping was done to compare RG cells grown in FBS or CG cells grown in PL using 60 Ab. The correlation between cultures was evaluated using the R function *corrplot* using the spearman method. A second set of phenotyping was done to compare the expression of markers by MSC grown in PL alone, upon PBMC conditioning and IFN- γ priming. A second set of Ab included the most informative ones assessed above, and markers identified through the RNAseq study. The whole Ab list is presented in **Supplemental Table 1**. Comparisons among independent MSC cultures were performed using unpaired t-test for RG and CG and ANOVA for resting and treated CG MSC comparisons. Statistical significance is recognized at $p < 0.05$.

Mass cytometry (CyTOF) phenotyping of MSC and PBMC

Design of the panel of CyTOF markers

CG MSC characterization was done using a 27 monoclonal anti-human metal-tagged Ab panel that included cell surface, cytoplasmic, and nuclear targets as detailed in **Supplemental Table 2**. Surface markers were selected after evaluation of their expression by MSC, with or without conditioning, by

flow cytometry. We retained the markers that showed important changes in FMI between conditions, and those presenting the most heterogeneous histograms (by comparing their robust coefficient of variation). Three intracellular markers (Prostaglandin-endoperoxide synthase 2 (PTGS2), indoleamine 2,3-dioxygenase (IDO1) and the proliferation-associated nuclear protein ki-67) were added to the panel as they are considered important markers of MSC conditioning. Metal-Ab matches were configured by the CyPS CyTOF platform, using the values of geometric mean established by flow cytometry. Ab were either purchased pre-conjugated from Fluidigm or purchased purified and conjugated in-house using MaxPar X8 Polymer Kits (Fluidigm, Les Ulis, France) according to the manufacturer's instructions.

Design of dedicated MSC surface barcoding

Barcoding allows gathering and analyzing of multiple samples within the same tube to reduce the technical variability of the results. As the classical barcoding method requires tough permeabilization protocols impacting the stability of extracellular antigens in our settings, we designed a gentler barcoding procedure based on CD90 expression, a well-known hallmark of MSC. In this approach, the antigen is recognized by several Ab linked to different isotopes. To limit steric hindrance, clones recognizing different epitopes of the CD90 antigen were used. Clones were coupled to distinct metal isotopes as defined in **Supplemental Table 2**, and a unique combination of 3 isotopes was attributed to each sample and allowed the study of 15 samples in the same tube.

Labeling of the cells

The methodology was followed according to (Dzangué-Tchoupou et al., 2018). Briefly, cells were thawed, washed in PBS (Maxpar, Fluidigm), suspended at 1×10^6 cells/ml and incubated with $5 \mu\text{M}$ of Cisplatin (5 min at room temperature (RT), Fluidigm). Then cells were washed in staining buffer (SB, Fluidigm). The 30 samples were splitted in 2 series of 15 samples for barcoding. Each sample was barcoded with a unique combination of 3 anti-CD90 Ab tagged with different isotopes and incubated (37°C , 40 min). After individual barcoding, samples were washed and gathered in their corresponding series and stained with cell surface antigens targeting antibodies (RT, 30 min). After washing, cells were fixed and permeabilized using the BD Cytotfix/CytoPerm kit (BD Bioscience) (RT, 60 min), and incubated with Ab directed against intracellular antigens (1 hour, RT). After washing, cells were fixed with paraformaldehyde (PAF) 1,6 % (Pierce, Thermo-Fisher) in SB (10 min, RT), washed and labeled with the DNA intercalator Iridium-125 (0,1 %, 4°C , overnight, Fluidigm) in MaxPar Fix and Perm buffer (Fluidigm) and frozen (-80°C) until acquisition. At acquisition day, cells were washed extensively in SB, resuspended with standardization/normalization Eq beads 4 elements. 200 000 to 300 000 cells were

acquired on the Helios 2 Mass Cytometer (Fluidigm). The 2 tubes were acquired successively in the same operating day.

Debarcoding and analysis of labeled MSC

FlowJo was used for data cleaning and debarcoding. For MSC, after beads removal (Ir193⁺/Ce140⁺), singlets (191Ir⁺/193Ir⁺) and viable MSC (CD105⁺/195Pt⁻) were gated manually. Boolean gating based on different metal-tagged CD90 was performed to obtain sample separation. Equal event sampling was selected, using 10 000 events per sample (the lowest common denominator across all) and each sample was saved as a separated FCS file. Non-supervised and supervised analysis were performed using Omiq software (www.omiq.ai, California, USA). First, we performed dimensionality reduction based on all markers (except for CD90, used for barcoding) and using the Optimized t-distributed stochastic neighbor embedding tool (Opt-SNE) (Max Iterations: 7500, Perplexity: 40 and Theta: 0,5), followed by single-cell data categorization/clusterization into ostensible cellular populations using ClusterX tool (Alpha: 0,001). The feature characteristics and abundance patterns of each cluster were analyzed using clustered heatmap.

Labeling of PBMC (MDIPA assay)

PBMC harvested after the coculture step and their counterparts incubated alone in culture media, for 72 hours, were frozen until all samples were prepared. When ready, samples were thawed, washed with PBS and incubated in 250U of Nuclease (Pierce, Thermo-Fisher, 37°C 30min). 1x10⁶ cells of each sample were then barcoded using the labeling of CD45 tagged to different isotopes. We used CD45 Ab tagged to Cd106, Cd111, Cd113, Cd116 (Clone HI30; Fluidigm) and pooled groups of 4 samples in a single tube containing a maximum of 3x10⁶ cells. Fc receptors of barcoded samples were blocked using Human TruStain FcX (Biolegend, Paris, France) and incubated for 10 min at RT. Cells were then stained using the Maxpar Direct Immune Profiling Assay (Fluidigm) which is optimized for labeling of 30 markers allowing the recognition of 37 PBMC populations (T, B, NK, dendritic cells, monocytes and granulocytes subsets). The list of markers included in the kit and the gating strategy is available in **Supplemental Table 3**. Briefly, FcR-blocked PBMC were diluted in cell staining buffer (CSB, Fluidigm) and stained. After 30-min RT incubation, the cells were washed twice in SB, followed by fixation in 1.6% PAF for 10 min. Then, the cells were spun to a pellet which was suspended in 1 ml of the 125 nM Cell-ID Intercalator-Ir (4°C, overnight, Fluidigm).

Immediately prior to acquisition on Helios XL (Fluidigm), samples were washed, counted and diluted with PBS mixed with EQ 4 Element beads at the CyPS core facility. A maximum of events was acquired per sample. Mass cytometry standard files produced by the HELIOS were normalized using the CyTOF

software v.6.7.1014. This method normalizes the data to a global standard determined for each log of EQ beads.

Debarcoding and analysis of PBMC

For PBMC, debarcoding was done as described for MSC and individual FCS files generated were analyzed using Maxpar Pathsetter, an automated analysis system powered by GemStone™ 2.0.41 (Verity Software House, Topsham, ME). This system is integrated with dimensionality-reduction mapping known as Cauchy Enhanced Nearest-neighbor Stochastic Embedding (Cen-se™), which generates a visual display of high-dimensional data labeled with the major cell populations. Cell subsets frequencies were compiled in tables and were used for statistical analysis.

Gene expression analysis

RNA Preparation

Total RNA from 1.5×10^6 – 2×10^6 rMSC (n=4), cMSC (n=16) and γ MSC (n=4) cells was extracted using the mirVana miRNA Isolation Kit (Thermo-Fisher). RNA concentration and purity were determined using NanoDrop ONE s (Thermo-Fisher). All samples presented ratios from 1.9-2.30 for 260/280 and 260/230. The RNA quality was assessed on gel using ARN FlashGel™ System (Lonza Bioscience, Basel, Switzerland). In samples used for RNAseq purposes (rMSC (n=3), cMSC (n=9) and γ MSC (n=3)), RNA integrity was further assessed with a Bioanalyzer 2100 (Agilent Technologies, California, USA), only samples with RNA integrity number > 9 were retained. RNA was reverse-transcribed using a concentration of 500ng/ μ L for 1h at 42°C using AMV (Roche, Merck, Fontenay-sous-Bois, France) with oligo-dT (Thermo-Fisher).

RNAseq analysis

RNA sequencing approach was carried out at the Genom'ic Core Facility at the Institut Cochin, University of Paris Descartes. Briefly, $\approx 1 \mu$ g of total RNA was isolated from cells and depleted of rRNA with the low Input RiboMinus Eukaryote System v2 (Ambion, Thermo-Fisher). The depleted RNAs were used to generate cDNA libraries according to the manufacturer's protocol (Ion total RNAseq kit V2, Thermo-Fisher). The sequencing was performed on NextSeq 500 from Illumina (Illumina, California, USA) using paired-end 150–base pair reads (at least 10 million reads per sample). After quality control of the run, adaptor and low-quality trimming and removal of contaminants, the reads were splice-aware mapped to a reference genome including annotations (GRCh38.p13, Ensembl release 101) using the STAR aligner v2.7.6a. Gene-level quantification was done with RSEM (v1.3.1). Raw counts were normalized, genes with low counts (≤ 10 in at least 3 samples) were discarded. Analysis for differential

expression between conditions, accounting for variations due to batch effect (Batch + Condition), was done fitting to a negative binomial generalized model implemented in the DESeq2 algorithm (version 1.26.0). The benefit for accounting for batch effect on the design formula was visualized by PCA, that showed variation in the data due to both batch and condition. Genes with adjusted pvalue < 0.05 were regarded as differentially expressed genes. General quality controls were performed, that showed low duplicated reads, excellent mapping percentages and complexity of the libraries (using Fastqc 0.11.9, picard 2.23.9, preseq 3.1.1, dupradar 1.18.0).

Hierarchical clustering of the top 80 most significant differentially expressed genes was performed on the normalized matrix. Distance between features was measured by (1 - Spearman correlation coefficient) and clustering was performed using the Ward.D2 method.

Annotation/research of cell markers

Differentially expressed genes were categorized according to their annotation on GO ontology at the release from 2021-09-01. Genes annotated as "integral components of membrane" (accession GO:0016021) (excluding genes products in this term that are also categorized as endoplasmic reticulum and respiratory chain components), as "catalytic activity" (accession GO:0003824), and as "extracellular space" (accession GO:0005615) were further studied.

Pathways analysis

clusterProfiler v4.2.0 package was used for all enrichment analysis. Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) was performed on the entire gene set, with a minimum size of gene set accepted of 10. All collections available in the Molecular Signatures Database (<http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp>) were queried. Redundant terms were manually removed and most interesting ones according to their biological activity were further studied.

Primers design and validation

Gene expression modulation was confirmed by qRT-PCR and a larger number of samples. We analyzed the expression of a panel of 22 candidate genes deduced from the RNAseq study as dysregulated by conditioning or by IFN- γ activation. The primer sets were obtained using primer3.ut.ee, and setting the following conditions: product size between 150-250 bp, max melting temperature (T_m) difference of 5, and an optimal primer T_m of 60°C. Primer sequences and details are shown in **Supplemental Table 4**. One μ g of RNA was reverse-transcribed for 1 h at 42 °C using AMV (Roche, Merck) with oligo-dT (Thermo-Fisher). PCR reactions were carried out in duplicates using the LightCycler 480 SYBR Green

Master Mix on the LightCycler® 480 System and following manufacturer's instruction (Roche Diagnostics, Meylan, France).

QPCR analysis

A total of 24 samples were assessed including rMSC (n=4), cMSC (n=16) and γ -MSC (n=4). The normalized expression levels were calculated according to the $\Delta\Delta C_t$ method. Briefly, ΔC_t values were determined as the difference between the cycle threshold (C_t) value of the gene of interest and the mean C_t value of the housekeeping gene GAPDH. Samples mRNA expression was normalized as specified in the figure legends. A One-way ANOVA test was performed to verify the significance of MSC gene expression modulation induced by the different treatments.

PBMC proliferation inhibition

The inhibition of leukocyte proliferation by MSC themselves or their conditioned media was evaluated using the carboxy fluorescein succinimidyl ester (CFSE) assay. Briefly, healthy PBMC were labeled with CFSE using CellTrace Cell Proliferation Kit (Invitrogen, Thermo-Fisher), re-suspended in complete media supplemented with anti-CD3, anti-CD28 T cell activation kit (Dynabeads, Gibco) and then transferred to rMSCs, cMSCs or γ MSCs containing wells in 1:10 MSC/PBMC ratio or to wells containing MSC supernatants in a concentration of 1×10^6 PBMC/mL. Non-activated PBMC and activated PBMC cultured in absence of MSC or their supernatants were used as controls. After 72 hours of culture, the percentages of CD4⁺ and/or CD8⁺ proliferating cells out of the total CD45⁺ live cells were determined using a flow cytometer by monitoring the CFSE fluorescent values. Briefly, PBMCs were harvested from each well, washed with PBS and stained with CD45-eFluor, CD4-PE, CD8-APC (Biolegend) and Fixable Near-IR Live/Dead Staining kit (Invitrogen).

To assess the role of soluble DPP4 (i.e. CD26) in the blockade of proliferation, saxagliptin (hydrochloride salt, Biotechne, R&D System), a potent inhibitor of this enzyme, was incubated in the conditioned media at 10^{-6} M for 3 days. Twenty-four conditions were tested (4 MSC cultures of either resting, activated by IFN- γ , or conditioned by 4 PBMC different donors).

Proteome samples preparation and analysis

Complete culture medium (n=2), supernatants produced by PBMC alone (n=3), rMSC alone (n=4), PBMC-MSC cocultures (n=4), and cMSC obtained after conditioning step (n=4) were harvested after cells incubation for 72 hours. Supernatants were centrifuged (650g, 10 min, 4°C) before sampling and aliquots were kept at -80°C. Samples were analyzed simultaneously for 609 unique protein biomarkers on seven pre-designed Proseek Multiplex® immunoassay panels: Cardiometabolic, Cardiovascular II,

Cardiovascular III, Development, Immuno-Response, Immuno-Oncology, Neurology (Olink Proteomics, Uppsala, Sweden). Processing, output data quality check and normalization were performed by Olink Proteomics. Raw data was normalized to the company's internal controls and delivered as Normalized Protein eXpression (NPX) values, a relative protein quantification arbitrary unit from Olink expressed on log₂ scale. Validation data and limits of detection (LOD) are available at the manufacturer's webpage (<http://www.olink.com>). Data values below LOD were removed from the dataset and proteins with >50% missing values were also excluded. Analysis of differentially secreted proteins (adjusted pvalue < 0.01) was performed using Olink® Insights Stat Analysis app (www.olink.com). Data visualization by PCA plots and heatmaps was done taking into account the 177 differentially secreted proteins and using *Clustvis* (Metsalu and Vilo, 2015), a web tool for visualizing clustering of multivariate data. Volcano plots showing potentially activating and immunomodulating proteins were prepared using all measured proteins and the *VolcanoseR* web app (Goedhart and Luijsterburg, 2020)

NSG-MG mouse model

Outline and grafting procedure

Animal experimentations have been designed to respect the 3R principles and received authorizations 02622.2 and 02638.02 under agreement of French Ethical Committees. They were performed under supervision of persons authorized by French Authorities in the animal facility of the University (authorization A751320). NOD-SCID IL-2R γ null (NSG) mice were obtained from Charles Rivers laboratories and bred in our facilities under specific pathogen-free conditions. Mice aged of 14 to 30 weeks were anesthetized (80 mg/kg ketamine and 4 mg/kg xylazine) and subcutaneously engrafted with 3 to 4 pieces of freshly collected human MG thymic biopsies following Sudres et al. protocol (Sudres et al., 2017). Sham-operated animals were used as controls. Clinical evaluation of mice was performed once a week and blood samples were obtained fortnightly. Globular fraction was used for humanization follow-up. Seven weeks after transplantation, the animals were euthanized and the spleen, the xenogeneic thymuses and the whole blood were recovered and used freshly for later analyses.

Mice humanization follow-up by flow cytometry

The percentage of human CD45⁺ cells in mice blood and in the spleen was assessed by flow cytometry. Briefly, after mechanical dissociation of spleen in PBS 3% FBS, cell filtration (70 μ m) and washing, obtained cell homogenates were treated with BD lysing buffer (BD biosciences) (RT, 10 min) to eliminate erythrocytes and keep splenocytes only. Same treatment was performed in blood samples

to obtain lymphocytes. After incubation, cells were washed and stained with anti-human Ab (4°C, 30 min). The list of antibodies is available on **Supplemental Table 5**. Viability of cells was assessed using the LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen). After washing, cells were resuspended in PBS, acquired using a CytoFlex S (Beckman-Coulter) and analyzed using FlowJo software.

Mice treatment

Variability was noted regarding the yield of humanization. Before randomization of the animals into the treatment groups, they were ranked according to their percentage of circulating human CD45+ cells in blood 2 weeks after grafting and categorized into 3 groups, (low, medium or high humanized mice) Treatments were distributed homogeneously inside each group. The pseudonymization of the cells was done after the step of cell culture and 2 weeks after transplantation of MG thymic fragments, 5×10^5 of rMSC or cMSC suspended in 50 μ L of isotonic NaCl, or NaCl alone, were injected into mice through the intra-caudal vein by an operator blinded to the treatment.

Clinical evaluation and scoring for NSG-MG experiments

The MG-like clinical status was assessed weekly as previously described using a composite score including behavior observation, weight loss, grip test and inverted grid test (Sudres et al., 2017; Tuzun et al., 2015). Mouse behavior was graded on a scale of 0 to 3: score 0, no sign; score 1, abnormal movements (walking with head and tail down); score 2, reduced motility and hunched posture; score 3, paralysis, dehydration or death (Sudres et al., 2017; Tuzun et al., 2015). The weight loss was scored from 0 to 3 according to the percentage of loss in grafted animals compared to sham mouse (<5% = score 0; 5-9.99% = score 1; 10-14.99% = score 2; \geq 15% = score 3). The muscle strength was analyzed by measuring the forelimb strength with a grip strength apparatus and comparing the weekly obtained values to the ones obtained during habituation (prior onset of symptoms). Muscle strength loss was scored from 0 to 3 (<10% = score 0; 10-19.99% = score 1; 20-29.99% = score 2; \geq 30% = score 3). The time mice resist to fall from inverted grid (Tr) was assessed for a maximum of 60 seconds and scored from 0 to 3 (Tr = 60 sec, score 0; Tr = 45 to 59 sec score 1; Tr = 44 -30, score 2; Tr < 30 sec, score 3). As clinical signs are not always obvious in resting mice, the grip test measurements were done after a 5-minute run on a treadmill. A weekly global clinical score was calculated as follow: Weight loss score + Strength loss score + Average obtained from (Behavior Score + inverted grid score). As the onset of the disease and the clinical signs vary according to the status of the MG donor, the clinical scores were normalized to the status at the time of cell injection.

Statistics

Differences between independent experimental groups were analyzed using GraphPad Prism 7 software. When more than 2 groups were compared, 1-way ANOVA test were used. All differences were calculated using a 2-tailed test. When only 2 groups were compared, unpaired T-test were applied. The test is specified in the figure legend. Statistical significance was recognized at pvalue inferior than 0.05. In all figures, the significance is displayed as asterisks, as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; or by pounds symbol #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 when precised.

Results

Basal characterization of research and clinical grade MSC

We assessed and compared the phenotypical profiles of MSC cultures grown using FBS (research grade experiments), or using PL (preclinical and clinical grade experiments). This first list of 60 markers tested was established in agreement with the literature, and included classical negative markers, strongly positive ones, and a series of markers known to be positive or increased under an activation process. After markers assessment by flow cytometry, the geometric mean for each one was calculated for each cell culture, and their correlations were explored using Pearson test.

The cultures of MSC prepared from different donors grew efficiently in FBS or in PL and expressed the classical sets of MSC antigens CD73, CD90, CD105, while not expressing the classically negative markers CD11b, CD14, CD34, CD45 (Supplemental Figure 1A). The labeling of most markers was remarkably homogenous, presenting a narrow FMI peak (Coefficient of Variation - CV up to 75), suggesting that most cells express the considered marker in the same range (Supplemental Figure 1B shows an example). Heterogeneities have been noted regarding some markers (CV from 75 up to 350), i.e. CD9, CD10, CD49a, CD54, CD59, CD140a, CD146, CD273, pinpointing the presence of cells with variable intensity of expression and suggesting the existence of MSC sub-populations.

A synthetic view of markers' expression is represented by a heatmap (Figure 1A) integrating the fluorescence mean intensity (FMI) for each one, and the differences observed between CG and RG cells. A large spectrum of expression intensities was observed. Twenty-one markers were not expressed in these culture conditions (white label). Then, 30 markers were expressed at low to medium intensity (red to dark orange label), and 9 were expressed at the highest intensity (from light orange to violet labels, CD59, CD49c, CD140b, CD55, CD166, CD49e, CD81, CD73, CD105). Additionally, we observed a general trend towards higher levels of expression in RG cells, i.e. using FBS, when compared to CG cells. Fifteen markers out of 60 were differentially expressed between CG and RG in a significant manner (Figure 1B). Among cell adhesion, cell-cell interaction and cell signaling molecules, significant differences were observed between RG and CG regarding CD29, CD44, CD49b, CD49c, CD49d, CD49e, CD61, CD146 and CD166. Differences have been noted regarding immunological regulators involved in complement metabolism (CD55, CD59), or in immunomodulatory mechanisms (CD73, CD105, CD200). The expression of the endopeptidase CD10 was also discriminant.

When comparing the expression of the totality of markers, we observed strong correlations within each group (i.e. PL-grown with PL-grown and FBS-grown with FBS-grown), these correlations were slightly diminished when the two categories were compared (Figure 1C, Pearson's correlation study).

However, the correlation coefficient was still very high, close to 0,8 for all cultures excepted for RG2, suggesting the overall preservation of phenotypical profile among cultures. We considered therefore that MSC grown in PL presented sufficient homogeneity to investigate their behavior and potency upon conditioning.

Figure 1

Comparison of RG and CG MSC phenotype

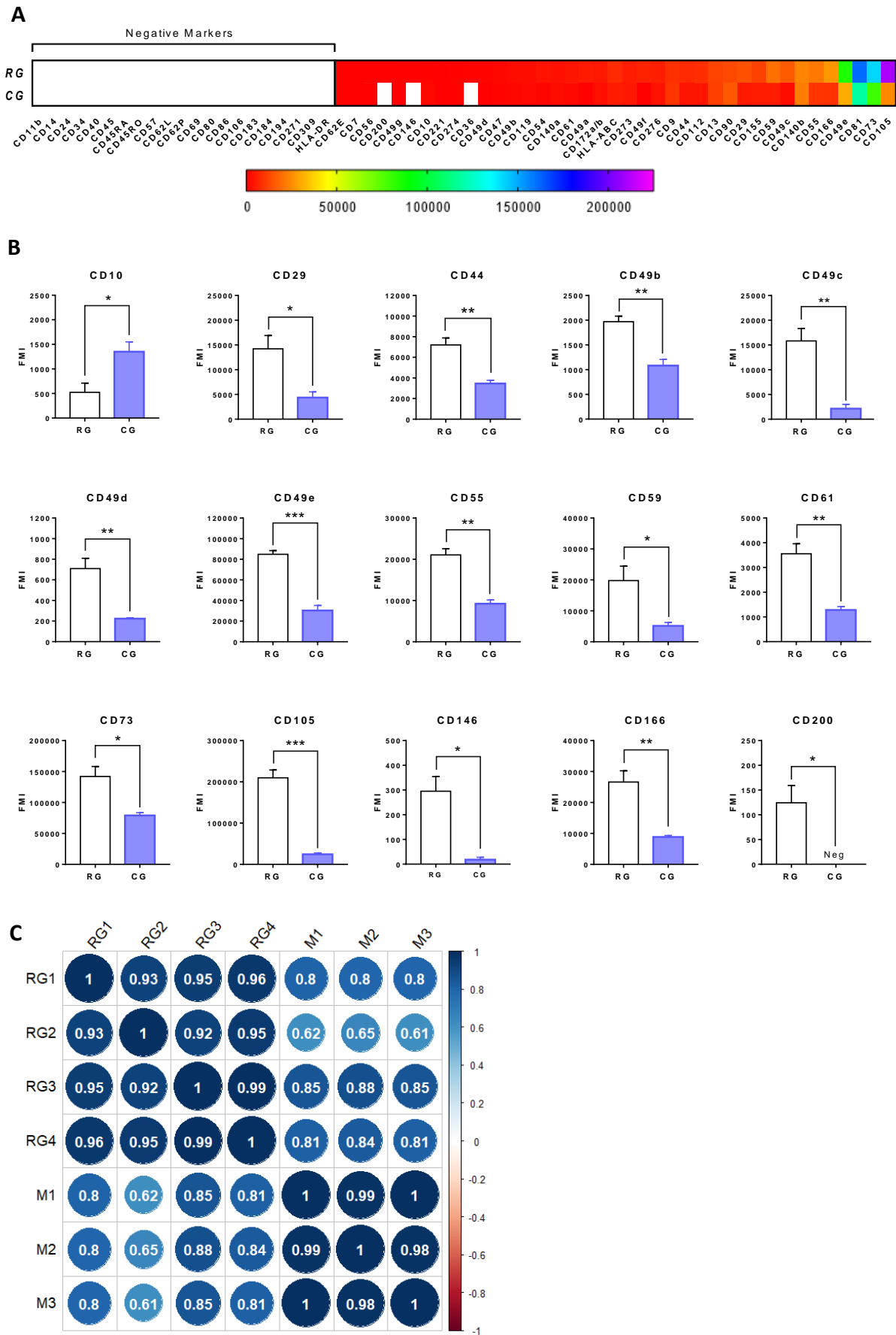
RG (n = 4) and CG (n=3) MSC cultures were analyzed for the expression of 60 extracellular markers using flow cytometry, and expression was plotted in terms of fluorescence mean intensity (FMI). (A) Heat-map integrating the mean FMI for each marker, in both culture condition. Non-expressed markers are shown in white, and the level of expression parallels the color gradient of intensity. (B) Bar plots comparing FMI levels of RG and CG cultures for the markers showing significant differential expressions. Data was analyzed using 2-tailed, unpaired Student *t* test and are presented as mean \pm standard error of the mean. **P* \leq .05, ***P* \leq .01, ****P* \leq .001. Neg: Not expressed marker. (C) Correlation matrix showing Pearson correlation coefficients between compared MSC cultures. The level of correlation parallels the color gradient of intensity. Correlation coefficients were calculated considering the expression of all tested markers and matrix was obtained using the R function *corrplot*, the maximum correlation coefficient being 1.

Supplemental Figure 1

Expression of MSC phenotypical hallmark

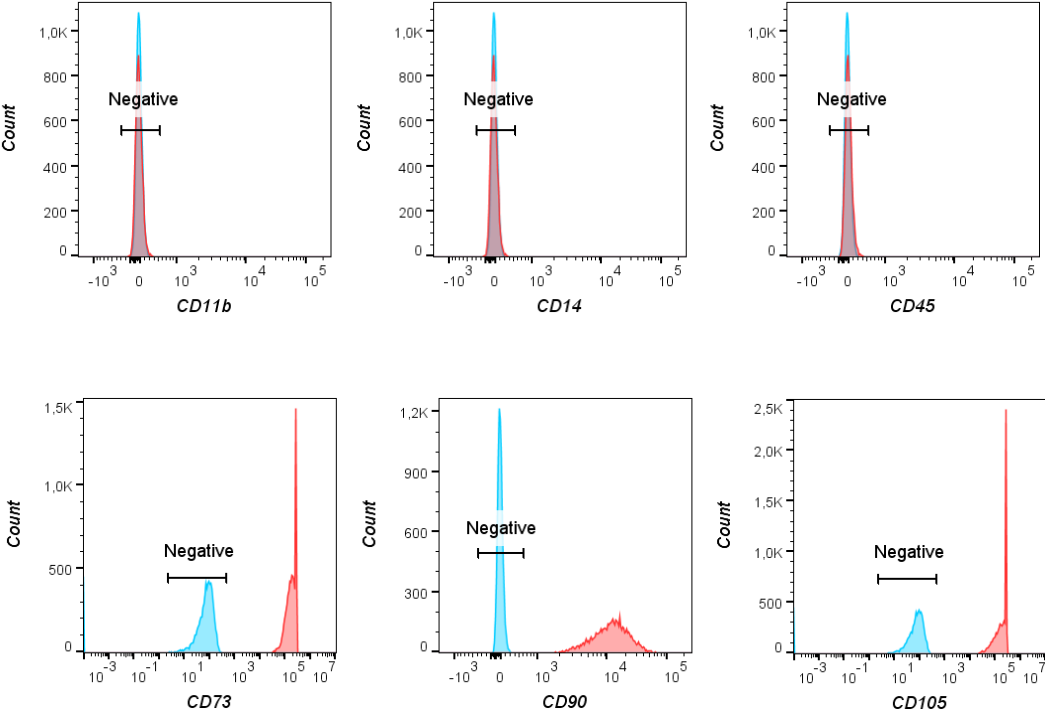
RG and CG MSC were screened for the expression of classically positive and negative MSC markers by flow cytometry. (A) Representative histograms of one MSC sample lacking the expression of CD11b, CD14, CD45 (Top histograms) and highly expressing CD73, CD90 and CD105 (Bottom histograms). (B) Representative histograms of markers with highly homogeneous expression, and presenting a narrow pic (CD166) and less homogeneous expression, presenting a larger pic (CD54). Blue pics represent MSC expression when staining with isotype controls and red ones, with dedicated antibodies. Values are expressed as FMI and vary from marker to marker.

Figure 1

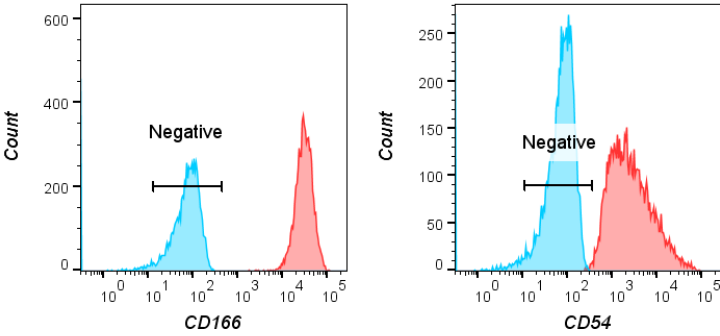


Supplemental Figure 1

A



B



Conditioning using PBMC triggers the expression of a specific panel of genes.

Primary adipose-tissue derived MSC cultures were grown for 3 days in coculture with PBMC preparations (cMSC, n = 9), activated for 48h by IFN- γ (γ MSC, n = 3), or left untreated (rMSC; n= 3) as outlined in **Figure 2A**. An RNAseq study compared genes dysregulation between these treatments.

To compare the general transcriptomic profile of samples, a non-supervised principal component analysis (PCA) was performed based on the expression of the 500 most variable genes. This first plot pointed-out the important genes' modulation induced by IFN- γ treatment, accounting for 75% of the dataset overall variability (**Figure 2B** left), which confirmed its strong effect on MSC described by several other groups. However, the analysis also pinpointed the effect of MSC donor in the analysis, as samples were regrouped mainly by this criterion in PC2, rather than cell's treatment. To demonstrate the benefit of accounting for donors, the PCA was re-plotted after removing donor effect (**Figure 2B** right). In this new representation, IFN- γ still represents the most important factor explaining our data set variability, but PC2 reflects now the effect induced by cellular conditioning in samples gene expression profile. Although PBMC 1 and 2 induced the same range of effects, PBMC 3 induced a more dramatic change, suggesting that the magnitude of conditioning is dependent on the PBMC donor.

To understand changes in gene regulation in MSC upon conditioning with PBMC or IFN- γ , we identified differentially expressed genes with an adjusted pvalue < 0.01. As shown in the Venn diagram (**Figure 2C**), when compared to the resting state, the number of genes dysregulated by IFN- γ was much higher (2089) than the number of genes dysregulated by PBMC conditioning (244). From them, 1103 and 124 genes were up-regulated, and 986 and 120 genes were down-regulated by IFN- γ and PBMC conditioning, respectively (data not shown). Only 52 differentially expressed genes (DEG) were common for both treatments. When comparing cMSC and γ MSC gene expression, the number of dysregulated genes was even higher (3614), the proportion of up- and down regulated genes were similar as described earlier, with 1838 genes up and 1776 down respectively. Together, these observations suggest treatment-dependent transcriptomic signatures and a molecular specificity of PBMC conditioning.

To visualize gene modulation by each treatment, volcano plots were made and representation cut-offs were set horizontally at $-\log_{10}(0.05)$ and vertically at $\log_2(1.5)$ (**Figure 2D**), the top 10 modulated genes with highest padj value were annotated. Results showed that IFN- γ induced strong highly significant dysregulations, when compared to rMSC transcriptomic profile, with modulations reaching values > 5 for \log_2 fold-change and > 100 for $-\log_{10}$ padj. Top 10 genes included those classically described for their involvement in classical IFN- γ pathways and related to host defense (*HLA*, *CD74*,

APOL1, IFITM1, IFI6, GBP2, LAP3, PLAAT4, WARS1, IRF1). On the other hand, the PBMC conditioning induced more subtle changes (log₂ fold changes between 1 and 5 and -log₁₀ padj < 10) and the most dysregulated genes were not involved in resistance to virus and bacteria, but in matrix modeling (*AOC3, PRR33*), migration (*AOC3, CEMIP*), cytokine production (*GPR176, CRLF1*), proliferation (*MCM4, WNT11, PKNOX2*) and chaperone protection (*CLU*).

Heatmap of the 80 most differentially expressed genes comparing samples of each pair of conditions (rMSC vs cMSC, γ MSC vs rMSC and cMSC vs γ MSC), underlined the homogeneity of gene expression within groups, and allowed a well-established segregation between treatments (**Supplemental Figure 2A-C**). Only few genes were shared by both treatments.

We then focused on genes coding for membrane-linked proteins in the list of DEG for each treatment, using a dedicated filter. When compared to resting, cMSC were found to express higher levels of *CD54, CD318, CD120b and CD26* (log₂ fold-changes from 2.1 to 0.8) and decreased levels of *CD344, CD143, CD271*. As expected, IFN- γ -treated cells expressed higher levels of *CD74, CD212, CD317, CD7, CD225, CD32a, CD257, CD353, CD253, CD274, CD215, CD40, CD106, CD136, CD270 and CD54* (log₂ fold-changes from 2.5 to 9.3) (data not shown). These lists provided several potential candidates to assess comparisons between treatments, at both transcriptomic and proteomic levels, if one assumes that gene expression is further reflected by differential protein levels at the cell membrane.

Among the genes that were highly dysregulated by conditioning, IFN- γ priming or both, as shown by our preliminary RNAseq study, we selected 22 candidates and analyzed their expression levels by quantitative RT-PCR. Gene expression was normalized to the housekeeping gene GAPDH and expressed as rMSC relative mRNA expression. We observed differences in gene expression modulation among the different conditions, and we could describe 5 scenarios, according to the type and the intensity of the modulation (**Supplemental Figure 2D**): (1) Up-regulated genes upon PBMC conditioning (*CCL2, CCL11, DPP4, IL6, PDCD1LG2, TNFRSF11B, TNIP1, TNIP3, and ZC3H12A*); (2) Down-regulated genes upon PBMC conditioning (*CILP, LGALS1*); (3) Up-regulated genes upon IFN- γ priming (*CCL8, CD74, CXCL9, CXCL10, CXCL11, HLA-DR, IDO1, TGFB1 and TNFAIP3*); (4) gene upregulated by both treatments (*ICAM1*); (5) gene with equivalent expression in all conditions (*PTGS2*).

These results confirm the effects produced by IFN- γ in cultures of MSC cells. The highest increases are noted regarding the group of CXCL chemokines, *HLA-DR* and *CD74*, and *IDO1* which is considered a central player in the anti-bacterial, antiviral and immunomodulatory roles of IFN- γ . Additionally, the genes pinpointed by the RNAseq study as involved in PBMC conditioning are validated by our PCR analysis (*CCL2, CCL11, DPP4, IL6, PDCD1LG2, TNFRSF11B, TNIP1, TNIP3 and ZC3H12A*).

Because the genes regulated by IFN- γ and PBMC conditioning are not always the same, these results help to delineate the mechanisms of action of the conditioning by PBMC.

Finally, we studied the functional and molecular mechanisms at play. For this, Gene set enrichment analysis (GSEA) were performed and enriched pathways were identified using $p_{adj} < 0.05$ as cut-off. The most informative databases were Reactome and GO for its “Biological process” category. The 10 most pertinent pathways for each treatment and on each category were represented as bar plots (**Figure 2E**). Unsurprisingly, principal enriched pathways in γ MSC included IFN- γ signaling, NF- κ B signaling, inflammatory response, signaling by interleukins and those that mimic the anti-viral response. Interestingly, these cells down-regulated pathways related to ECM remodeling. In cMSC, enriched pathways included those related to an active cellular and metabolic state (DNA replication, rRNA processing and translation), to ECM remodeling and migration (ECM disassembly, activation of MMP, cells’ chemotaxis and migration), to paracrine and autocrine communication (signaling by several different interleukins and chemokines) and to immunomodulation (regulation of defense response, positive regulation of inflammatory response).

As described, enriched pathways were frequently different, depending on the treatment, and this suggests different or complementary mechanisms exerted by PBMC or IFN- γ .

Figure 2

Gene signature of MSC under different treatments

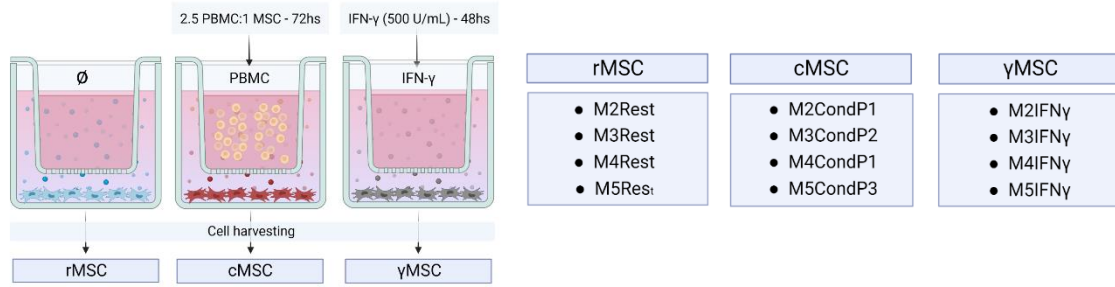
RNAsequencing analysis and RT-PCR were conducted on CG MSC under different treatments. (A) Schematic representation of the different cell samples used for RNAseq study: non-stimulated MSC (Resting MSC or rMSC), MSC obtained after coculture with PBMC for 72h (Conditioned MSC or cMSC) and MSC after stimulation with 500U/ml of INF- γ for 48h (γ MSC). (B) PCA analysis of 500 most variable genes in the transcriptome before (left) and after (right) removal of unwanted batch effects from donor using the *removeBatchEffect* from the *limma* v3.50.3 package. (C) Venn diagram showing the number of shared and unique differentially expressed genes (DEG) found between compared conditions. The total number of DEG is shown in bold, over each diagram label. (D) Volcano plots visualization of RNAseq data comparing γ MSC versus rMSC (left) and cMSC versus rMSC (right). The top 10 DEG with highest padj were annotated. Padj = p adjusted value. (E) Most pertinent up- or down-regulated pathways, determined from GSEA. Each plot displays pathways in Reactome and Gene Ontology Biological process (GO_BP) collections in cMSC (top) and γ MSC (bottom) when compared to rMSC. Bar height represents gene ratio and bar color the padj value.

Supplemental Figure 2

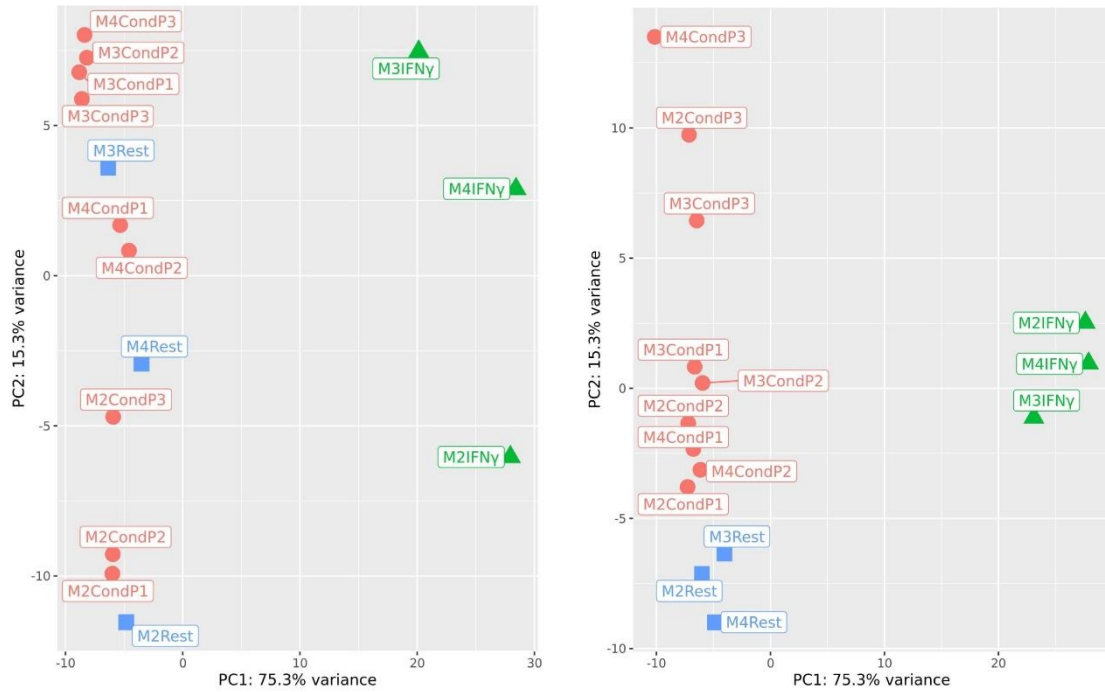
Heat map representing the top 80 DEG between (A) rMSC and cMSC, (B) rMSC and γ MSC and (C) cMSC and γ MSC. These maps display the names of the genes as well as the extent of the expression from low (green) to red (high). (D) Gene expression assessment by RT-PCR. Gene expression was first normalized to the reference gene GAPDH and then expressed as mRNA expression relative to rMSC of each culture. The graphs are grouped in categories, according to the expression of the given gene under the effect of treatment. Data was analyzed using One-way ANOVA and are presented as mean \pm standard error of the mean. * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, **** $P \leq .0001$.

Figure 2

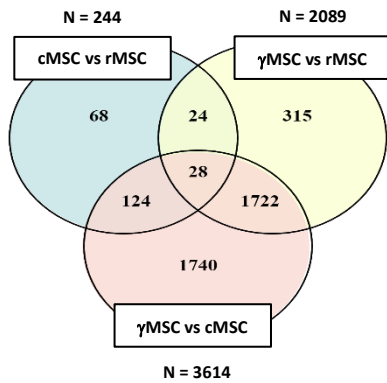
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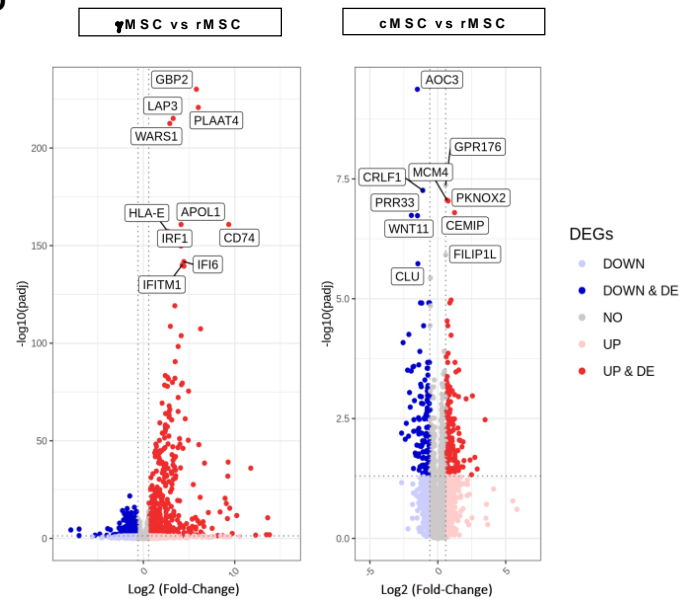
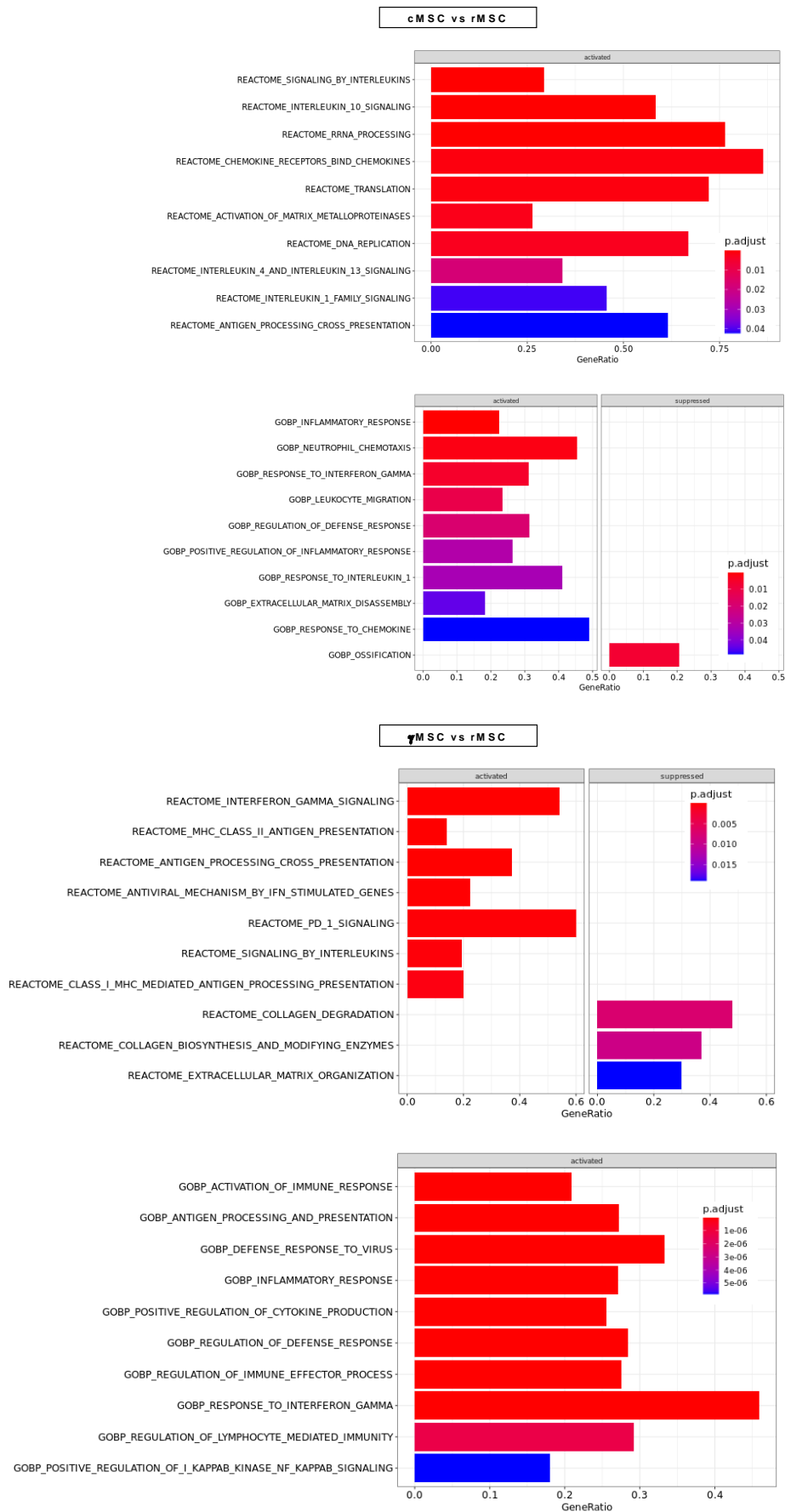


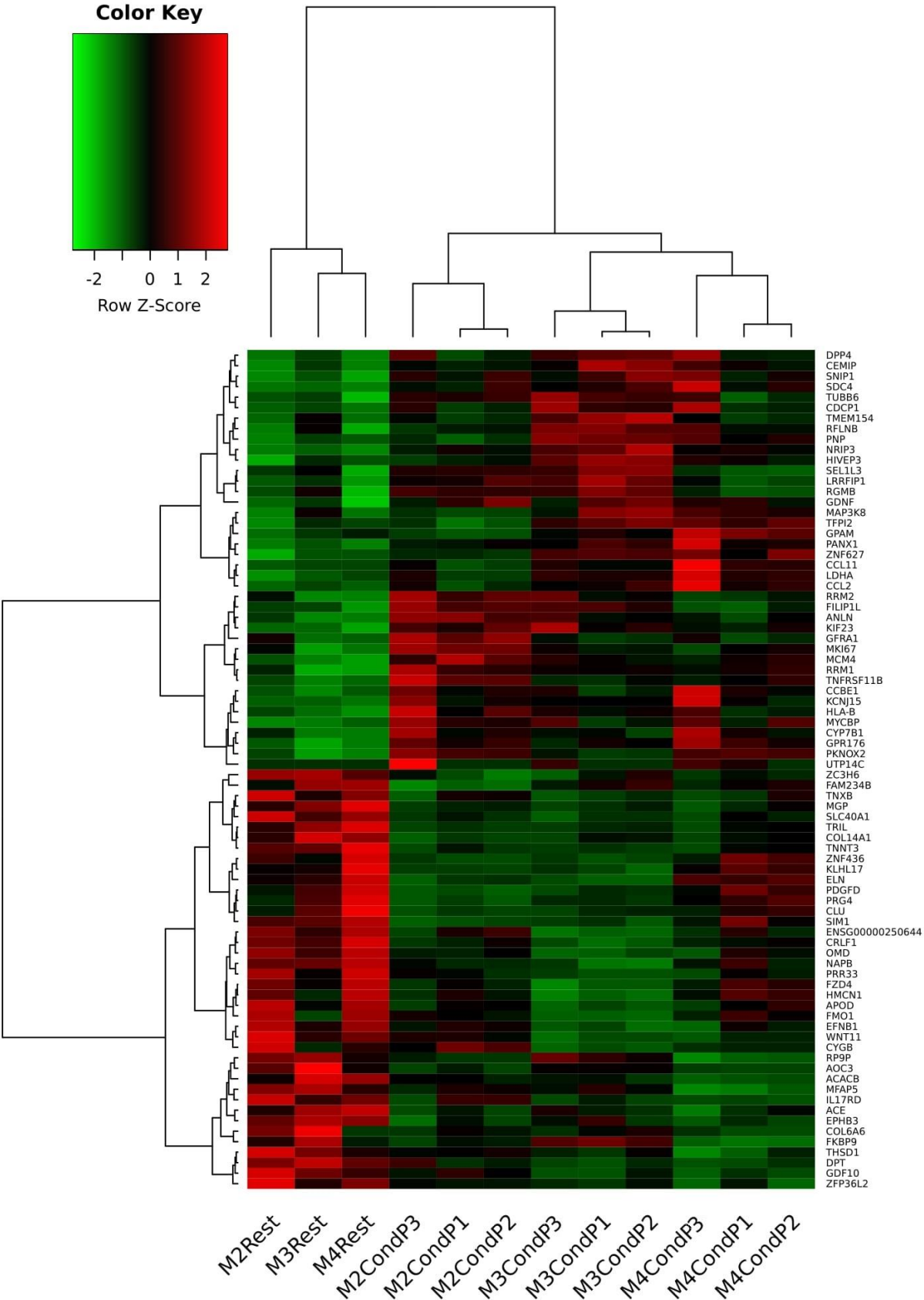
Figure 2 (Continuation)

E



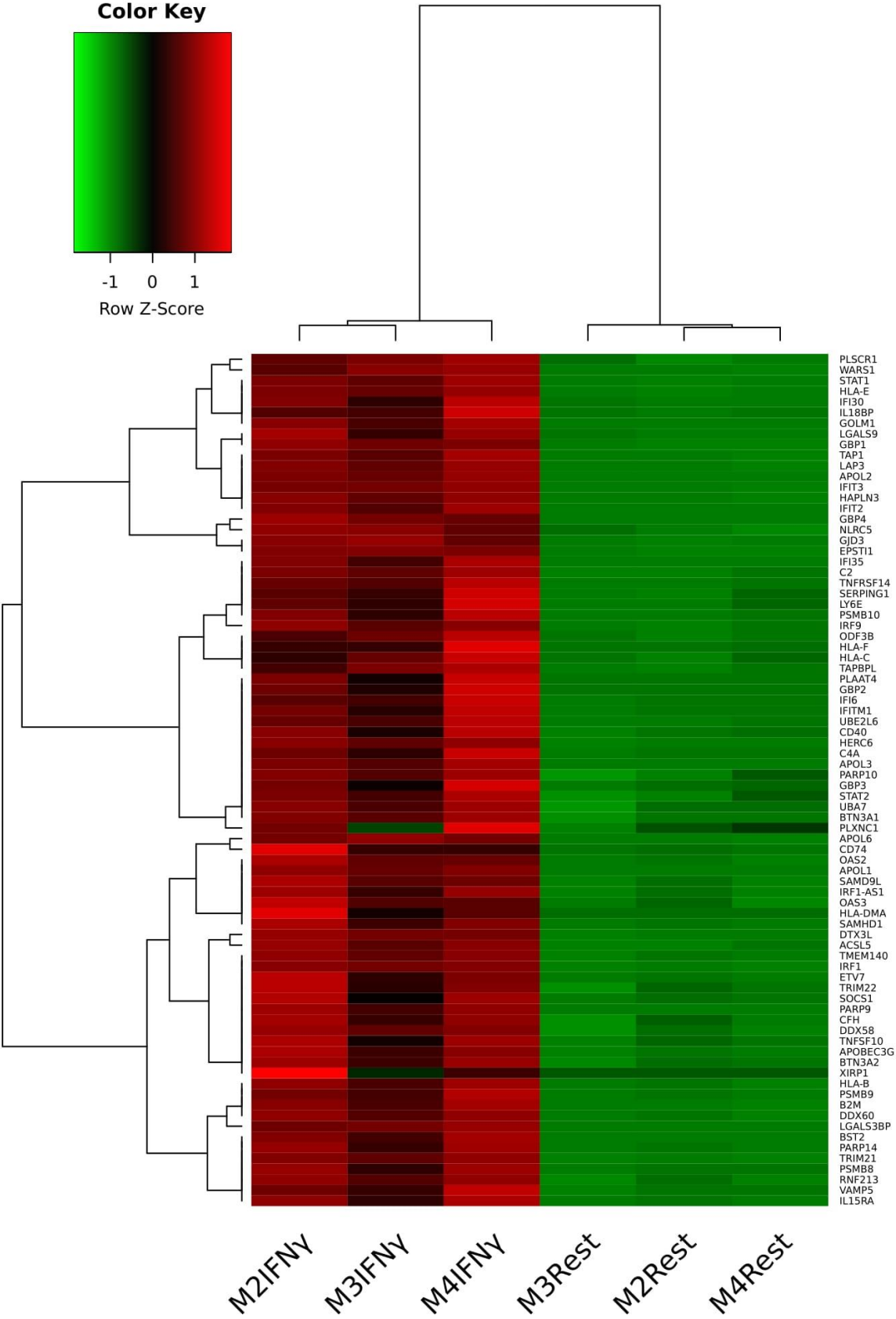
Supplemental Figure 2

A



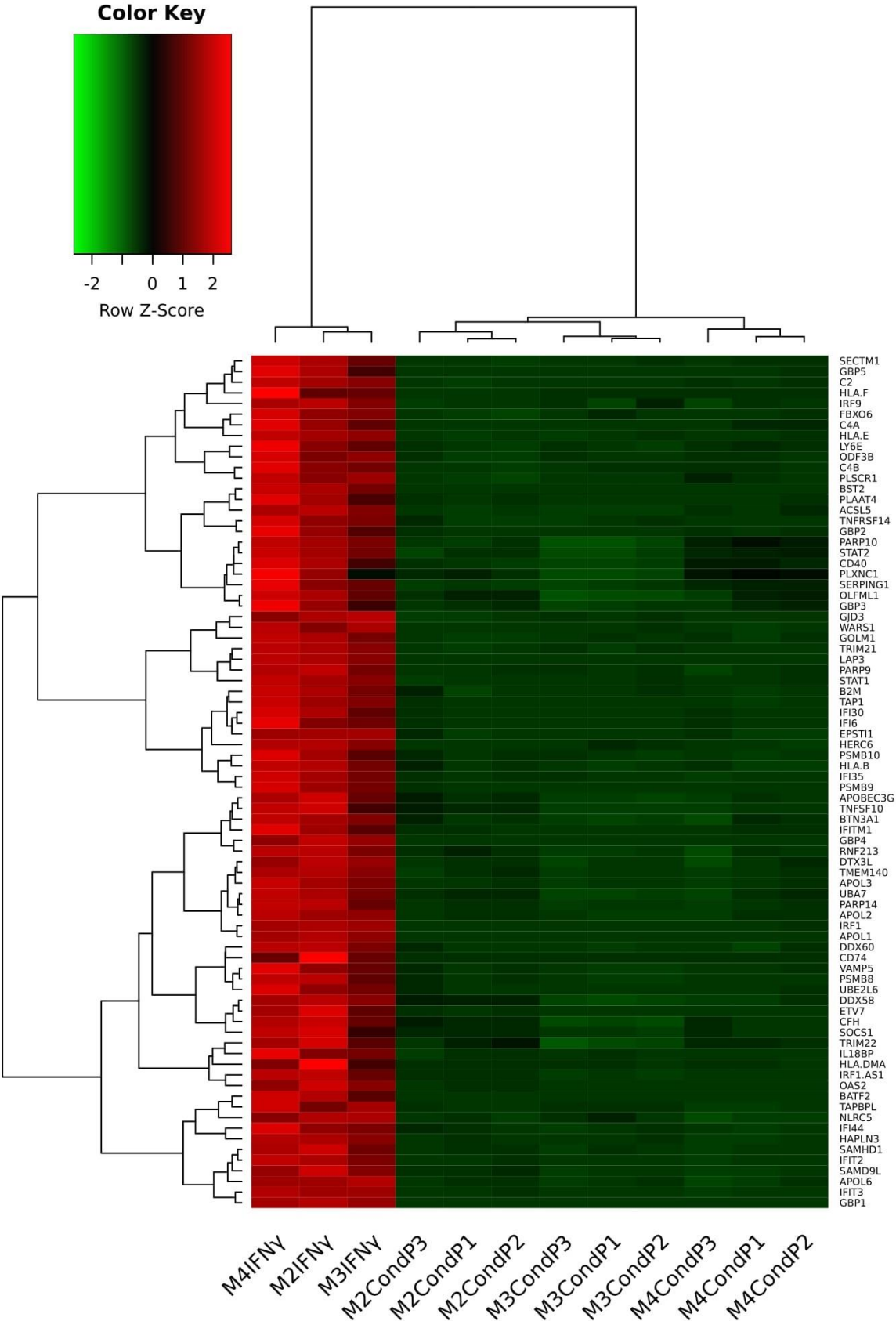
Supplemental Figure 2

B



Supplemental Figure 2

C

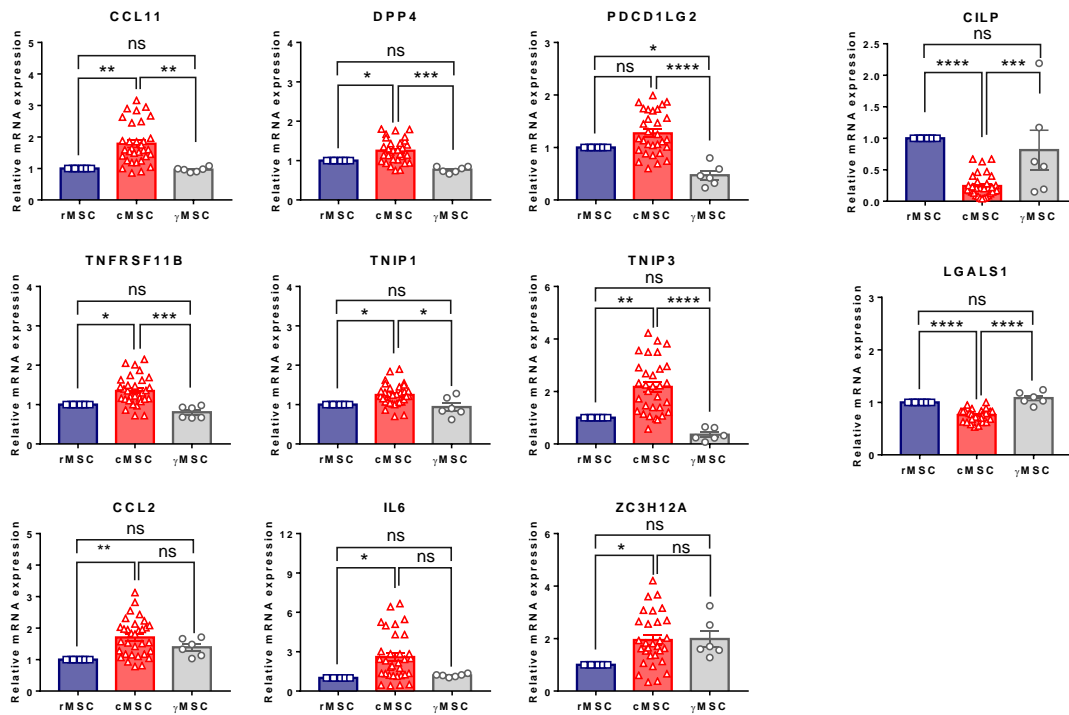


Supplemental Figure 2

D

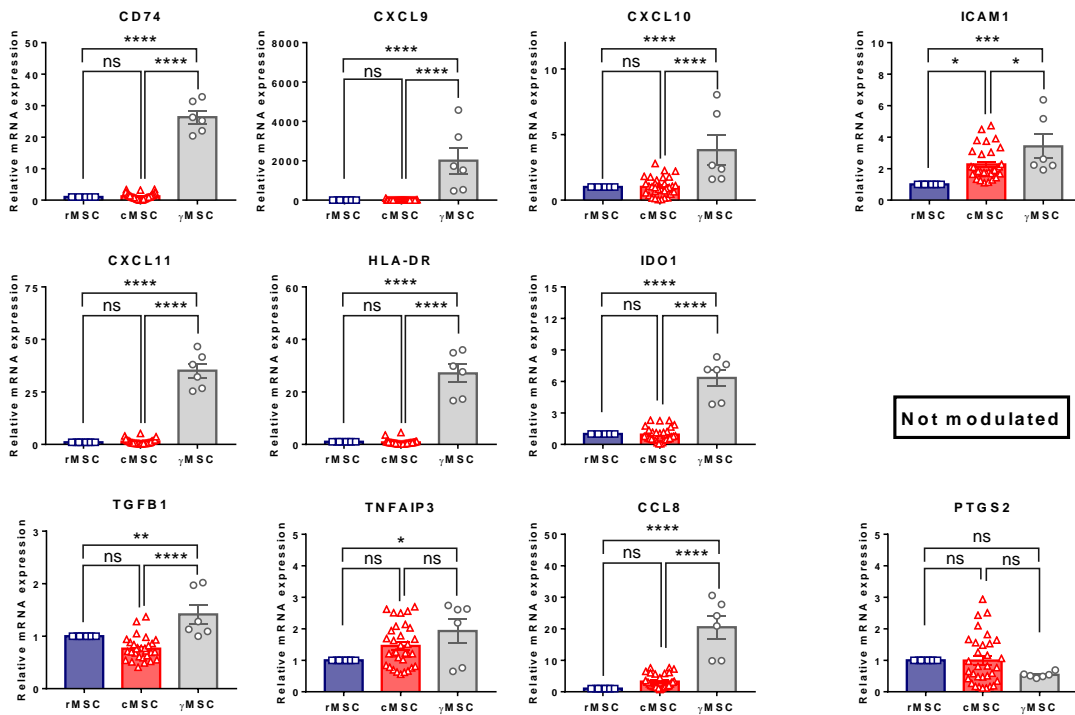
Up-regulated by conditioning

Down-regulated by conditioning



Up-regulated by IFN γ

Up-regulated by IFN γ and conditioning



Not modulated

Differential phenotypical hallmarks of MSC conditioning by PBMC

Using 28 samples (4 rMSC, 20 cMSC, 4 γ MSC), we compared the expression of a list of 21 markers established from the literature and from the ones whose changes were suspected from our gene expression studies. Indeed, we compared especially the expression of CD13, CD26, CD47, CD49a, CD54, CD55, CD59, CD61, CD73, CD90, CD105, CD112, CD120b, CD172ab, CD194, CD273, CD274, CD317, CD318, HLA-ABC, HLA-DR. **Figure 3** presents treatment-related differentially expressed markers when reported to the resting state and grouped by categories. Interestingly, we observed that CD26, CD105, CD273 and CD318 were specifically increased following conditioning (**Figure 3A**), while CD49a and CD59 were specifically decreased (**Figure 3B**). On the other hand, CD274, CD317, HLA-ABC and HLA-DR were increased by IFN- γ treatment, while CD55 was decreased (**Figure 3C**). CD54 was increased by both treatments, while CD73 was impacted differently by both treatments (**Figure 3D**). Significant differences between cMSC and γ MSC were noted for CD47, CD61, CD112 (**Figure 3E**). Important variability was reported regarding CD120b and CD172ab expressions, and no conclusion could be drawn.

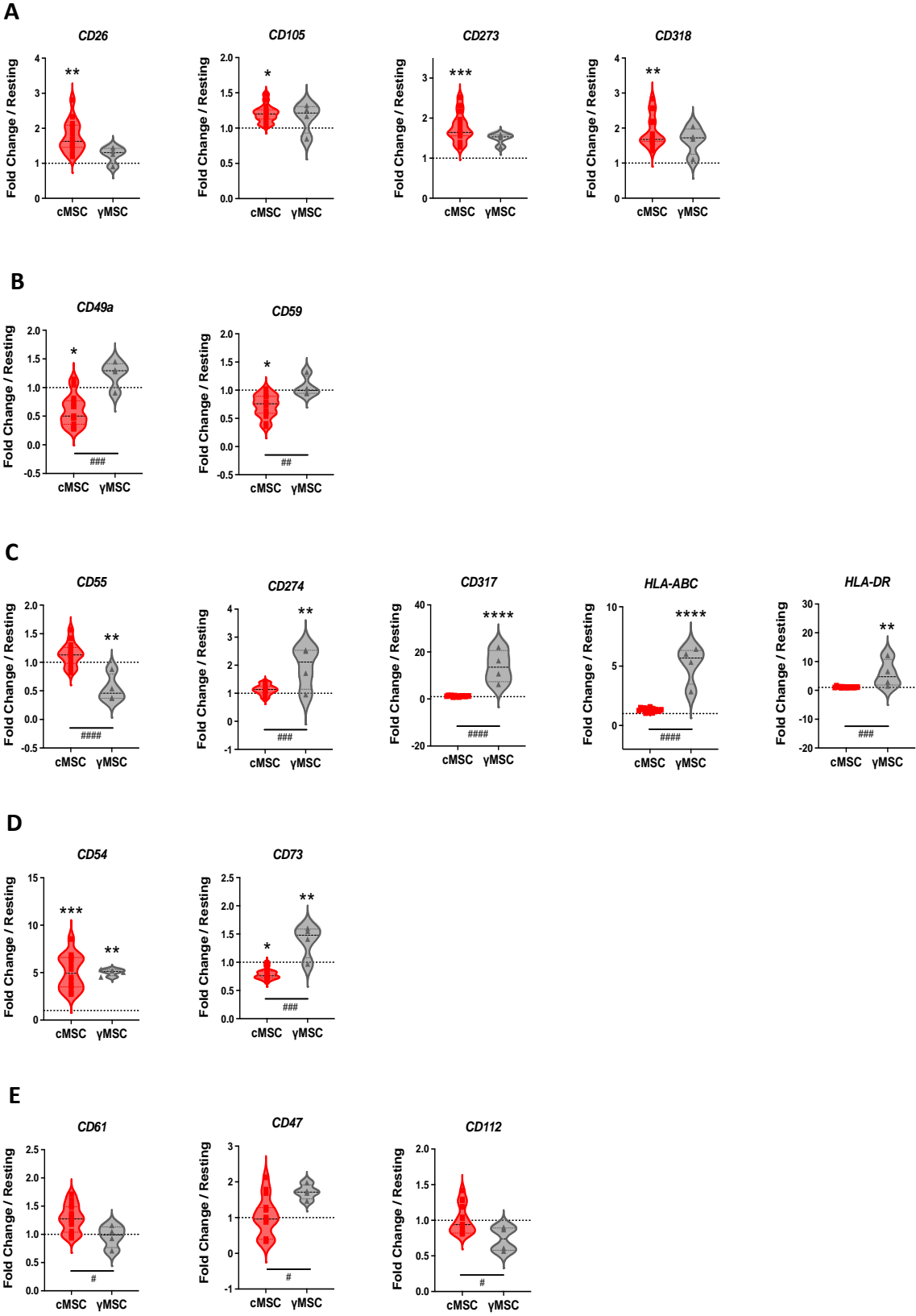
These results are in global agreement with the RNAseq dataset outputs and with the PCR validation above, and it provides phenotypical specific markers (CD26, CD273, CD318) for PBMC conditioning characterization that may be used for their validation. Results also underline the differential pathways used by IFN- γ and PBMC conditioning during MSC priming. Importantly, HLA markers were increased by IFN- γ treatment but not by PBMC conditioning.

Figure 3

cMSC phenotypical signatures established by flow cytometry

rMSC (n=4), cMSC (n=16), and γ MSC (n=4) were analyzed for the expression of 21 extracellular markers using flow cytometry. Differences in the expression of markers between conditions are represented using violin plots and shown as fold-changes of their respective rMSC cultures, represented by dashed lines. Data was analyzed with One-way ANOVA test. Statistical significance between stimulated cells and rMSC are represented by stars: * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, **** $P \leq .0001$. Statistical significance between cMSC and γ MSC are represented by hashtags: # $P \leq .05$, ## $P \leq .01$, ### $P \leq .001$, #### $P \leq .0001$. (A) Up-regulated, and (B) down-regulated markers in cMSC. (C) Markers modulated exclusively in γ MSC. (D) Markers modulated by both treatments. (E) Markers with significant differential expression between cMSC and γ MSC.

Figure 3



Characterization of metaclusters by mass cytometry (CyTOF)

The previous flow cytometry experiments allowed the identification of informative markers to build-up an MSC-dedicated mass cytometry panel. Markers with high reported CV in CG MSC and the ones modulated upon treatments were included. After testing, 24 functional metal-tagged Ab were retained, to which 3 Ab directed against the intracellular targets PTGS2 (prostaglandin E2 synthase), IDO-1 (indoleamine-2,3-dioxygenase) which are especially involved in MSC immunomodulation capacity, and Ki67 (a proliferation marker) were added to configure the final panel. We used 30 cell preparations (4 rMSC, 22 cMSC, 4 γ MSC) to study MSC clusters and the changes in their representation upon treatments.

To limit the experimental variabilities, samples were barcoded using an original approach based on combination of Ab directed against CD90 tagged with specific metals (**Figure 4A**), since CD90 expression is a hallmark of MSC. Each sample was attributed a unique labeling code composed of 3 different metal-tagged CD90 Ab. To limit steric hindrance, different clones of CD90 Ab were used.

Dimensional reduction of the 27 analyzed parameters was performed taking all samples into account and using the optimized T-distributed stochastic neighbor embedding (opt-SNE) algorithm; the resulting map is presented in **Figure 4B**. In this figure, samples were contour-colored according to their condition (rMSC, cMSC and γ MSC) and we observed that the general phenotypic profile of rMSC and cMSC was close as demonstrated by their highly overlapping representations, while γ MSC had a completely different profile which did not superposed to the any of the former ones. We then categorized single-cell data into ostensible cellular metaclusters using ClusterX algorithm. The projections of 4 rMSC samples, 22 cMSC samples and 4 γ MSC samples defined 10 different metaclusters (**Figure 4C**). This number was relatively low, and the clusters were tightly connected when compared to the situation classically observed in whole PBMC analysis. Indeed, as observed above by classical flow cytometry, MSC grown in culture are usually homogenous. While cMSC and rMSC clustering profiles were closely related, γ MSC one presented different distributions and intensities. This underlines and confirms the differential effects of PBMC conditioning and IFN- γ priming on MSC.

Upon IFN- γ treatment, the homogeneity of MSC increased (**Figure 4D**). Indeed, 75% and 17% of cells were contained in clusters 2 and 7, respectively, totalizing 92%. Upon PBMC conditioning, most metaclusters were conserved when compared to the rMSC ones, nevertheless one metacluster (number 10) was over represented.

The **Figure 4E** underlines the significances attributed to these metaclusters modulation: the number 10 is significantly enhanced when comparing cMSC to rMSC (left), similarly clusters number 2 and 7

are enhanced when comparing γ MSC to rMSC (middle) and cMSC to γ MSC (right) and most of the remaining clusters are significantly diminished.

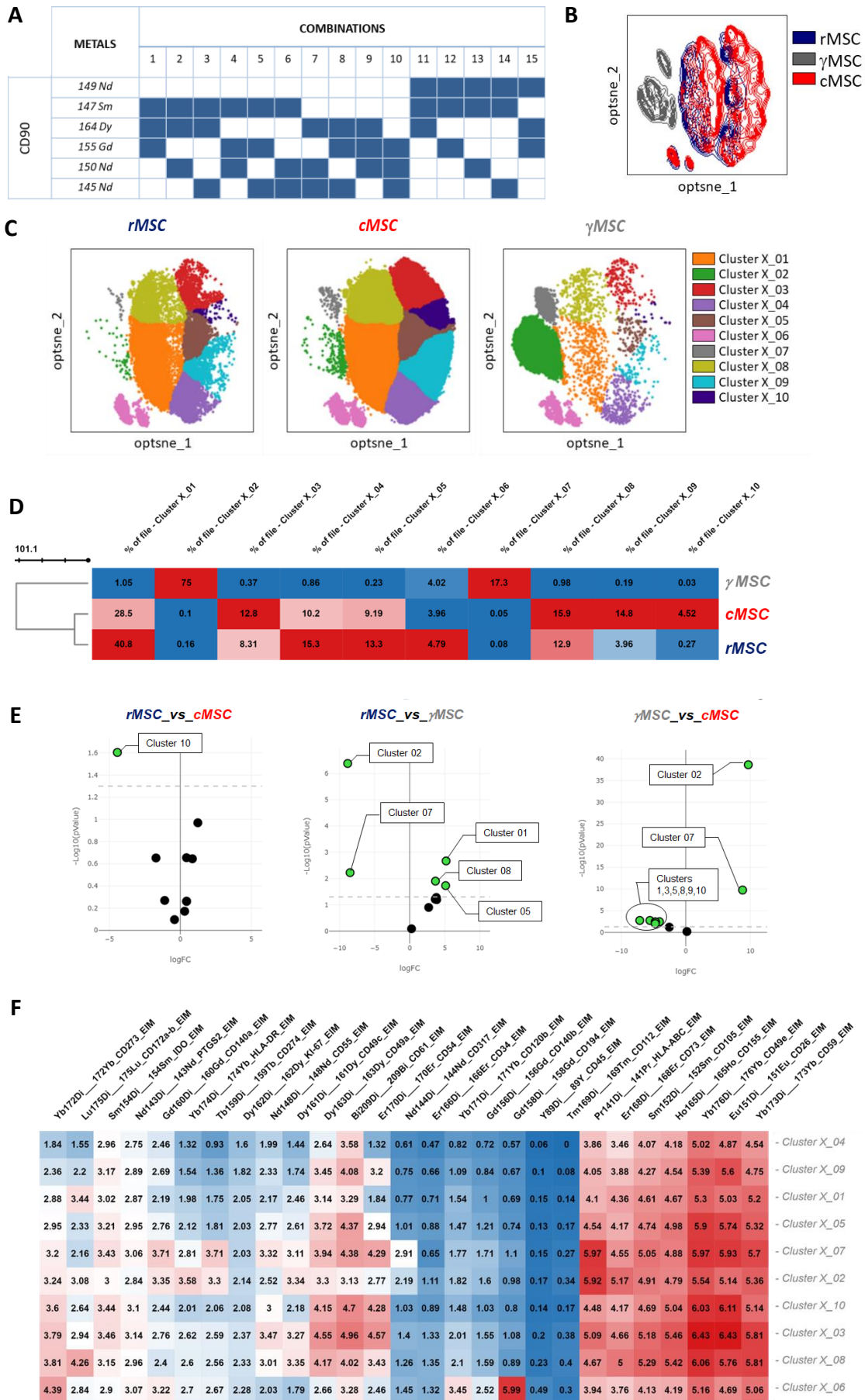
Finally, in order to define clusters identity, a heat map showing markers expression for each cluster is presented in **Figure 4F**. As observed, markers expression among the different clusters varies mostly regarding their intensity and not the presence or absence of expression, which explains the intrinsic connection between most of them. The clusters 2 and 7 are mainly characterized by high expression of CD73, CD140a and b, CD274, CD317, and HLA-ABC and DR. Differences between both clusters include higher expression of CD54, CD61 and CD55, and lower levels of CD172a/b in cluster 7 compared to cluster 2. The cluster 10, which was induced after PBMC conditioning, contained cells expressing among the highest levels of CD26, CD49a, CD49e, CD54, CD61, CD155, CD273, IDO and PTGS2. These results are coherent and complementary to previous results obtained by flow cytometry.

Figure 4

Unsupervised and supervised analysis of MSC phenotypic profile by CyTOF

rMSC (n=4), cMSC (n=22), and γ MSC (n=4) were barcoded and stained with a dedicated home-made panel and analyzed by CyTOF. Unsupervised and supervised data analysis was performed using Omiq software. (A) Table showing barcoding strategy based on CD90 staining. Each sample was identified by a unique combination of 3 different metal-tagged CD90 antibodies, then samples were distributed in 2 tubes gathering 15 each. (B) Unsupervised optimized t-Distributed Stochastic Neighbor Embedding (Opt-SNE) plot overlaying rMSC, cMSC and γ MSC samples. (C) Scatterplot showing metaclusters yield by each MSC condition. Metaclusters were identified using ClusterX analysis tool and each one is represented by a different color. (D) Heat-map showing the frequency of cells in each of the identified metaclusters and for each MSC condition. (E) Volcano plot presenting the significantly modulated clusters between 2 given conditions: rMSC *versus* cMSC (left), rMSC *versus* γ MSC (middle) and γ MSC *versus* cMSC (right) (cut-off p value ≤ 0.05). (F) Heat-map showing the expression of markers for each metacluster, expressed in mean mass intensity (MMI) units.

Figure 4



Differential characterizations of the secretomes

The supernatants of cell cultures outlined in **Figure 5A** were harvested and their contents analyzed using the proximity extension assay developed by Olink. This study was focused on PBMC conditioning and did not apprehend the secretome modulation induced by IFN- γ . The first analysis step consisted in filtering, from the global list, the differentially secreted proteins between two or more studied conditions (culture media, PBMC alone, rMSC, MSC-PBMC coculture and conditioned MSC). By establishing $\text{padj} < 0.05$ as the cut-off, 177 proteins were identified. We then performed PCA based on these 177 proteins (**Figure 5B**) and we observed that samples belonging to the same category nicely clustered together. The most important variance was linked to the presence or absence of MSC, since medium alone and PBMC cultured alone clustered at one side, while differences still existed between them. In parallel, each condition associated to MSC (rMSC, cMSC, coculture) clustered remarkably, thus pointing the differential contents of these compartments. We then plotted a heatmap comparing the expression of these 177 proteins (**Supplemental Figure 3**), and confirmed the clustering according to each condition. An overall homogeneity was observed within each condition, suggesting little variability inside each group. Variations between conditions appeared clearly. Culture medium alone and PBMC alone shared similar contents, but were drastically different from conditions containing MSC. Interestingly, the coculture condition did not result in the simple composition of PBMC and rMSC alone supernatants, suggesting that the interaction between these cell types induced changes in the production of some proteins. Similarly, the supernatant from cMSC was, both, distinct from the supernatants of rMSC and MSC in coculture, suggesting that conditioned cells newly produce a unique set of proteins.

Based on the profile of expression provided for each of the 177 proteins, we proposed to categorize them among 6 distinct classes (exemplified in **Figure 5C**). As presented, (1) 7 proteins highly present in culture medium and in PBMC alone but decreased in rMSC, cMSC and coculture supernatants were presumably consumed by adherent cells, *e.g.* EGF. (2) 65 proteins equally present in rMSC, cMSC and coculture supernatant but reduced in medium and PBMC alone were presumably constitutively produced by MSC, *e.g.* HGF. (3) 20 proteins were found more abundant in PBMC supernatants and in coculture than in medium alone, rMSC and cMSC and may be involved in the conditioning of MSC by PBMC, or in the immunomodulation by MSC, *e.g.* CCL24. (4) 40 proteins were found more abundant in cMSC (and eventually in coculture) than in the other conditions and may be involved in the immunomodulatory capacities of cMSC, *e.g.* Gal-1. (5) 10 proteins less abundant in cMSC than in coculture and rMSC supernatants were presumably inhibited after the coculture conditioning, *e.g.* CXCL11. Finally, (6) 33 proteins were not classified in any of the former categories due to their particular and not patterned expression profiles (not shown).

Conditioning molecules and proteins involved in MSC immunomodulation were the center of the interest of the proteomic study. We first focused on the category containing the molecules potentially involved in the conditioning of MSC by PBMC. Volcano plot in **Figure 5D** shows the increased expression of each of the candidate proteins in coculture as compared to its level in rMSC supernatant (cut-offs were set at $-\log_{10}(0.05)$ and $\log_2(1.5)$). Of note, as the supernatants of coculture condition were obtained after 72hs of PBMC-MSc interaction, the category contained proteins that may promote the activation of MSC, as well as, proteins released by MSC which already exert immunomodulatory activities. This category included AZU1, CCL3, CCL4, CCL24, CD5, CHI3L1, CTSS, CXCL16, GNLy, GZMA, IL-1RA, IL-16, KYNU, LOX-1, MMP7, MPO, TNF, TNF-R2, TR-AP. On the other hand, volcano plot in **Figure 5E** pinpointed some of the differentially secreted molecules when comparing cMSC to rMSC supernatants. The up-regulated proteins (in red) correspond to the ones categorized as potential immunomodulatory and they include CD59, FAS, FS, Gal-1, Gal-3, GAS6, GDNF, HNMT, IDUA, IL-6, Thy-1, OPG, among others. The figure also highlighted down-regulated proteins (in blue) such as CCL11, ADM and SNAP29 that are examples of inhibited proteins after coculture, and that may also play a role in MSC immunoproperties.

Figure 5

Characterization of MSC secretome

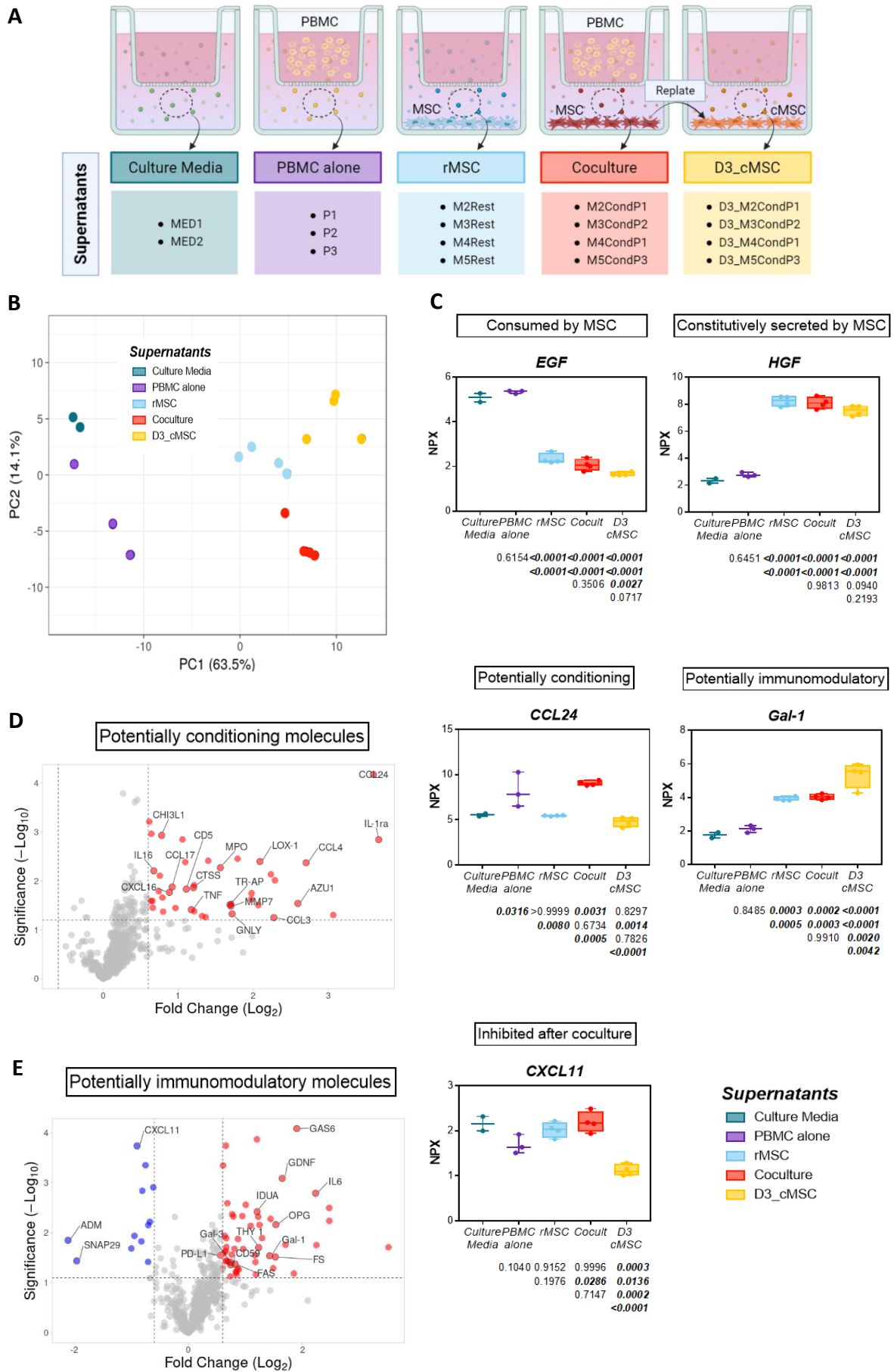
Supernatants produced by MSC before, during and after coculture with PBMC, by PBMC alone and the culture media alone were analyzed for the secretion of 609 different proteins using proximity extension assay methodology (Olink). Data was obtained as NPX value (Normalized Protein eXpression, Olink's arbitrary unit expressed in Log₂ scale) and explored using Olink® Insights Stat Analysis app (www.olink.com). (A) Representation of the different samples set-ups used for supernatant preparation, the categories and the samples' identity attributed to each. Color codes were established for each sample type and are used throughout this figure. (B) PCA plot of samples based on the differentially secreted proteins identified between at least two categories (n=177). Data analysis was performed using One-way ANOVA and adjusted p value of 0.01 as cut-off. (C) Typical representations of the different groups in which the differentially secreted proteins were classified. Each group is illustrated by an example protein whose secretion profile is characteristic of the group profile, *i.e.* EGF plot shows the typical general secretion profile of molecules identified as “consumed by MSC”. Values depicted under the plot represent the pvalue obtained for the comparison of categories. pvalues ≤ 0.05 are shown in bold. (D) Volcano plot showing differentially expressed molecules when compared coculture and rMSC supernatants, and (E) D3_cMSC and rMSC supernatants. Representation's cut-offs were set at log₂ (1.5) and -log₁₀ (0.05). Significantly upregulated proteins are shown in red, significantly down-regulated proteins in blue and non-significantly modulated proteins in grey.

Supplemental Figure 3

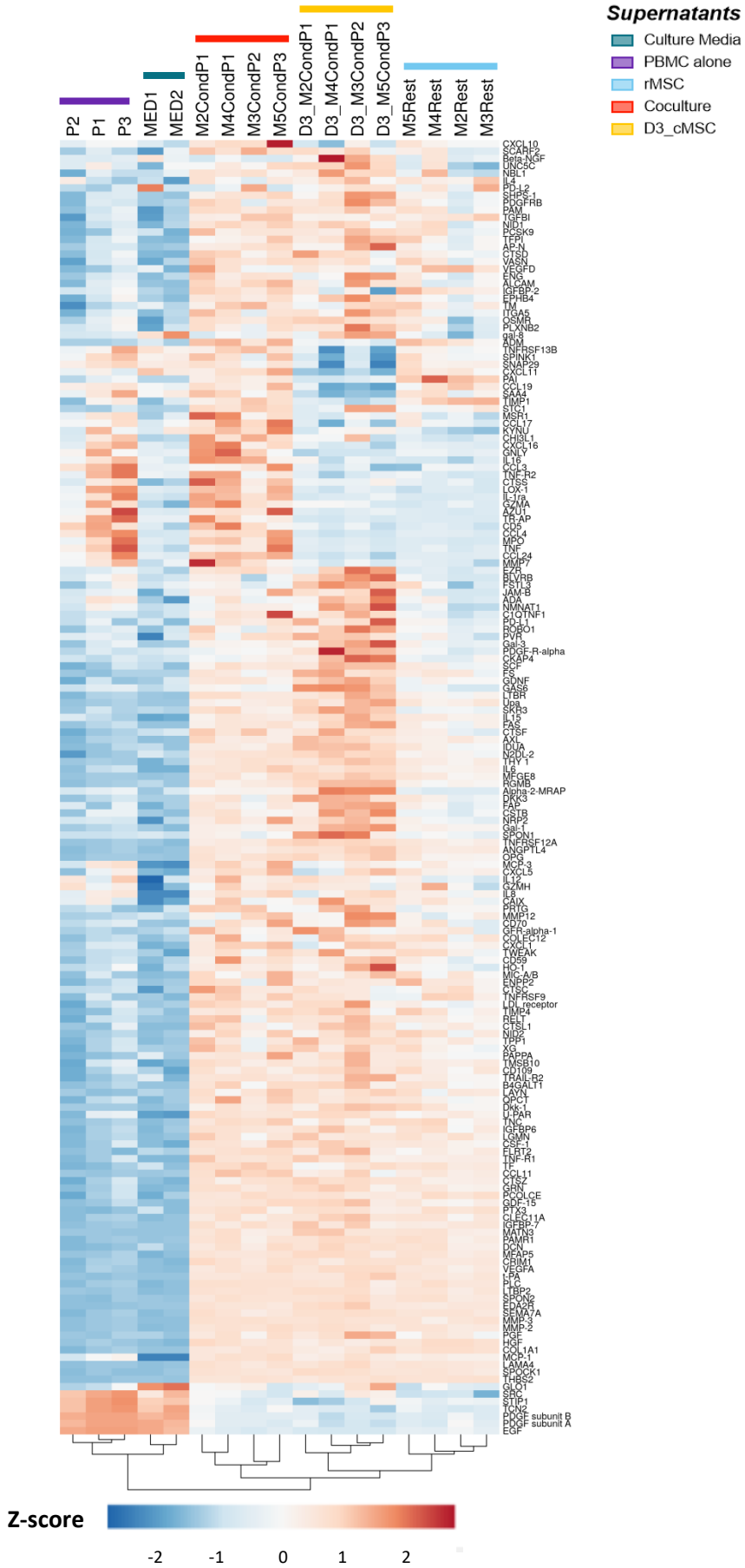
Comparison of the differentially secreted proteins in different samples

Heat-map showing the detection of the 177 differentially secreted proteins in the different samples. The columns correspond to the samples while the rows correspond to the proteins. Rows are centered and unit variance scaling is applied to them. Both rows and columns are clustered using correlation distance and average linkage, only columns dendrograms are shown for simplification. Color intensity of each grid represents the numeric differences expressed as Z-score.

Figure 5



Supplemental Figure 3



Phenotypical changes of PBMC populations during the conditioning

Until now, only the unilateral effect of PBMC conditioning on MSC transcriptomic, phenotypic and secretory profile was assessed. However, during coculture, paracrine interactions between both cell types are bidirectional, then not only PBMC will impact on MSC status but MSC will also act on PBMC. To address the functional changes induced in the PBMC populations during the conditioning step, we used the Maxpar Direct Immune Profiling Assay (Fluidigm), a ready-to-use CyTOF panel, to profile 37 immune cells subsets including T cells (Th1, Th2, Th17, Treg, CD4 and CD8 subsets of naïve, activated, central or effector memory), B cells (total, naïve, memory, plasmablasts), monocytes (classical, non-classical, transitional), NK cells (total, early, late), dendritic cells (plasmacytoid, myeloid), basophils and neutrophils. Modifications in cell subsets were expressed as fold-changes to their respective PBMC donor that has not been cocultured with MSC but grown in culture medium alone, for 72hs using the Transwell system.

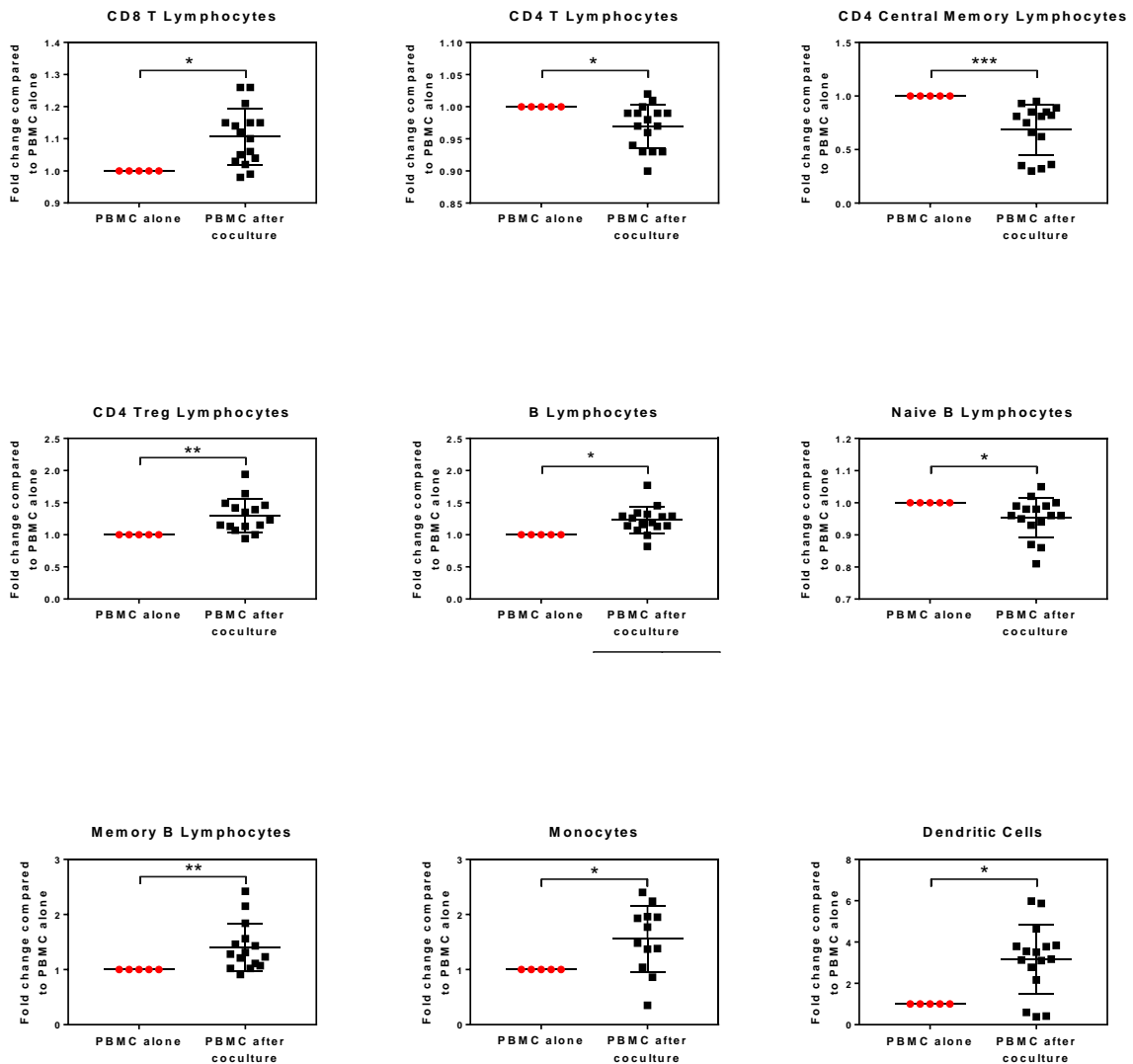
We observed (**Figure 6**) that the conditioning increased significantly the proportions of CD8⁺ (CD3⁺CD8⁺) cells, the population of CD4 Treg (CD4⁺CD25⁺CD127^{low}CCR4⁺), while the proportion of CD4⁺ (CD3⁺CD4⁺) and CD4 central memory (CD4⁺CCR7⁺CD27⁺) were decreased. The proportion of B cells (CD19⁺CD20⁺HLA-DR⁺) was increased, among them naïve B lymphocytes (CD27⁻) were slightly decreased while memory B lymphocytes (CD27⁺) were increased. The proportions of monocytes (HLA-DR⁺CD11c⁺) and dendritic cells (HLA-DR⁺CD123⁺ and HLA-DR⁺CD11c⁺CD38⁺) were increased. These changes in proportions and activation of sub-populations provide both readouts for functional efficacy of the conditioning step and mechanistic clues of immunomodulation.

Figure 6

Changes in PBMC subsets during the conditioning

PBMC used for MSC conditioning (n=16) were harvested after the coculture step and analyzed by CyTOF using the Maxpar Direct Immune Profiling Assay. PBMC from the same donors (n=4) but cultured in growth medium alone (without MSC) were used as controls. Changes in PBMC cell subsets after coculture are shown as fold-change relative to control PBMC. Statistical significance between conditions are represented by stars: * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, **** $P \leq .0001$.

Figure 6



Inhibition of T cell proliferation by MSC

To determine if MSC treatments impact MSC immunomodulation properties, we challenged the functional efficacy of rMSC, cMSC, γ MSC and of their secretomes to block the proliferation of activated T cells. Following CFSE labeling of PBMC, and their activation by anti CD3/CD28 microbeads-coupled Ab, T cells proliferation was assessed by gathering CD4⁺ and CD8⁺ simple positive cells. After 72 h of activation, T cells divided prolifically and up to 5 generations of daughter cells were observed, while in absence of activation cells did not proliferate (**Figure 7A**, upper histograms). When activated PBMC were placed in medium produced by MSC of any condition (**Figure 7A**, lower histograms), inhibition of the proliferation was observed. Both, the proportion of proliferating T cells and the number of daughter cells' generations were drastically reduced by cMSC supernatant, while the effect of rMSC and γ MSC produced media were moderate. **Figure 7B** summarizes and compares the quantitative effect of rMSC (n=4), cMSC (n=20), γ MSC (n=4) supernatants on T cell proliferation. cMSC were more efficient in reducing the number of proliferating T cells (mean of measures were close to 50%) and the effect was significantly stronger when compared to the other two conditions. Same scenario was observed when gating separately CD4⁺ and CD8⁺ T cells, both cells subsets were similarly sensitive to the inhibition (**Figure 7C**).

On the other hand, when activated PBMC were grown in direct contact with rMSC, cMSC and γ MSC, the proportion of proliferating T cells decreased equally in the three groups (**Figure 7D**). These results suggest that, while MSC inhibited the proliferation of activated T cells upon direct contact whatever the treatment, the supernatant produced by cMSC was the most efficient in inhibiting T cell proliferation.

As one of the differentiating signatures of cMSC was the expression of CD26 and as soluble CD26 has been detected in the supernatant (data not shown), we assessed the potential role of this molecule in the cMSC inhibitory capacity by adding saxagliptin, an inhibitor of the enzymatic activity of CD26, to the supernatant. We observed a partial but significant reversion of the cMSC supernatant inhibitory effect in the presence of saxagliptin (**Figure 7E**). No significant effects were reported for rMSC and γ MSC supernatants in presence of saxagliptin.

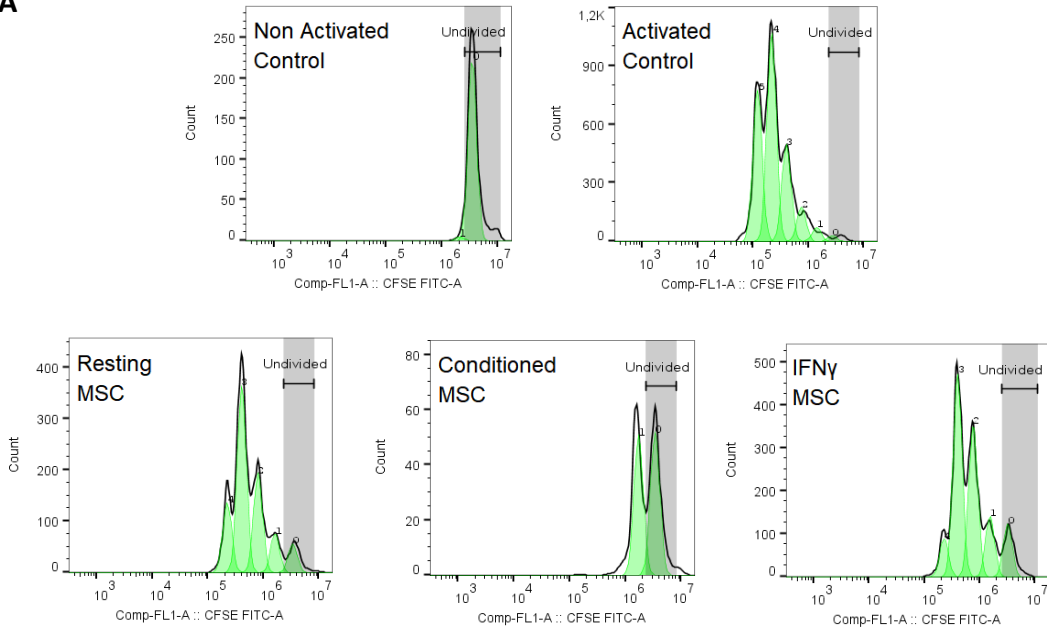
Figure 7

Assessment of functional capacities of MSC *in vitro* under different conditions

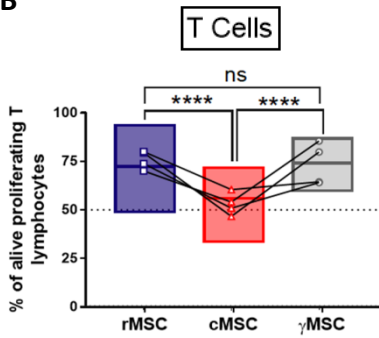
PBMC were incubated with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with microbeads-coupled anti CD3⁺/CD28⁺ antibodies. The inhibition of T cell proliferation, induced by MSC themselves or their supernatants, was assessed by flow cytometry. Analyzed read-out was the percentage of proliferating cells compared to control condition (PBMC activated), higher the inhibition, lower the percentage of proliferating cells. (A) Histograms showing representative proliferation profiles of non-activated and activated PBMC alone (experimental control conditions, Top histograms), or in contact with supernatants produced by rMSC, cMSC, and γ MSC respectively (Bottom histograms). Each green pic represents a daughter cell generation. (B) Floating bars showing the maximum, minimum and median values of proliferating T cells after culture with supernatants from rMSC, cMSC and γ MSC. Each point represents the mean value obtained from samples of a given MSC donor (M2, M3, M4, M5). (C) Box-and-whiskers plots showing proliferation of CD4⁺ T cells and CD8⁺ T cells subsets separately, after culture with MSC supernatants. The “+” represents means values. (D) Proliferation of T cells in absence of, or in direct contact with, MSC from different conditions. (E) Proliferation of T cells when incubated with MSC supernatants from different conditions in presence or absence of saxagliptin (SAX), an inhibitor of CD26/DDP4. Data was analyzed using One-way. ** $P \leq .01$, *** $P \leq .001$, **** $P \leq .0001$

Figure 7

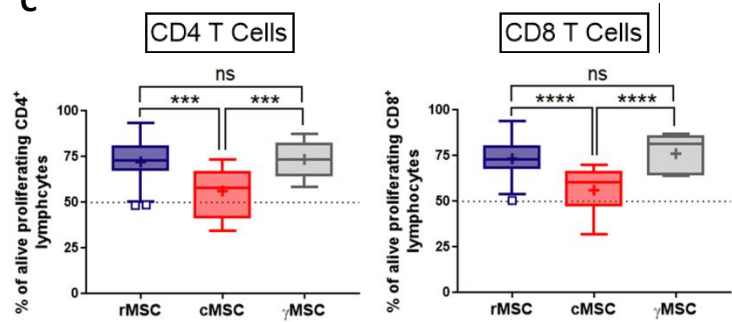
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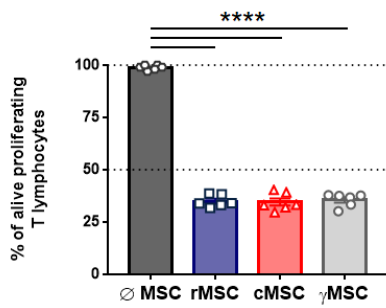
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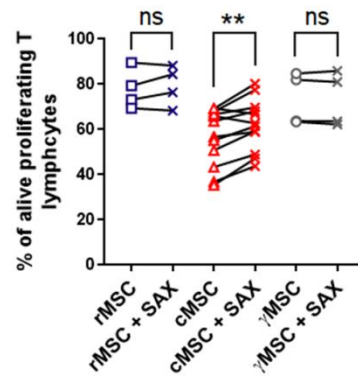
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D



E



Modulation of clinical and immunological status in the NSG-MG mouse model

As described in the outline (**Figure 8A**), NSG mice were grafted with fragments of thymic tissue obtained from MG patients and symptoms development was followed by weekly clinical test. Every two weeks, blood collection documented the presence and proportions of human blood cells in the NSG mice, i.e. their humanization. Human cells present in the thymus fragments reached the blood system of the mouse, and were identified as CD45⁺ cells, among which CD3⁺ subsets (CD4⁺, CD8⁺ and CD4⁺CD8⁺) were predominant and CD19⁺ B cells represented a smaller proportion. All mice presented significant humanization; although the proportions of human cells varied from mouse to mouse (**Figure 8B** illustrates a representative analysis of intermediate level). rMSC, cMSC cells and placebo were injected following randomization of the mice in groups homogenous for humanization and clinical signs.

The MG-like symptoms were assessed using a composite score based on 4 complementary tests, including clinical observation, inverted grid test, grip test and weight monitoring. In this model, the onset of the disease and the clinical signs vary according to the status of the MG donor. Therefore, the clinical scores were normalized to the status at the time of cell injection. A total of 27 animals (3 groups of 9 mice) were treated. We observed that cMSC improved the clinical status of mice, as compared to placebo group and they reached statistical significance on weeks 4, 5 and 7 (**Figure 8C**). Four mice in the placebo-treated group, 1 in the cMSC-treated group and none in the rMSC group died before the completion of the study. Upon sacrifice, we analyzed the proportions of human cell sub-populations contained in these humanized mice. No differences were found in humanization levels between the 3 groups but we observed a trend towards a decrease in Th17⁺ cells proportion and an increase in Treg cells proportion (**Figure 8D**). This trend did not reach significance; however, the power of the analysis was reduced by the small number of surviving animals in the non-treated group. Of note, at the time of sacrifice, the remnants of the thymic fragments did not contain any more human T and B cells (data not shown).

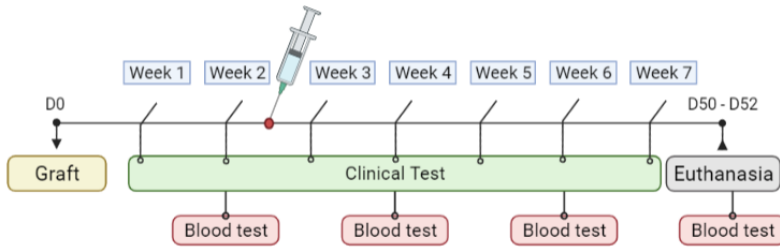
Figure 8

cMSC treatment effect in the NSG-MG mouse model.

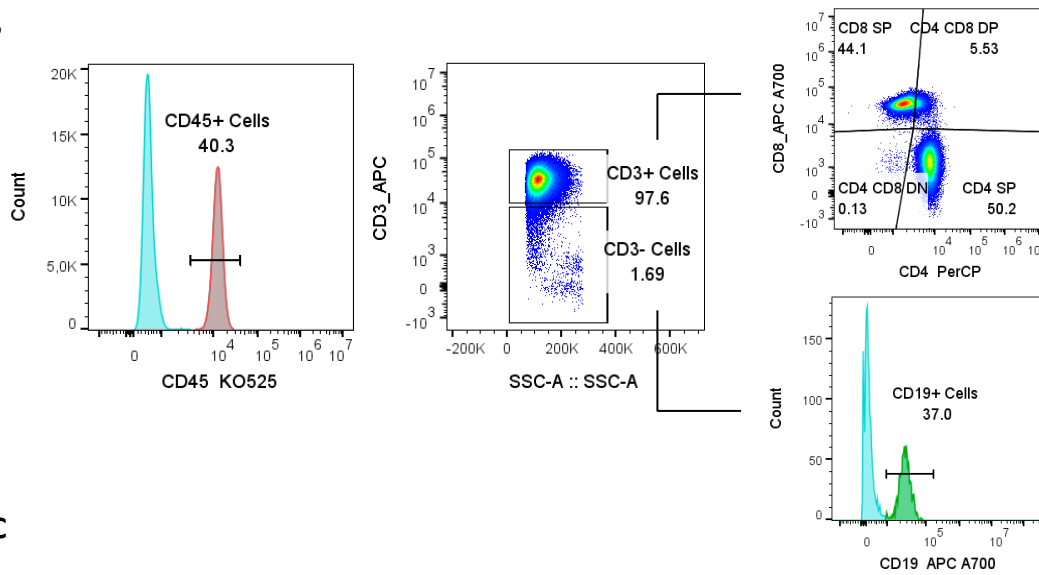
rMSC or cMSC cells (500.000 cells) or vehicle (NaCl) were injected in NSG-MG mouse model and the effect of treatment was tracked through composite clinical score assessment. Composite score is calculated based on 4 tests: weight loss, grip test, inverted grid test and behavior observation. (A) Experimental outline indicating frequency of clinical and blood tests, and treatment of mice relative to thymus fragment grafting. (B) Description of human cell populations using blood tests for assessing mice humanization; a representative example is presented. (C) Integration of the weekly general clinical score (GCS) evolution in treated and non-treated mice. For each mouse the weekly registered GSC was normalized to its initial GSC (obtained at the onset of the disease, week 2). (D) Percentages of circulating Treg (left) and Th17 (right) measured in mice blood at euthanasia. Data was analyzed using One-way ANOVA and are presented as mean \pm standard error of the mean. * $P \leq .05$, *** $P \leq .001$.

Figure 8

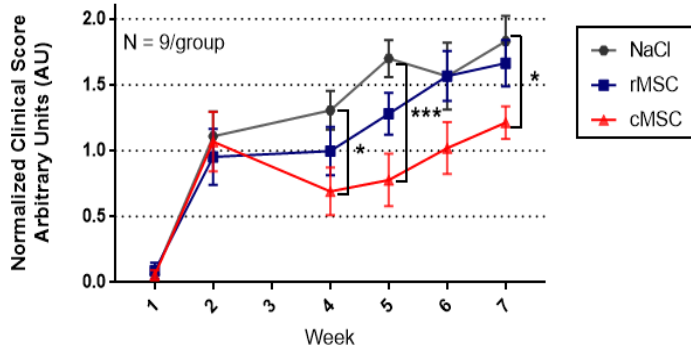
A



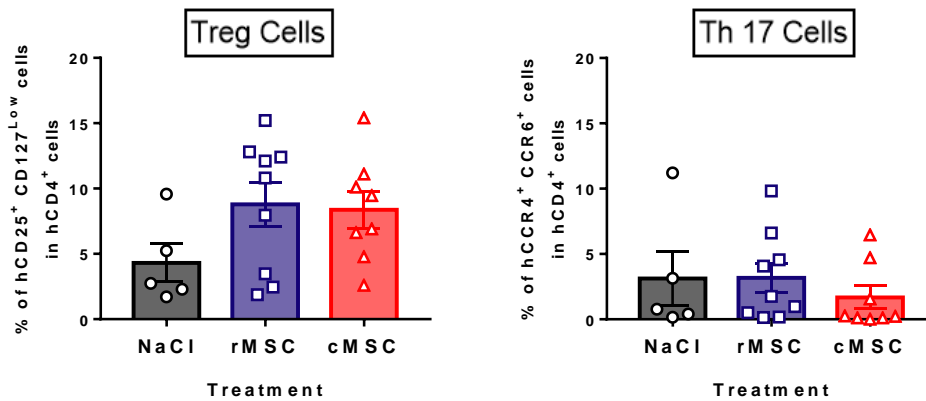
B



C



D



Discussion

Although some treatments are proposed to MG patients, they trigger long-term severe side effects and this mandates the search of new therapeutic solutions. Of note, any progress made in the field of this auto-immune disease may benefit to the whole field, in which disequilibrium of the immune homeostasis threatens the life or the quality of life of patients. The multifaceted mechanisms of action of MSC may facilitate immunomodulation. Their use has been investigated in hundreds of trials, without toxicity, but frequently with low efficacy. Recent rationale evidence reshapes the methodology for their use on a large scale (Galipeau and Sensébé, 2018b; Guess et al., 2017; Krampera and Le Blanc, 2021). There is agreement on the fact that conditioning, or priming, or licensing of the cells before use will improve their biological and immunomodulatory capacities (Giri and Galipeau, 2020). The nature of the conditioning agent to be used is not yet ascertained, because the cytokines presently used may have broad activities and may increase the immunogenicity of the cells (Griffin et al., 2013; Sivanathan et al., 2014). Conditioning MSC through PBMC may appear more physiological, and with a wider specificity. The use of cells as therapeutic agents, however, requires this product to be robustly characterized, and its main mechanisms of action unveiled. In this study, we suggest that the conditioning of MSC by PBMC induces phenotypic changes, triggers the expression of genes and the production of proteins involved in inhibition of proliferation, population changes and immunomodulation, which show therapeutic benefit in a preclinical MG model. We propose, therefore, robust signatures and potency assays to promote the use of this kind of conditioning.

In this setting, MSC are grown in coculture with PBMC that were not previously activated. These PBMC, have been extracted from healthy donors' blood, they were conserved frozen and thawed prior to use. The coculture lasts 72h and enables prolonged exchanges between both cell types, which are metabolically active. Some molecules detected using the Olink assay, which are produced by PBMC, present a pro-inflammatory profile, such as AZU, CCL3, CCL17, CCL24, GNLV, GZMA, IL-16, MPO or TNF- α , and may provide to the MSC the pro-inflammatory environment necessary to trigger their immunomodulatory capacities (Bernardo and Fibbe, 2013; Crop et al., 2010a; Sayegh et al., 2019).

IFN- γ is recognized as a potent activator of immunomodulation, and its use has been proposed recently to prepare human cell therapy products (Guan et al., 2017a; Mebarki et al., 2021b). IFN- γ indeed is directly or indirectly involved in the activation of immunomodulation pathways through expression of *CD74*, *CD274*, *CXCL9*, *CXCL10*, *CXCL11*, *Galectins*, *HGF*, *HLA-DR*, *ICAM1*, *IDO1*, *IL6*, *MCP1*, *PDL1*, *PTGS2*, *VCAM* as observed in the literature (Guan et al., 2017a; Noronha et al., 2019; Sivanathan et al., 2014) and in this study. However, IFN- γ activates several hundreds of genes involved in cellular defense against pathogens (virus, bacteria, parasites) and likely useless regarding immunomodulation, and it

notably triggers the strong expression of HLA class II molecules which participate in the immunogenicity of cells (Guess et al., 2017; Sivanathan et al., 2014) and facilitate their clearance. It also triggers the expression of several cytokines of the CXCL family (CXCL9, CXCL10, CXCL11) involved in the activation of the immune system (migration, extravasation, differentiation), which may be avoided in the context of autoimmune or inflammatory diseases. The use of PBMC previously activated by mixed lymphocyte reaction or primed by biological materials also triggers the immunomodulation capacity of MSC through the expression of CCL5, CCL7, CIITA, CXCL9, CXCL10, CXCL11, ICAM-1, IL-8, TRAIL and HLA-DR (Chinnadurai et al., 2018), and/or of CCL20, COX2, CXCL1, CXCL5, CXCL6, IL-1a, IL-1b, IL-6, IL-8, IL-33 (Crop et al., 2010a) maybe through the paracrine secretion of IFN- γ . Here, by gathering the results of gene dysregulation study, of the differential secretomes' characterization and of phenotypic changes, we unveiled some actors of immunomodulation that may be involved at different degrees in the immunomodulation triggered by PBMC and performed by MSC. Indeed, we observed at gene and/or protein level that CCL2, CHI3L1, CTSS, CXCL16, DPP4, IL1-RA, LOX-1, MMP9, PD-L2, *TNIP1*, *TNIP3*, TNF-R2, *ZC3H12A* expressions are increased upon conditioning. These molecules are involved in several pathways of immunomodulation. Nevertheless, some molecules related to classical MSC immunomodulation pathways, but not the least, are also triggered by PBMC conditioning such as IDO-1, ICAM-1, IL-6, PGE2, PD-L1. These results therefore suggest both markers and mechanisms useful for cMSC characterization.

To validate the RNAseq study, we selected candidate genes either up or down-regulated by PBMC conditioning, by IFN- γ treatment or both. Genes coding for extracellular proteins, that could become convenient phenotypic markers of conditioned cells, were also assessed. We confirmed that *DPP4*, *PDCD1LG2*, *TNFRSF11B*, *TNIP1*, *TNIP3*, *CCL2*, *IL6* and *ZC3H12A* genes were upregulated specifically through PBMC conditioning. These genes are involved in cleavage of several cytokines and inhibition of T cell proliferation (*DPP4* i.e. CD26) (Metzemaekers et al., 2016; Ou et al., 2013), checkpoint inhibition (*PDCD1LG2* i.e. CD273), inhibition of NF- κ B pathway (*TNIP1*, *TNIP3*, *ZC3H12A* i.e. *MCPIP1*) (Dang et al., 2016; Matsushita et al., 2009; Peng et al., 2020; Pu et al., 2020; Verstrepen et al., 2014; Yin et al., 2022) and some present pleiotropic activities, among which immunomodulation (*ZC3H12A*, *CCL2* and *IL6*) (Bouffi et al., 2010; Philipp et al., 2018). Meanwhile, *CILP* and *LGALS1* (i.e. galectin 1) gene expression was down-regulated by conditioning. Interestingly, some genes that were upregulated by PBMC conditioning were also upregulated by IFN- γ such as *ICAM-1* which is a cell adhesion molecule playing an important role in cell-cell contact and involved in immunomodulation (Espagnolle et al., 2017; Liu et al., 2020b; Ren et al., 2010a). Conditioning also modified the expression of genes involved in matrix modeling (*AOC3*, *PRR33*, *TNFRSF11B*), migration (*AOC3*, *CEMIP*), cytokine production (*GPR176*, *CRLF1*), proliferation (*MCM4*, *WNT11*, *PKNOX2*), and chaperone protection (*CLU*).

Together, these results suggest that PBMC conditioning and IFN- γ use different pathways, and that some partially overlap, which correlates with the pathway enrichment analysis results presented in this study

Using classical cytometry and information provided by the RNAseq study, we proposed a phenotypical signature of cMSC grown in PL. Although slight variability was noted, the cultures remained very robust using PL, as attested by the inter-group and intra-group correlation studies (Camilleri et al., 2016; Guiotto et al., 2020; Karlsen and Brinchmann, 2019; Schallmoser et al., 2007; Viau et al., 2019)

In a first step, we observed and confirmed differences in expression of markers according to growth in FBS or in PL. We observed statistically significant differences between RG and CG for 15 out of 60 markers, essentially regarding adhesion molecules (CD29, CD44, integrins, CD61, CD146, CD166) and immunological regulators involved in complement metabolism (CD55, CD59), co-stimulation, antigen presentation and immunomodulation molecules (CD73, CD105, CD200, HLA-DR), or enzymes (CD10). Our study confirms overall recent works (Camilleri et al., 2016; Chinnadurai et al., 2018; Reis et al., 2018b). Even though we did not test exactly the same panel, we noted that CD140b, CD273, CD274, CD276 were positive and CD200, CD271 were very low or negative in Camilleri's study, as in ours; discrepancy was noted regarding the expression level of CD36 and CD146 which were negative in our hands. These results still underline the potential important effect of PL in the culture medium, which is rich in growth factors and may activate specific pathways (ERK, PI3K, p38, MAPK) (Karlsen and Brinchmann, 2019; Viau et al., 2019). Our results suggest or confirm that CD10, CD49c, CD55, CD200 could constitute a signature differentiating CG and RG cells (Camilleri et al., 2016; Najar et al., 2012).

In a second step, we analyzed the differences between rMSC, cMSC and γ MSC using a set of markers combining those with wider range of expression (larger CV) identified in the first study above, and the new markers suggested from the RNAseq study. We observed the specific increased expression of some proteins under conditioning by PBMC, which coding-genes were identified by RNAseq (CD26, CD120b, CD318, CD273), and we confirmed that CD54 is increased by both, IFN- γ and PBMC conditioning. The expression of integrins, intercellular adhesion molecules (ICAM-1/CD54), and other glycoproteins (CD318/CD31) on their surface, enable MSC to bind to T lymphocytes with high affinity (Haddad and Saldanha-Araujo, 2014; Liu et al., 2020b; Ren et al., 2010a; Ruth et al., 2021), then higher expression of these molecules could benefit MSC-T lymphocytes interaction and immunosuppressive capacity of MSC over their target. The increased expression of PD-L2 after MSC conditioning could also increase their immunosuppressive capacities through interaction with PD-1 (Haddad and Saldanha-Araujo, 2014). We confirmed the specific activation of some proteins by IFN- γ (HLA-ABC, HLA-DR, CD317, CD274), as expected (Chinnadurai et al., 2018; Guan et al., 2017a; Ménard and Tarte, 2013).

We also observed that molecules known for their role in regulating the complement pathways (CD55 and CD59) were modulated by IFN- γ and PBMC conditioning with opposite effects: CD55 is decreased by IFN- γ but conserved by conditioning and CD59 is decreased by PBMC but conserved by IFN- γ . This finding indicates that MSC upon both treatments may regulate complement but privileging different mechanisms.

Finally, we propose new combinations of markers as signatures for the validation of resting, PBMC-conditioned or IFN- γ -activated MSC. Compared to the resting status, the cMSC signature includes up-regulations of CD26, CD54, CD105, CD273, CD318 and down-regulation of CD49a and CD59. The γ MSC signature includes up-regulations of CD54, CD73, CD274, CD317, HLA-ABC, HLA-DR, and down-regulation of CD55. These markers could be mandated to follow the MSC status throughout their production for clinical application.

The CyTOF analysis confirmed and extended these results. Classical CyTOF barcoding requires tough fixation/permeabilization protocols that can alter surface antigens by introducing conformational changes and irreversibly modifying antigenic epitopes recognized by Ab. Indeed, we observed that the fixation/permeabilization of MSC induced a drastic shift in the expression of some markers and provided a surface phenotypic profile that did not reflect actual one (data not shown). The design of a barcoding based on the labeling of CD90 by combinations of Ab allowed limiting technical variabilities and batch effects. Surface barcoding strategy has the advantage to preserve cell phenotypic profile. The CyTOF analysis documented the homogeneity of MSC grown in culture, since few metaclusters were observed. Of note, IFN- γ treatment homogenizes even more the MSC, since most of the cells were gathered in only two metaclusters (number 2 and 7), a property of IFN- γ licensing previously reported (Szabó et al., 2015). We did not observe, using this methodology, the reported multilineage heterogeneity of MSC which may be due to culture conditions or the differentiation status (Freeman et al., 2015).

CytoF allows to test several Ab at the same time, and to assess markers' concomitant expression on cells. The comparison of metaclusters contents suggests that the signature metacluster of cMSC highly express CD26, CD49a, CD49e, CD54, CD61, CD155, CD273, IDO1 and PTGS2. The γ MSC signature would harbour CD73, CD140a and b, CD274, CD317, IDO1 and HLA-ABC and DR. Interestingly, several markers suggested by the RNAseq study and the subsequent Q-PCR validation, were expressed by the MSC populations, and/or were specific for treatments. Indeed, we observed CD26 and CD273 highly expressed in the metaclusters formed by cMSC, and, CD274, CD317, HLA-DR in the metaclusters formed by γ MSC. CD54 was increased by both treatments. IDO1 was found significantly increased at the RNA level only in γ MSC, while it is observed increased at the protein level in both γ MSC and cMSC

characteristic clusters. PTGS2 was not found increased at the RNA level, but its expression at the protein level was important in characteristic metaclusters of both treatments.

The culture media are deeply modified by the different cell types, alone or in coculture. On one hand, the 3 days-long culture may mask some components with short half-life such as interleukins or prostaglandins. On the other hand, this duration allows bidirectional exchanges in paracrine fashion. The study of these secretomes is constitutively different (and complementary) from the analysis of RNAseq, since it provides information regarding proteins whose production and stability may not be reflected by gene expression. To avoid biases due to the presence of specific proteins in the PL, the secretomes were compared to culture medium alone incubated for 3 days in the same conditions as the MSC cultures. We also retained proteins for which the *p*_{adj} value was below 0.01. The PCA indicated that groups of proteins were clearly separated according to their culture of origin. The differential comparisons of the protein contents allowed to propose some molecules that could be ranked in different categories.

Some proteins, mainly growth factors, were consumed by the MSC (not by PBMC) during culture (EGF, PDGF subunits A and B). An important proportion of proteins, known to be constitutively produced by MSC, were observed in all cultures containing these cells, such as collagen (Col1A1), metalloproteinases (MMP3), growth factors (HGF, PGF, VEGF, GDF-15), laminins (LAMA4), receptors (TNF-R1, LDL receptor, TNFRSF9), membrane markers (CD59, CD70, CD109) and others.

It is subtler to determine the function of proteins detected in the medium obtained from the PBMC- MSC coculture. Indeed, some of them may be conditioning ones, some may be already produced to promote immunomodulation, and both cell types may participate. Both functional proteins may co-exist in the medium. Because of their known pro-inflammatory function, AZU1, CCL3, CCL4, CCL24, GNLY, GZMA, IL-16, Kynureninase, MPO, and TNF α , may participate to create a pro-inflammatory environment leading to the conditioning of MSC (Dorner et al., 2002; Glass et al., 2006; Wensink et al., 2015; Yan et al., 2018a). On the other side, CHI3L1, CTSS, CXCL16, IL1-RA, LOX1, MMP7, TNF-R2 would participate to the immunomodulatory functions devoted to MSC (Andrews et al., 2022; Beldi et al., 2020; Harrell et al., 2020b; Liu et al., 2021; Murase et al., 2000; Ragni et al., 2020; Rodriguez et al., 2019; Rozier et al., 2021; Wang et al., 2018b; Zhang et al., 2013).

Proteins which may play a role in MSC immunomodulatory capacity were also detected in the medium produced by cMSC, once they were trypsinized after the coculture step and replated for 72hs. These molecules include Gal-1, Gal-3, PD-L1, GAS6, IL-6, Fas, which are well reported in literature for their involvement in inflammation regulation, cell adhesion and migration and apoptosis inhibition in diverse cell types including MSC (Baillly et al., 2021; Fajka-Boja et al., 2016; Ferreira et al., 2018b; Harrell

et al., 2021; Sioud et al., 2010; Tanaka and Siemann, 2020). Finally, some proteins were down-regulated upon conditioning such as ADM, CXCL11, CCL17, CCL19 and SNAP29. Interestingly these proteins were described as stimulators of proinflammatory cytokines release (Mastrodonato et al., 2018; Ozelik et al., 2019) or highly involved in response to IFN- γ .

As a note of caution, we detected the presence of molecules classically associated to membranes (mainly CD, *e.g.* CD59, CD70, CD90, CD109, CD274) whose physiological presence in a soluble form and effects are not always documented. Additionally, this proteomic study cannot discriminate the pure role played by extracellular vesicles.

Phenotypical markers are useful for quality control or even underline mechanisms of action. However, they do not necessarily reflect the biopotency of the cells and, because functional markers are mandated (de Wolf et al., 2017), they were explored.

In coculture of MSC and immune cells, bidirectional effects are observed, and they may evolve according to the duration of this interaction (Andreeva et al., 2017; Cuerquis et al., 2014). We took advantage of the Maxpar Direct Immune Profiling Assay (Fluidigm) using 30 markers to characterize 37 populations of immune cells by CyTOF. We implemented the barcoding of samples based on CD45 that allowed the reduction of technical and batch effect. To avoid biases due to donor intrinsic variability, samples of the different PBMC placed in coculture were compared to samples of the same donors but grown in wells devoided of MSC. We observed that, in coculture, the proportions of CD8⁺, CD4⁺ Treg, total and memory B cells, monocytes and dendritic cells increased, while total and central memory CD4⁺ T lymphocytes and naïve B lymphocytes are slightly decreased. To our knowledge, this is the first technical evidence of the simultaneous involvement of several populations of immune cells in the effect of MSC, especially in the absence of prior activation. This result confirms the validity of assessing population changes as functional readouts of the conditioning step, and may become the first element of a “functionality card” to be paralleled to the “phenotypic card” of cMSC.

The functional immunomodulatory capacities of MSC were also assessed *in vitro* through the inhibition of T cell proliferation. Direct contact between MSC and PBMC was sufficient to trigger inhibition, whatever the treatment of the cells. This is generally considered as a hallmark of immunomodulation capacities (Galipeau et al., 2016; Ramasamy et al., 2008). Inhibition is generally mediated by the activity of IDO1, the depletion of available tryptophan and the extracellular release of its metabolites (Chinnadurai et al., 2014; Meisel et al., 2004b). However, media conditioned by cMSC were able to produce stronger inhibitions than media conditioned by γ MSC and rMSC, suggesting that IFN- γ treatment of the cells does not add a benefit regarding this inhibitory effect in rMSC conditioned media. The observation that conditioned medium is efficient, is appealing in a clinical perspective.

Indeed, upon injection in the body and during their survey, MSC have little probability to contact directly several immune cells, while they will continuously produce and diffuse their secretome.

Of note, the inhibitory capacity of MSC, when placed in direct contact with PBMC, or even when producing conditioned media, was strictly dependent upon the biological status of the cells. Indeed, immediately after thawing, frozen cells did not show the inhibitory capacity, neither the medium they produced (data not shown). It is mandatory to expand the cells in culture for a few days to recover the integrality of their potency as recently suggested (Chinnadurai et al., 2016; François et al., 2012b; Giri and Galipeau, 2020).

Our gene and protein expression studies suggested the specific involvement of DPP4 in immunomodulation mediated by cMSC. DPP4 (i.e. CD26) may act through the induction of IL-10 (Hatano et al., 2015) and through its dipeptidase catalytic activity cleaving several chemokines, interleukins and growth factors involved in immune pathways (including the CCL and the CXCL family, IGF, TNF...) and converting them in inactive or antagonistic molecules that act in a negative feedback fashion (Metzemaekers et al., 2016; Ou et al., 2013; Rafei et al., 2008). DPP4 may act in soluble form and can be inhibited by saxagliptin, indeed Olink analysis showed that DPP4 was detectable in cMSC supernatants. We observed that conditioned medium produced by cMSC, when incubated with saxagliptin, loose a small but significant part of its inhibitory effect. This effect is not observed with conditioned medium produced by rMSC or γ MSC. This observation strengthens the hypothesis that DPP4 is involved in the immunomodulating pathways deployed by cMSC, even if it is not solely responsible for this inhibition.

Finally, the capacities of cMSC were evaluated *in vivo*. The NSG-MG model was developed to closely resemble the pathophysiology of the human disease. Although the triggering events responsible for MG are unknown and cannot be translated into a model yet, the other components are present in the NSG-MG (T and B cells, thymic inflammatory micro-environment and production of Ab). In this model, the multiple immune dysregulations may be counterbalanced by the multiple immunomodulation targets brought by MSC. One drawback of the model lies in its fidelity to the human clinical context, *i.e.* the reflection of the individual status of the patients. As previously reported (Sudres et al., 2017), we observed that the clinical status of mice groups reflected the severity of the disease presented by the MG donor of the thymus fragments, *i.e.* the most affected MG patient gave rise to the mice group showing the most severe clinical scores. For this reason, the clinical results were normalized to the initial status of the mice at the onset of the disease. We also observed variability in the level of humanization obtained after grafting of thymic fragments, potentially due to the quality of individual fragments which may depend on the surgical approach for exeresis, the implantation and the success

of the vascularization. This was considered in the randomization of mice for the preparation of homogenous groups of mice before treatment. We could not document a statistical link between the level of humanization and the severity of the clinical score. Among the components of the clinical score, the weight and the strength loss measurement were more informative and objective, than observational behavior or the capacity to cling to the inverted grid. The composite clinical scores documented significant differences brought by the injection of cMSC. This confirmed and extended the observations obtained previously using MSC of research grade (Sudres et al., 2017). We observed a trend towards an increase in the proportion of the Treg cells and decrease in Th17 subsets upon treatment with cMSC, which may represent a mechanistic clue of the effect of cells, as suggested by other groups in other contexts (Duffy et al., 2011; Negi and Griffin, 2020), especially at early stages of the disease (Luz-Crawford et al., 2013).

Final Conclusions

This study provides tools to elaborate mechanisms of action regarding the conditioning of MSC and their consecutive immunomodulation capacities. This study suggests that the conditioning of MSC by PBMC modulates the expression of particular genes, induces phenotypic changes, and the production of proteins involved in inhibition of proliferation and modulation of immune cell subsets. The results suggest that PBMC conditioning and IFN- γ treatment operate differently on MSC, and cMSC trigger classical and potential novel immunomodulation mechanisms with stronger effect *in vitro* and with demonstrated therapeutic benefit, *in vivo*, in a preclinical MG model. We then propose robust transcriptomic, phenotypical, and functional signatures to follow the MSC status throughout their production for clinical application and promote the use of this kind of conditioning.

Supplemental

Table S 1. Antibodies used for flow cytometry MSC characterization

Antibody	Clone	Isotype	Reference N°	Supplier
CD7	DK24	IgG2b, κ	F0789	Dako
CD9	M-L13	IgG1, κ	555372	BD
CD10	W8E7	IgG2a, κ	347503	BD
CD11b	94	IgM	6602573	Immunotech
CD13	WM15	IgG1, κ	561698	BD
CD14	M5E2	IgG2a, κ	555399	BD
CD24	ML5	IgG2a, κ	555428	BD
CD26	BA5b	IgG2a, κ	302709	Biolegend
CD29	MAR4	IgG1, κ	559883	BD
CD34	563	IgG1, κ	561209	BD
CD36	CB38	IgM, κ	555455	BD
CD40	5C3	IgG1, κ	560963	BD
CD44	DF1485	IgG1	F7083	Dako
CD45	HI30	IgG1, κ	555485	BD
CD45RA	HI100	IgG2b, κ	555488	BD
CD45RO	UCHL1	IgG2a, κ	11-0457-42	eBiosciences
CD47	B6H12	IgG1, κ	556045	BD
CD49a	SR84	IgG1, κ	559596	BD
CD49b	AK-7	IgG1, κ	555498	BD
CD49c	C3 II.1	IgG1, κ	556025	BD
CD49d	HP2/1	IgG1, κ	IM1404U	Immunotech
CD49e	IIA1	IgG1, κ	555617	BD
CD49f	GoH3	IgG2a, κ	555736	BD
CD49g	3C12	IgG1, κ	130-102-716	Miltenyi Biotec
CD54	HA58	IgG1, κ	555511	BD
CD55	IA10	IgG2a, κ	561901	BD
CD56	MY31	IgG1, κ	556647	BD
CD57	HCD57	IgM, κ	322306	Biolegend
CD59	H19	IgG2a, κ	560954	BD
CD61	VIPL2	IgG1, κ	555754	BD
CD62E	68-5H11	IgG1, κ	551145	BD
CD62L	DREG-56	IgG1, κ	555544	BD
CD62P	AK-4	IgG1, κ	555524	BD
CD69	FN50	IgG1, κ	555530	BD
CD73	AD2	IgG1, κ	550257	BD
CD80	2D10	IgG1, κ	305219	Biolegend

CD81	JS-81	IgG1, κ	561957	BD
CD86	IT2.2	IgG2b, κ	305412	Biolegend
CD90	5E10	IgG1, κ	F7274	Dako
CD105	SN6	IgG1, κ	12-1057-73	eBiosciences
CD106	51-10C9	IgG1, κ	555647	BD
CD112	TX31	IgG1, κ	337411	Biolegend
CD119	GIR-94	IgG2b, κ	308703	Biolegend
CD120b	367A02	IgG2a, κ	358403	Biolegend
CD140a	ϐR1	IgG2a, κ	556002	BD
CD140b	28D4	IgG2a, κ	558821	BD
CD146	P1H12	IgG1, κ	550315	BD
CD155	SKIL4	IgG1, κ	337617	Biolegend
CD166	3A6	IgG1, κ	559263	BD
CD172a/b	SE5A5	IgG1, κ	323809	Biolegend
CD183	49801	IgG1, κ	FAB160A	R&D
CD184	12G5	IgG2a, κ	555974	BD
CD194	1G1	IgG1, κ	561110	BD
CD200	MRC OX-104	IgG1, κ	552475	BD
CD221	1H7	IgG1, κ	555999	BD
CD271	ME20.4-1.H4	IgG1, κ	130-091-917	Miltenyi Biotec
CD273	24F.10C12	IgG2a, κ	329605	Biolegend
CD274	MIH3	IgG1, κ	374513	Biolegend
CD276	MIH42	IgG1, κ	351005	Biolegend
CD309	89106	IgG1, κ	FAB357P	R&D
CD317	R538E	IgG1, κ	348405	Biolegend
CD318	CUB1	IgG2b, κ	324017	Biolegend
HLA-ABC	G46-2.6	IgG1, κ	555553	BD
HLA-DR	L243	IgG2a, κ	307610	Biolegend

Table S 2. Antibodies used for mass cytometry MSC characterization

Extracellular Antigen	Metal tag	Clone
CD26	151 Eu	BA5b
CD34	166 Er	581
CD45	89 Y	HI30
CD49a	163 Dy	TS2/7
CD49c	161 Dy	ASC-1
CD49e	176 Yb	IIA1
CD54	170 Er	HA58
CD55	148 Nd	JS11
CD59	173 Yb	H19
CD61	209 Bi	VI-PL2
CD73	168 Er	AD2
CD105	152 Sm	266
CD112	169 Tm	TX31
CD120b	171 Yb	3G7A02
CD140a	160 Gd	D13C6
CD140b	156 Gd	18A2
CD155	165 Ho	1C6
CD172 a/b	175 Lu	SE5A5
CD194	158 Gd	L294H4
CD273	172 Yb	24F.10C12
CD274	159 Tb	29E.2A3
CD317	144 Nd	RS38E
HLA-ABC	141 Pr	W6/32
HLA-DR	174 Yb	L243

Intracellular Antigen	Metal tag	Clone
IDO1	154 Sm	703808
PTGS2	143 Nd	Polyclonal
KI-67	162 Dy	B56

Barcoding (CD90 metal tag)	Clone
145 Nd	5E10
147 Sm	5E10
149 Sm	F15-42-1
150 Nd	Thy-1A1
155 Gd	Thy-1A1
164 Dy	5E10

Table S 3. Markers assessed by MDIPA kit and PBMC subsets identification strategy

Antibody	Clone	Metal	Antibody	Clone	Metal
CD45	HI30	89Y	CD183 (CXCR3)	G025H7	156Gd
Live/dead indicator	N/A	103Rh	CD185 (CXCR5)	J252D4	158Gd
CD196 (CCR6)	G034E3	141Pr	CD28	CD28.2	160Gd
CD123	6H6	143Nd	CD38	HB-7	161Dy
CD19	HIB19	144Nd	CD56 (NCAM)	NCAM16.2	163Dy
CD4	RPA-T4	145Nd	TCRγδ	B1	164Dy
CD8a	RPA-T8	146Nd	CD294	BM16	166Er
CD11c	Bu15	147Sm	CD197 (CCR7)	G043H7	167Er
CD16	3G8	148Nd	CD14	63D3	168Er
CD45RO	UCHL1	149Sm	CD3	UCHT1	170Er
CD45RA	HI100	150Nd	CD20	2H7	171Yb
CD161	HP-3G10	151Eu	CD66b	G10F5	172Yb
CD194 (CCR4)	L291H4	152Sm	HLA-DR	LN3	173Yb
CD25	BC96	153Eu	IgD	IA6-2	174Yb
CD27	O323	154Sm	CD127	A019D5	176Yb
CD57	HCD57	155Gd			

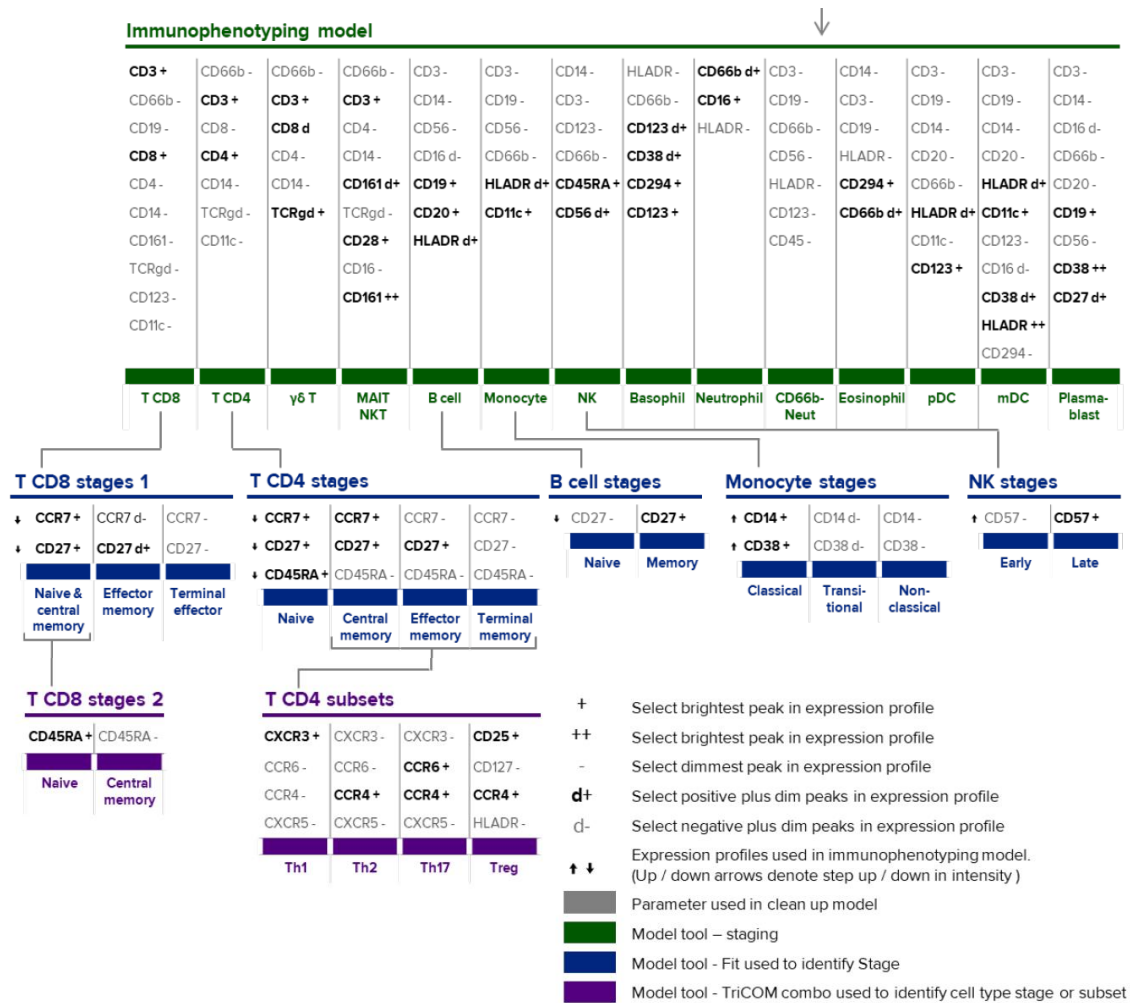


Table S 4. List of primers used for RT-qPCR

<i>Gene</i>	<i>Left Primer</i>	<i>Right Primer</i>	<i>Product size (bp)</i>	<i>Elongation time (sec)</i>	<i>Annealing T° (°C)</i>
CCL2	AGCAGCAAGTGTCCTCAAAGA	TCTGGGGAAAGCTAGGGGAA	195	14	60
CCL8	GGGACTTGCTCAGCCAGATT	CATCTCTCCTTGGGGTCAGC	186	18	60
CCL11	CCCAGAAACCACCACCTCTC	TGCCACTGGTGATTCTCCTG	216	14	60
CD74	AGACAGATCCCCGTTCTGA	GGGAAAGGGAAGAGAGTGGC	211	18	60
CILP	AGGCTGGGGAGTACTTTTGC	AGTCTTAACAGGGCAGCGTC	200	14	62
CXCL9	TGAGAAAGGGTCGCTGTTCC	GCTGACCTGTTTCTCCACT	206	14	62
CXCL10	CTGCCTCTCCCATCACTTCC	GCAGGGTCAGAACATCCACT	227	18	60
CXCL11	CTCCTTCCAAGAAGAGCAGCA	GCGTCTCTTTTGAACATGGG	156	14	62
DPP4	GCCACTTACCTTGCAAGCAC	CCGATCCCAGGACCATTGAG	238	14	60
HLA-DRA	AGACAAGTTCACCCACCAG	AGCATCAAACCTCCAGTCT	220	18	60
ICAM1	TTGGGCACTGCTGTCTACTG	GAAGTCCCAGCCCCATTTGA	226	14	62
IDO1	ACATGCTGCTCAGTTCCTCC	CTGGCTTGAGGAATCAGGA	223	14	60
IL6	TACCCCAGGAGAAGATTCC	GCCATCTTTGGAAGGTTGAG	199	14	62
LGALS1	AAACCTGGAGAGTGCCTTCG	GGAAGGGAAAGACAGCCTCC	205	14	62
PDCD1LG2	GCAATGTGACCCTGGAATGC	GTCCTTCGTCCTCACTTGG	189	14	60
PTGS2	CATCCCCTTCTGCCTGACAC	GCTCTGGTCAATGGAAGCCT	204	18	60
TGFB1	GGGACTATCCACCTGCAAGA	CCTCCTTGGCGTAGTAGTCG	239	14	62
TNFAIP3	TCGACAGAAACATCCAGGCC	AACAGCGCCTTCCTCAGTAC	175	14	62
TNFRSF11B	TGAACAACCTGCTGTGCTGC	ACGGTCTTCCACTTTGCTGT	181	14	62
TNIP1	TCAAAACCTCCCGAAGTGG	GCAAGGCTACTGTGAGTGGT	220	14	60
TNIP3	ACCAGCAATGGGATCAGCAA	TCTCTGCTGCTGCTGCTT	154	18	60
ZC3H12A	TTGTGAAGCTGGCCTACGAG	TGAGTGGCTTCTTACGCAGG	207	18	60

Table S 5. Antibodies for NSG-MG humanization follow-up

Taget	Clone	Isotype	Reference N°	Supplier
CCR4	1G1	IgG1 k	565391	BD
CCR6	11A9	IgG1 k	565925	BD
CD3	OKT3	IgG1 k	317317	Biolegend
CD4	SK3	IgG1 k	566321	BD
CD8	RPA-T8	IgG1 k	561453	BD
CD14	MφP9	IgG2b k	560270	BD
CD16	3G8	IgG1 k	560996	BD
CD19	HIB19	IgG1 k	560994	BD
CD25	M-A251	IgG1 k	563700	BD
CD38	HIT-2	IgG1 k	12-0389-42	eBiosciences
CD45	HI30	IgG1 k	560779	BD
CD56	NCAM 16.2	IgG2b k	345811	BD
CD90	5E10	IgG1 k	561971	BD
CD127	HIL-7R-M21	IgG1 k	560822	BD
CXCR3	1C6	IgG1 k	564032	BD

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III. Conclusion and perspectives

Conclusions

MG is a rare autoimmune disease with unmet medical needs

MG is due to dysregulations of the immune system involving several actors and finally mediated by auto-Ab targeting, mainly, the AChR at the NMJ. While the pathophysiology of the disease has made big advances in unraveling the altered biological process, identifying the targets and describing the different existing forms, the etiology is still not identified, hampering the possibility to predict or prevent the disease through medical or genetic screening or vaccination. The treatment is still based on pharmacological drugs. MG is invalidating, and maybe life-threatening. Treatments are symptomatic, or based on immunosuppression, they trigger long-term side effects or are poorly tolerated, some patients become intolerant or refractory, which worries their doctors in quest of new solutions (Gilhus et al., 2019). Novel biotherapies are arriving in the field, and proposed on the medical market, some encouraging results deserve full attention but long-term efficacy and side-effects are still not well-documented, and should be further followed-up. In this context, it is appealing to conceive immunomodulation through cell therapy, instead of immunosuppression through pharmacotherapy.

MSC nature and biological properties are better understood and exploited

After a period of strong enthusiasm almost 20 years ago, the first clinical applications turned out frustrating, until new discoveries and concepts made possible to improve the yields and uses. New sources of MSC, bearing higher proportions of cells and with increased immunomodulating capacities, have been identified and proposed, up to the stage of clinical grade production (Galipeau and Sensébé, 2018b). Efforts in science unveiled the pleiotropic mode of action of MSC immunobiology and allowed to sort-out the complementary mechanisms underlying MSC's biological properties. Efficient poly-targeting capacity of MSC made them very attractive tools to treat AID, in which immune dysregulation are usually complex and multifaceted.

Conditioning may change, or improve, the biological properties of MSC

Several molecules or specific microenvironments indeed modulate the expression of different gene panels, among which, some involved in immunomodulation, impacting on MSC functional capacities. Although the use of these molecules is appealing, the plethora of mechanisms triggered, simultaneously, may be responsible for molecular side effects. It was therefore challenging to set up more specific conditioning strategies.

Conditioned MSC preclinical proof of concept

In this context, the laboratory assessed the therapeutic efficacy of MSC conditioned by coculture with allogeneic, unstimulated PBMC, in a non-direct contact set-up. A proof of concept has been obtained using the new animal model developed in the laboratory, the NSG-MG mice humanized by the transfer of MG thymic fragments. In this model, MSC prepared from adipose tissue and grown in research grade and conditioned using PBMC, improved the clinical status of the animals (Sudres et al., 2017).

How to move towards clinical perspectives?

It is mandatory to consider some general aspects related to cells' production, and to the comprehension of their mechanisms when conceiving a potential clinical product. Indeed, (1) the cells should be produced avoiding the use of animal-derived products, considered as potential biohazards; (2) the cells may be easily characterized, at all steps of their production, using dedicated markers and providing cut-offs for validation; (3) the biopotency of the cells may be ascertained, and verified along their production; this may include *in vitro* and/or *in vivo* assays; (4) mechanisms of action may be delineated, to anticipate potential side effects; and (5) whenever possible, the sources of variability should be discarded or substituted by more standardized tools.

The PhD Thesis Project

To answer and fulfill previous cited requirements, along the PhD Thesis elaborated around this emerging project, (1) we characterized and compared RG and CG MSC profiles, (2) we explored the effects and mechanisms deployed by the interactions between PBMC and MSC, as compared to that of IFN- γ at transcriptomic and phenotypic levels, (3) we analyzed the secretomes to identify the main proteins responsible for conditioning effect, (4) we assessed the functional efficacies of conditioned MSC and their supernatants *in vitro*, and (5) we evaluated their efficacy *in vivo* in the NSG-MG model.

The homogeneity of CG MSC

The so-called clinical grade cells were prepared in a cell therapy facility using GMP grade reagents and medium, and PL. This substitute is equivalent, or even more potent to promote growth of MSC, and it avoids the biohazard issues related to the use of reagents derived from animals (Guiotto et al., 2020; Schallmoser et al., 2007; Viau et al., 2019). Preliminary tests (personal communication) comparing PL and FBS, using the same cultures, indicated that PL promoted a faster growth, but also a faster exhaustion of MSC, since cells' growth was limited to 11 passages and senescence apparition

was evident. In clinical practice, such situation should not be encountered, because MSC would now be used after a maximum of 3 passages.

One recommendation of ISCT is the testing of the differentiation potential of MSC. Indeed, these cells are able to differentiate into adipogenic, chondrogenic or osteogenic cells, depending on the supplements used to trigger these processes. We did not assess these properties in the laboratory during the PhD project, but the group of Pr. C. Martinaud, who provided the CG MSC, did so for 3 out of the 5 cultures. The results were positive (personal communication), therefore we did not repeat these experiments.

Our selection of 60 markers, based initially on literature compilation, confirmed the results obtained by others (Camilleri et al., 2016; Chinnadurai et al., 2018; Reis et al., 2018b). A few differences were noted regarding the expression of CD36 and CD146. We cannot exclude differences in the preparation of cells and reagents, between these studies and ours.

When grown in PL, MSC generally presented lower expression of markers than when grown in FBS, and the reason for this difference is unknown. FBS contains several classes of proteins, some of which may participate to cell adhesion and spreading, hence increasing the expression of extracellular markers involved in cell-cell contact or cell-substrate contact. Indeed, more than half of the 15 markers that were up-regulated in FBS, were involved in adhesion (CD29, CD44, integrins, CD61, CD146, CD166), co-stimulation, antigen presentation and immunomodulation (CD73, CD105, CD200, HLA-DR). Nevertheless, the use of PL did not substantially modify the ranking of expression of the markers between cultures, excepted for one culture (RG2) for which the correlation coefficient when compared to CG cells fell to 0,62. For the other cultures the correlation study revealed a very good homogeneity between the cultures. The use of PL was also reputed to homogenize, stabilize the cultures, however its preparation requires also standardization (Guiotto et al., 2020; Viau et al., 2019).

The transcriptomic signature of conditioning

We assessed the genes and patterns deregulated by PBMC conditioning, and compared it to the well-documented deregulations induced by IFN- γ (Guan et al., 2017a; Noronha et al., 2019; Sivanathan et al., 2014). A preliminary study (personal communication) underlined that the effect of IFN- γ was dose-dependent, and that its kinetics varied according to the gene under consideration, which formed the basis for the selection of the doses and timing used in the project. This dose allowed to confirm the results obtained in several studies regarding gene expression modifications (Guan et al., 2017a; Noronha et al., 2019; Sivanathan et al., 2014).

As indicated in the Venn diagram, the PBMC conditioning changed significantly the expression of 244 genes, *i.e.* almost 10 times less than DEG induced by IFN- γ treatment. A few, but some genes were modulated by both treatments. Interestingly, the PCA analysis underlined clearly the effect of IFN- γ treatment, but also pinpointed so-called batch effects. After correction, delimitation between rMSC and cMSC was recognizable and PBMC donor effect was identified (effects with P5 were stronger). This kind of biological variability may be due to intrinsic differences in the proportions of the categories of cells (granulocytes, monocytes, lymphocytes...), or their level of activation due to the particular status of the donor. Such difference has been pointed (Chinnadurai et al., 2018). To get rid of this kind of variability, or attenuate it, it would be possible to mix PBMC from different donors; however, this may activate mixed lymphocyte reactions prone to trigger different mechanisms of activation. One solution would consist in replacing the PBMC by the molecules they produce. This solution is being considered presently in the laboratory.

Among the genes deregulated by PBMC conditioning, IFN- γ treatment or both, we selected a panel to constitute a robust transcriptomic signature. The panel was not specifically based on fold change expression, but merely on the involvement in immunological pathways as indicated in **Table 10** below.

We could confirm that *CCL2*, *DPP4*, *IL6*, *PDCD1LG2*, *TNFRSF11B*, *TNIP1*, *TNIP3*, and *ZC3H12A* genes were upregulated specifically through PBMC conditioning. These genes employ several mechanisms: cleavage of several cytokines and inhibition of T cell proliferation (*DPP4*) (Metzemaekers et al., 2016; Ou et al., 2013), checkpoint inhibition (*PDCD1LG2*), inhibition of NF- κ B pathway (*TNIP1*, *TNIP3*, *ZC3H12A*) (Dang et al., 2016; Matsushita et al., 2009; Peng et al., 2020; Pu et al., 2020; Verstreppe et al., 2014; Yin et al., 2022) and pleiotropic activities (*CCL2*, *IL6*) (Bouffi et al., 2010; Philipp et al., 2018). Meanwhile, we confirmed that *CCL8*, *CD74*, *CXCL9*, *CXCL10*, *CXCL11*, *HLA-DR*, *IDO-1*, *TGFb1*, *TNIP3*, were upregulated upon IFN- γ treatment as described previously (Guan et al., 2017a; Noronha et al., 2019; Sivanathan et al., 2014). Interestingly, *ICAM-1*, a cell adhesion molecule playing an important role in cell-cell contact and involved in immunomodulation (Espagnollet et al., 2017; Liu et al., 2020b; Ren et al., 2010a) was upregulated by both PBMC conditioning and IFN- γ priming.

Taken together, and along with the analysis of the pathways in both treatments, these results document specific mechanisms used by PBMC conditioning, and they provide a set of genes to be assessed as a signature, or as readouts, for the control of proper conditioning in the process of production (**Table 11** below).

Table 10. Panel of genes assessed by qPCR and their function according to GeneCards.org

Gene name	Function
CCL2 (MCP-1)	C-C Motif Chemokine Ligand 2. Involved in immunoregulatory and inflammatory processes. Displays chemotactic activity for monocytes and basophils.
CCL8 (MCP-2)	C-C Motif Chemokine Ligand 8. Displays chemotactic activity for monocytes, lymphocytes, basophils, and eosinophils.
CCL11 (Eotaxin)	C-C Motif Chemokine Ligand 11. Displays chemotactic activity for eosinophils, but not mononuclear cells or neutrophils.
CD74 (class II γ chain)	Associates with class II MHC. Important chaperone that regulates antigen presentation.
CXCL9	C-X-C Motif Chemokine Ligand 9. Involved in T cell trafficking. Chemoattractant for lymphocytes but not for neutrophils, affects the growth, or activation state of immune and inflammatory cells.
CXCL10	C-X-C Motif Chemokine Ligand 10. Involved in stimulation of monocytes, NK and T-cell migration, and modulation of adhesion molecule expression. Involved in chemotaxis, differentiation, and activation, regulation of cell growth, apoptosis, and angiostatic effects.
CXCL11	C-X-C Motif Chemokine Ligand 11. Chemotactic for interleukin-activated T-cells but not unstimulated T-cells, neutrophils, or monocytes.
DPP4 (CD26)	Dipeptidyl Peptidase 4. Highly involved in glucose and insulin metabolism, as well as in immune regulation. Regulates various physiological processes by cleaving peptides in the circulation. Acts as a positive regulator of T-cell coactivation.
HLA-DR	Plays a central role in the immune system and response by presenting peptides derived from extracellular proteins.
ICAM1 (CD54)	Intercellular Adhesion Molecule 1. Cell surface glycoprotein typically expressed on cells of the immune system. Required for several interactions between various cell types.
IDO1	Indoleamine 2,3-Dioxygenase 1. Plays a role in antimicrobial and antitumor defense, neuropathology, immunoregulation, and antioxidant activity. Modulates T-cell behavior by catabolism of tryptophan. Involved in the peripheral immune tolerance.
IL-6	Interleukin 6 (previously IFNβ2). Functions in inflammation and the maturation of B cells. Essential for the development of T follicular helper (Tfh) cells required for the induction of germinal-center formation. Required to drive naive CD4(+) T cells to the Th17 lineage.
PDCD1LG2 (CD273)	Programmed Cell Death 1 Ligand 2. Involved in negative regulation of activated T cell proliferation, of IFN- γ and interleukin-10 production. Involved in the costimulatory signal, essential for T-cell proliferation in a PDCD1-independent manner.
TGFβ1	Transforming Growth Factor Beta 1. Regulates cell proliferation, differentiation, and growth, modulates expression and activation of growth factors. At high concentrations favors Treg cell development while at low concentrations, favors differentiation to Th17 cells.
TNFAIP3 (A20)	TNF Alpha Induced Protein 3. Involved in the cytokine-mediated immune and inflammatory responses. Inhibitor of programmed cell death. Has a role in the function of the lymphoid system.
TNFRSF11B (OPG)	TNF Receptor Superfamily Member 11b (osteoprotegerin). Negative regulator of bone resorption and osteoclast development. Plays a role in lymph-node organogenesis.
TNIP1	TNFAIP3 Interacting Protein 1. Plays a role in autoimmunity and tissue homeostasis through the regulation of nuclear factor kappa-B activation. Involved in the prevention of autoimmunity. Involved in the anti-inflammatory response of macrophages.
TNIP3	TNFAIP3 Interacting Protein 3. Involved in cellular response to lipopolysaccharide; negative regulation of I-kappaB kinase/NF-kappaB signaling; and TLR signaling pathway.
ZC3H12A (MCP-IP1)	Zinc Finger CCCH-Type Containing 12A. Endoribonuclease involved in cellular inflammatory response and immune homeostasis, adipogenesis and angiogenesis. Prevents aberrant T-cell-mediated immune reaction in controlling T-cell activation. Negatively regulates macrophage-mediated inflammatory response. Plays a role in the regulation of macrophage polarization.

The phenotypic signature of conditioned cells

Phenotyping cells helps to characterize them, distinguish their status, understand some mechanisms of action, and validate them for further uses when cut-offs are established. Hence, the ISCT validated recommendations include the phenotypical characterization of MSC using a minimal set of positive and negative markers (Dominici et al., 2006). Similarly, we searched to establish a specific signature characterizing cMSC by assessing the expression of markers known or foreseen to be expressed by MSC, and CD markers revealed among the dysregulated genes. As positive control of cell activation, we also validated the signature for MSC treated by IFN- γ , gathering informations from the literature (Chinnadurai et al., 2018; Guan et al., 2017b; Krampera et al., 2013; Ménard and Tarte, 2013). Singular markers expression assessment was complemented by CyTOF analysis, which allowed the simultaneous study of more than 30 ones.

We observed the specific increased expression of CD26, CD105, CD273 and CD318 under conditioning by PBMC, CD120b being more difficult to interpret because of modulation variability among donors. We confirmed that CD54 is increased by both IFN- γ and PBMC conditioning. These markers are involved in immunological functions through cell-cell or cell-substrate communication such as CD54, integrins, CD318 (Haddad and Saldanha-Araujo, 2014; Liu et al., 2020b; Ren et al., 2010c; Ruth et al., 2021) and through immunosuppressive capacity such as CD273. Some markers were down-regulated (CD49a, CD59) but the use of down-regulated markers is more difficult to consider when designing cut-offs and validations. We also confirmed the specific induction of some proteins (CD274, CD317, HLA-ABC and HLA-DR) by IFN- γ , as expected.

The CyTOF analysis confirmed these results. This methodology is not useable in routine procedure but it allows the simultaneous analysis of several markers at the single cell level. Technical difficulties and highly impacting limitations were encountered in several steps of classical CyTOF protocol, especially regarding the barcoding strategy. Then, replacements, adaptations and the set-up of an original barcoding procedure based on MSC's CD90 expression, which is a hallmark of resting and activated MSC, was performed. The resulting surface barcoding approach allowed reduction of technical variabilities while having a minimal impact on cells phenotype by preserving the conformation of MSC membrane antigens. In this study, we further observed that IDO1 and PTGS2, which were not screened by classical flow cytometry, were found expressed by CyTOF. Both molecules are importantly related to MSC immunomodulation capacity through enzymatic activities and production of active catabolites such as IDO1, and through direct activity on several receptors and substrates such as PTGS2 (Aggarwal and Pittenger, 2005; Meisel et al., 2004a).

Taken together, these markers integrate the proposed phenotypic signature (**Table 11** below).

Table 11. Conditioned MSC Specific signature

Signature nature	Read-outs
Transcriptomic signature (Q-PCR)	<i>CCL2, CCL11, DPP4, ICAM-1, IL6, PDCD1LG2, TNFRSF11B, TNIP1, TNIP3, ZC3H12A</i>
Phenotypic signature (Flow cytometry)	Increased: CD26, CD54, CD105, CD273, CD318 Not increased: HLA-DR Intracellular: IDO1, PTGS2
Functional assessment <i>in vitro</i> (Flow cytometry)	Inhibition of activated T cell proliferation Increased Treg proportion
Proteomic signature	To be confirmed and validated

The secretomes of conditioning and conditioned cells

We attempted to open the “black box” constituted by the culture supernatants produced by PBMC and MSC cells before, during and after the steps of conditioning. Indeed, we tried to identify and discover the proteins involved in MSC conditioning by PBMC and the molecules produced consequently by cMSC to exert immunomodulation, in order to understand some mechanisms, and then to try to mimic them later. The study of these secretomes is constitutively different (and complementary) from the analysis of RNAseq, since it provides informations regarding proteins whose production and stability may not be reflected by gene expression.

Among the techniques available, we have chosen the Olink proximity extension assay methodology, which allows the screening of a large number of molecules, without the preconceived idea brought by ready-to-use multiplex assays which are as expensive but contain a limited number of candidate proteins restricted to a small number of functions. The Olink methodology has been previously tested and compared in the laboratory to classical ELISA assays and to the Somalogic methodology and it was concluded that the Olink approach was very reproducible, robust and sensitive, providing a very large linear range of analysis (Sonia Berrih-Aknin, personal communication). Then, this method offers the advantage to allow research and discovery. However, our strategy for this research was still slightly oriented. Indeed, the test was performed using plates dedicated to more or less specific pathways, *i.e.* pathways including immune processes. A preliminary search performed by Olink also documented the identity of plates containing the highest number of candidates linked to MSC or PBMC biology. We therefore selected the plates containing the highest numbers of candidate proteins susceptible to be involved in immune modulations, and cell-cell interactions, they were named: Cardiometabolic, Cardiovascular II, Cardiovascular III, Development, Immuno-Response, Immuno-Oncology, Neurology. Some proteins were redundant from one plate to the other, and this

allowed us to challenge the robustness of the methodology, in comparing the results obtained with different plates regarding the same candidate protein.

We are aware that we have been finally limited by the number of plates (i.e. the number of proteins) to be tested, since new methodologies allow now to assess more than 3000 proteins. Also, the Olink PEA methodology is based on relative comparisons, and does not provide quantitative results. If required, absolute quantifications must be done separately.

In a first step, we performed a pilot study, whose aim was to validate the search in culture supernatants prepared in our conditions, to validate the appropriate dilution of samples, and to assess the robustness. This step was passed successfully using 3 plates, 3 dilutions, and 12 samples (personal communication). The results obtained in this pilot study were perfectly reproduced when performing the complete study several months later with new samples.

Gathering the results obtained for each group (medium alone, PBMC alone, resting MSC, coculture PBMC-MSC, and conditioned MSC grown for 3 days), we observed a good segregation of treatment groups, confirming the notion that each condition participated to produce a specific medium within three days in culture. Bidirectional exchanges occurred in paracrine fashion. The study was not designed to assess the kinetics of these changes but still provided several innovative informations.

The major objective of PEA was circumscribed to the identification of molecules belonging to the 2 previous cited categories. However, the differential comparisons of the protein contents provided interesting complementary categories and molecules that could be ranked (arbitrary) in them. These categories were named: consumed, constitutively produced by MSC, conditioning, potentially immunomodulatory and inhibited by conditioning. (1) Proteins still present in culture medium alone but disappearing in culture with cells were consumed or degraded. (2) Proteins highly enriched in culture containing MSC in any form were considered as constitutively expressed by MSC. (3) Proteins present in coculture and in PBMC supernatants were either conditioning, or already immunomodulatory ones. Because of their known pro-inflammatory function, AZU1, CCL3, CCL4, CCL24, GNLY, GZMA, IL-16, Kynureninase, MPO, TNF α may participate to a pro-inflammatory environment prone to the conditioning of MSC (Dorner et al., 2002; Glass et al., 2006; Wensink et al., 2015; Yan et al., 2018a). Interestingly, traces of IFN- γ were observed and may be produced in small quantity by monocytes, but variability among PBMC donors did not allow attaining the stringent statistical cut-off that we imposed. On the other hand, CHI3L1, CTSS, CXCL16, IL1-RA, LOX1, MMP7, TNF-R2 would participate to the immunomodulatory functions devoted to MSC (Andrews et al., 2022; Beldi et al., 2020; Harrell et al., 2020b; Liu et al., 2021; Murase et al., 2000; Ragni et al., 2020; Rodriguez

et al., 2019; Rozier et al., 2021; Wang et al., 2018b; Zhang et al., 2013). (4) Proteins enriched in cultures of cMSC upon replating and growth for 3 days were playing a role in MSC immunomodulatory capacity; this included Gal-1, Gal-3, GAS6, IL-6, VEGF-A, Fas, which are involved in inflammation regulation, cell adhesion and migration, and apoptosis inhibition (Bailly et al., 2021; Fajka-Boja et al., 2016; Ferreira et al., 2018b; Harrell et al., 2021; Sioud et al., 2010; Tanaka and Siemann, 2020). (5) Proteins down-regulated in cultures of cMSC upon replating and grown for 3 days were considered inhibited; this included ADM, CXCL11, CCL17, CCL19 and SNAP29, described as stimulators of proinflammatory cytokines release (Mastrodonato et al., 2018; Ozcelik et al., 2019).

Of note, the exploratory power of Olink methodology is weaker compared to the RNAseq study and relatively more supervised, since with Olink methodology we measured only 609 proteins that went through a “selection filter”, while the RNAseq study evaluated the expression of 16 000 genes. Finally, RNAseq measures gene expression, while Olink measures secreted proteins, which are different entities and not necessarily transposable (e.g. case of Gal-1, which at transcriptomic level show reduced expression in cMSC, however its secretion is upregulated in cMSC supernatants compared to the other assessed conditions).

The characterization of these secretomes will allow us to define the proteins involved in conditioning and in immunomodulation, in future steps of this project.

The functional capacities of MSC *in vitro*

The phenotypical characterization is generally considered a required, but not completely sufficient, criteria to validate cells for use in cell therapy. Their biopotency, indeed, has to be documented. Since we are looking at the immunomodulatory capacity of MSC, and not at their differentiation capacity into a regenerative product such as bone, cartilage or adipose tissue, we investigated functions involved in the immunomodulation with a widely used method.

One hallmark of immunomodulation efficacy is the inhibition of the proliferation of activated T cells, and the methodology uses flow cytometry and the principle of successive dilutions of an incorporated dye through the divisions of activated cells (Ramasamy et al., 2008). We observed that direct contact between MSC and activated PBMC was sufficient to trigger inhibition of proliferation with the same efficacy, regardless of the (resting or activated) state MSC. At variance, when activated PBMC were incubated in medium produced by MSC of different conditions, we observed that the media conditioned by cMSC were able to produce stronger inhibitions than media conditioned by γ MSC and rMSC. Therefore, both direct contact and secretome were efficient. This observation is

especially reassuring in clinical perspective, since injected cells may continuously produce and diffuse their secretome *in vivo*, while their direct contact may be limited to a few cells.

Taking advantage of this methodology, and as a proof of validity, we assessed the potential involvement of DPP4 in immunomodulation, a possibility that was emerging from our RNAseq and phenotypic studies. DPP4 can be expressed as a membrane-bound form, but also as a soluble molecule. Indeed, Olink analysis showed that DPP4 was present in cMSC supernatant. DPP4 acts through different mechanisms, but its dipeptidase catalytic activity cleaves several chemokines, interleukins and growth factors involved in immune pathways, converting them in inactive or antagonistic molecules blocking these respective pathways (Metzemaekers et al., 2016; Ou et al., 2013). DPP4 catalytic activity can be inhibited by saxagliptin. We observed that conditioned media produced by cMSC, when incubated with saxagliptin, lost a significant part of their inhibitory effects. This observation reinforces the hypothesis that DPP4 is involved in the immunomodulating pathways deployed by cMSC, even if it is not solely responsible for this inhibition. This also constitutes an original validation of the inhibition assay to complete a “functionality card” of the cells.

It seems very important to remind, that freshly thawed MSC do not display their immunomodulation capacities. This was described previously (Chinnadurai et al., 2016; François et al., 2012b; Giri and Galipeau, 2020) and we could only confirm this loss. Growing cells in culture for a few days is sufficient to restore their capacities. In clinical practice, in several assays, MSC were produced in huge amounts, then frozen, and thawed a few minutes or hours before to be injected. This may be one reason for the lack of success of many trials in the past. In the same vein, it is counterproductive to use cells grown for several passages, hence getting closer to senescence. It is now essential to take into consideration the biological requirements, the viability and fitness of the cells during the conception of a new generation of clinical trials (Giri and Galipeau, 2020).

The inhibition of proliferation of activated T cells frequently goes hand in hand with the increase in proportion of Treg cells, which is part of the mechanism of immunomodulation activity (Negi and Griffin, 2020). We took advantage of the new MDIPA kit associated to the CyTOF technology, to screen the potential changes in proportions, phenotypes, or activation status of PBMC when placed in coculture with MSC. This should offer a snapshot of the biological paracrine interaction of MSC on PBMC. We observed that, in coculture, the proportions of CD4⁺Treg, CD8⁺T cells, B cells and especially memory B cells, monocytes and dendritic cells increased; while CD4⁺ T cells, especially central memory CD4⁺ and naïve B lymphocytes, are slightly decreased. This constitutes a strong evidence of the simultaneous involvement of several populations of immune cells in the effect of MSC, especially in the absence of prior activation.

Once populations of interest have been proposed (*i.e.* Treg, here) it is easy to use the most specific markers to build a rapid and easy assay by classical flow cytometry. This assay therefore provides useful readouts.

An overview of potential mechanisms

By gathering the results of gene deregulation study, of the differential secretomes' characterization and of phenotypic changes assessed by flow cytometry and CyTOF, we unveiled some actors of immunomodulation that may be involved at different degrees in the immunomodulation triggered by PBMC and performed by MSC. Some of them have impacts on the proliferation of T cells, and on the nature and activation status of cell populations contained in the PBMC preparations. It is therefore possible to propose the general synthetic **Figure 46** below.

Preclinical study in MG animal model

The laboratory has set a new animal model of MG, based on the active transfer of MG thymus fragments into immunodeficient mice (NSG-MG), for the purpose of studying the physiopathology of the disease and potential new treatments (Sudres et al., 2017). Upon humanization, the multiple immune dysregulations may be counterbalanced by the multifaceted immunomodulation mechanisms exerted by MSC, and the functional capacities of cMSC were therefore evaluated *in vivo*.

Despite variabilities in the yields of humanization, it was possible to constitute homogenous groups. The clinical score was a composition of weight evolution, observation of behavior, capacity to cling to an inverted grid, and strength measurement. Among them, weight and strength loss measures were the most informative and less subjective tests and received the most important weighting coefficients in the calculation of the global clinical score. We observed significant differences brought 2 weeks after the injection of cMSC, and lasting up to the time of euthanasia. All mice produced anti-AChR Ab, in low quantities, but we could not observe a decrease in the titers after any treatment; however, the technique chosen for this measurement was based on ELISA (commercial kit), not on radioimmunoassay or cell-based assay, and it was clearly lacking sensitivity (personal communication). As a whole, the functional efficacy of cMSC, thus, confirmed the results obtained previously in the laboratory using RG cells (Sudres et al., 2017), and this was one major aim of the Thesis Project. We observed a trend towards an increase in the proportion of the Treg cells and decrease in Th17 subsets upon treatment with cMSC, which may represent a mechanism of immunomodulation (Duffy et al., 2011; Luz-Crawford et al., 2013; Negi and Griffin, 2020).

The animal model, however, is difficult to establish, mainly due to the shortage of MG donors of thymic fragments. During the sanitary crisis linked to COVID 19, surgical interventions on MG patients were rare and a few pieces were transmitted to the laboratory, and furthermore animal experimentations were restricted. Also, as the model reproduces the disease status of the donor, a small proportion presented important clinical signs. For these reasons, we attempted to implement methodologies to valorize and maximize, in the future, the amount of information that can be obtained from every mouse. We assessed the follow-up of electromyography (EMG), and the quantification of voluntary exercise. These methodologies are still time-consuming but may be less operator-dependent, and easier to standardize.

EMG is directly focused on the modifications of the neuromuscular transmission. It reports early changes, *e.g.* the so-called myasthenic decrement during the acute phase (Pachner and Kantor, 1982; Plomp et al., 2015). EMG testing has been set to allow the longitudinal, weekly follow-up of the animals (either controls or cMSC-treated) in a minimally invasive way, thanks to the platform for evaluation of animal models in the Core Facility (UMS28). The methodology was inspired from that used for studies in Human, as described in the Introduction of this dissertation. While it was possible to observe myasthenic decrements in the classical EAMG model, especially using the snake venom bungarotoxin, an irreversible blocker of the AChR, for sensitization (Kennel et al., 1993), we only rarely observed decrements in NSG-MG mice. We explained this relative failure by the genetic background of the NSG mice, that would not be sensitive enough to the MG onset (Christadoss et al., 1985), and to the safety factor present in mice that protects their NMJ from the loss of an important proportion of AChR (Plomp et al., 2018).

Wheel running is a spontaneous activity performed by most animal species, which can complete long night runs (up to 20 km) without displeasure (Sherwin, 1998). The analysis of different parameters of the running session (average speed, daily cumulated time of exercise, average distance) allows discriminating several pathological models (Grajales-Reyes et al., 2017) but has never been used to assess MG onset, progression, or treatment, although the disease is characterized by fatigability worsened by effort and ameliorated by rest. We have set some conditions using the classical model of EAMG. We observed huge differences in the running performances of the animals, even healthy, and we observed that there were “good runners” and “bad runners”, maybe due to uncertain physiological or psychological aspects. In this asset, while we reduce the impact of operator dependent factors in the measures and we won in terms of “objectivity” compared to other tests, unfortunately we lose power interpretation due to mice intrinsic behavior variability. We would need high number of animals to get statistically valuable results, which is not yet possible with the NSG-MG mice. However, the laboratory is still working on the conditions of use of running wheels to explore the behavior of animals

developing a form of MG. Once the conditions are set, we will compare the running behavior of control mice and of our myasthenic models, upon treatment with cMSC.

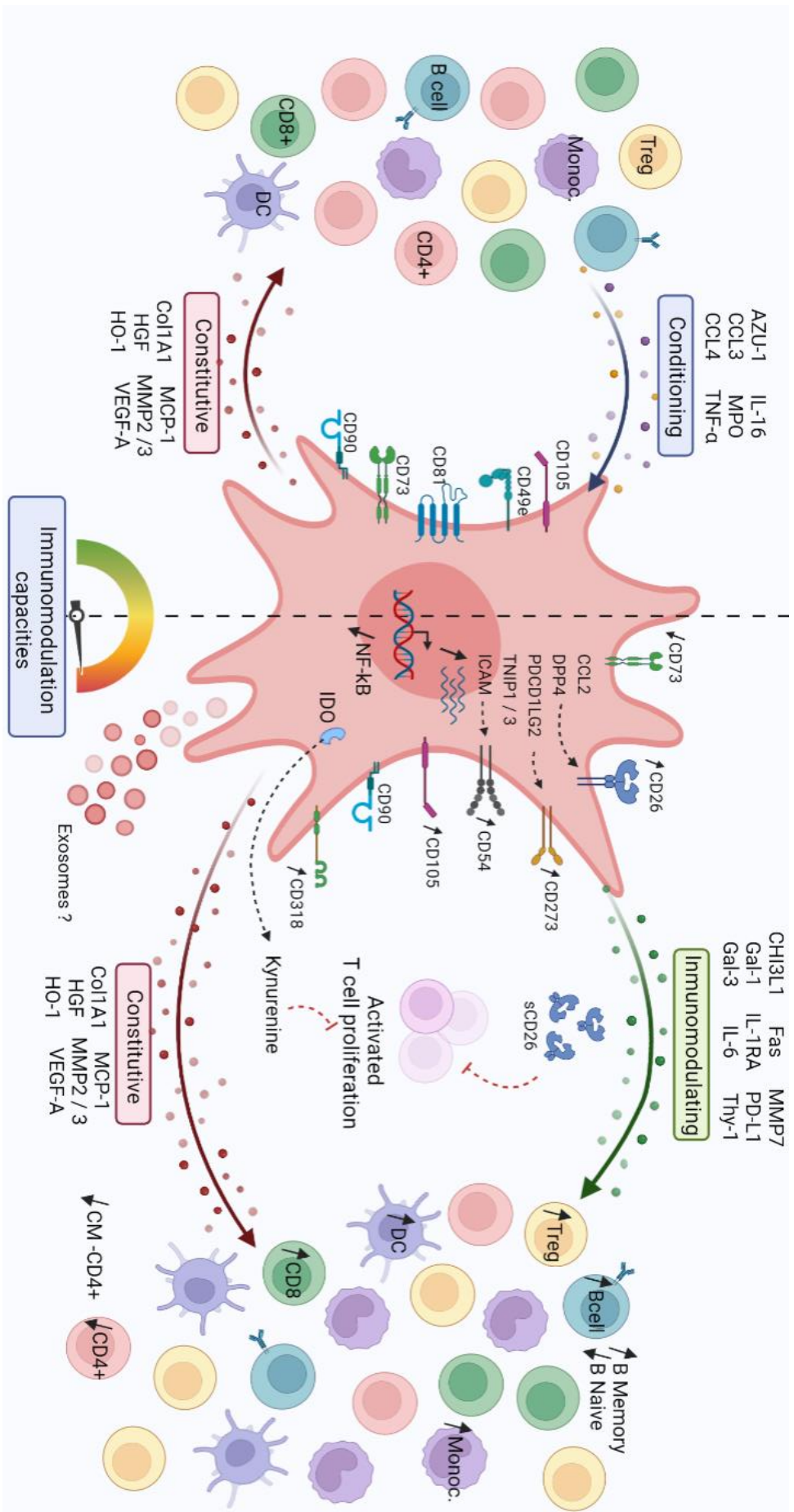


Figure 46. Schematic representation of events taking place upon conditioning of MSC with PBMC

Limits of the study

We are aware that the study presented some limitations, mainly technical or methodological, and some have been addressed along the dissertation.

We used 5 different cultures of clinical grade MSC, and 4 of research grade. We initially performed a correlation study, using 60 markers, and observed a very high correlation between cultures. We decided not to increase the number of MSC cultures, but to produce more “couples” associating one MSC to several PBMC while still keeping in workable numbers of associations.

We observed, as others (Chinnadurai et al., 2018), that variability induced by PBMC donors was present, probably due to the variable cellular composition of each batch. This, however, did not preclude statistical evaluations. One possibility to reduce this impact would be to mix several donors to perform the conditioning. However, in this context, allogeneic PBMC would cross-react and create mixed lymphocyte reactions which should be avoided in our approach of conditioning. A second possibility, which also substantiate the secretome study, is to identify the proteins that may be responsible for conditioning, and to replace PBMC by the recombinant proteins they produce. This approach is under development in the Laboratory.

The use of PL has been, initially, problematic. Indeed, due to a shortage of a provider, we had to change the nature of the PL that was used in a first set of experiments, and to repeat the experiments with the new PL product, which we then used all along. These step would, probably, have been avoided if recent data, demonstrating that PL was relatively stable and always provided the same kind of production (Guiotto et al., 2020), was available at that time. Nevertheless, our cultures are now well-characterized and the present signatures may serve as references for using new kinds of PL.

Regarding the secretome study, the proteins that have been identified and categorized in the different groups correspond, for sure, to a non-exhaustive list of their content, considering the fact that the number and nature of proteins assessed using the PEA methodology was limited and their choice was oriented. However, screening more than 600 by PEA represent a good compromise between the amount of obtained information, the time spent in sample preparation and data analysis and mostly the robustness of measures when compared to other techniques with higher exploratory power.

By the end of this project, perspectives are considered regarding methodological or conceptual aspects, for the short or long term. We proposed two last sections gathering the ideas and wishes to extend the project beyond this doctoral work.

Complementary studies: that could be done as a direct extension of the project

The phenotypic and functional signatures have been obtained using classical methodologies. By now, they could be more standardized, incorporated in a unique restricted panel allowing simultaneous assessment of markers modulation. While all markers assessed by classical flow cytometry are extracellular, CyTOF has shown that intracellular markers were expressed and detectable too (IDO1, PTGS2). Therefore, the methodology for assessing these intracellular markers in a routine fashion should be designed.

Along the same line, while populations of modified PBMC have been described using the MDIPA kit using CyTOF, populations of interest should be individualized (*e.g.* Treg, monocytes...) and dedicated panels to analyze these populations by classical flow cytometry should be designed. These steps will allow PBMC modulations track in an easier and standardized basis.

The transcriptomic study was centered on the MSC, whether resting, conditioned or activated by IFN- γ . However, documenting the transcriptomic modifications of the PBMC before, during and after conditioning would enlighten about the mechanisms used by PBMC to produce conditioning and the ones explaining the effects of the bidirectional dialog with the MSC during coculture.

The “omic” studies, *i.e.* transcriptomic, phenotypic and proteomic, provided distinct and complementary results. The statistical analyses of these studies have been done on the basis of individual omics, but it may be very interesting to integrate, and identify if complementary information can be obtained by crossing, merging and comparing omics outputs through bioinformatics tools.

As indicated above, collaborations are being set to establish the conditioning or the immunomodulatory capacities of the molecules identified within the secretomes. This will be done using the readouts established (transcriptomic and phenotypic signatures), using recombinant molecules individually. The assessment of molecules combinations will require high throughput screenings technics that are under study.

The search of the mechanisms of actions deployed by PBMC or MSC to exert their respective effects led to propose candidate genes and proteins. Using the readouts set previously, it will be of great interest to validate or infirm the pathways using either pharmacological molecules or molecular activators or inhibitors (*i.e.* siRNAs...). We started such a study, in exploring the potential place of DPP4

in the inhibition of activated T cells, using the pharmacological inhibitor saxagliptin. However, several pathways exist, that deserve explorations.

New concepts to explore, perspectives that could be considered as strategic avenues

One next step may be very useful when considering a potential translation towards clinical applications: how would a MG patient react to these cell therapy approaches? The preparation of MG patient PBMC would document to what extent these cells are responsive to conditioned MSC, *in vitro*. But obtaining MG patients MSC would help to consider the use of a patients' own MSC to accomplish immunomodulation, in view of an autologous use, if these MSC are as capable of immunomodulation as MSC from healthy donors.

One next step towards clinical application will rely on the documentation of biodistribution of MSC in immunodeficient mice under GLP-like conditions. Evaluation of the biodistribution of infused cells relies on the detection of human cells in several organs using human-specific sequences by qPCR. This study will go hand in hand with a toxicity study, which is mandated to trigger clinical trials. Of course, such studies would be done in collaboration with specialized teams.

While the project was focused on cellular therapy, the world of acellular therapy is opening and rapidly growing. We did not explore, here, the component of the secretomes represented by extracellular vesicles. These EV may be produced by cMSC, isolated, individualized, characterized, evaluated and compared to the cMSC. Would it be possible finally to replace the effective cMSC by the EV that they produce?

Finally, in this study, MG has been considered as a model system for studying AID. However, several of them share numerous mechanisms of action, and many would benefit from immunomodulatory therapeutic approaches. It would be interesting, then, to collaborate with complementary teams developing animal models of other AID, to assess the cMSC efficacy. The validation of this innovative therapy in MG may therefore pave the way for the development of this treatment for other pathologies involving immune dysregulations.

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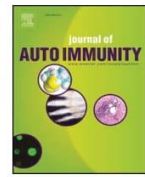
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IV. Other publications



IL-23/Th17 cell pathway: A promising target to alleviate thymic inflammation maintenance in myasthenia gravis

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ABSTRACT

IL-23/Th17 pathway has been identified to sustain inflammatory condition in several autoimmune diseases and therefore being targeted in various therapeutic and effective approaches. Patients affected with autoimmune myasthenia gravis exhibit a disease effector tissue, the thymus, that harbors ectopic germinal centers that sustain production of auto-antibodies, targeting proteins located in the neuromuscular junction, cause of the organ-specific chronic autoimmune disease.

The present study aims to investigate the IL-23/Th17 cell pathway in the thymic inflammatory and pathogenic events.

We found that thymuses of MG patients displayed overexpression of Interleukin-17, signature cytokine of activated Th17 cells. This activation was sustained by a higher secretion of Interleukin-23 by TEC, in addition to the increased expression of cytokines involved in Th17 cell development. The overexpression of Interleukin-23 was due to a dysregulation of interferon type I pathway. Besides, Interleukin-17 secreted, and Th17 cells were localized around thymic ectopic germinal centers. These cells expressed podoplanin, a protein involved in B-cell maturation and antibody secretion. Finally, production of Interleukin-23 was also promoted by Interleukin-17 secreted itself by Th17 cells, highlighting a chronic loop of inflammation sustained by thymic cell interaction.

Activation of the IL-23/Th17 pathway in the thymus of autoimmune myasthenia gravis patients creates an unstoppable loop of inflammation that may participate in ectopic germinal center maintenance. To alleviate the physio-pathological events in myasthenia gravis patients, this pathway may be considered as a new therapeutic target.

1. Introduction

Myasthenia gravis (MG) is an organ-specific chronic autoimmune disease caused by auto-antibodies that target proteins located in the neuromuscular junction. Most patients have antibodies directed against acetylcholine receptor (AChR). These patients commonly present thymic abnormalities such as follicular hyperplasia or thymoma in early and late onset patients, respectively [1]. Sudres et al. have

demonstrated that MG thymuses contain the pathological factors, including antibodies and defective T cells, required to induce MG symptoms in an immunodeficient mouse model [2]. A randomized clinical trial showed that thymectomy allows an amelioration of MG patient symptoms. However, this procedure is still not a cure since it does not fit to all patients, and most patients need other long-term therapies [3].

In AChR⁺ MG patients, inflammation occurs in the thymus and is sustained by interferon-related molecules. This inflammation is

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Abbreviations			
AChR	acetylcholine receptor	IFN	interferon
AID	activation-induced cytidine deaminase	IL	interleukin
CD	cluster of differentiation	MG	myasthenia gravis
CCL	chemokine (C-C motif) ligand	mRNA	messenger ribonucleic acid
CCR6	C chemokine receptor 6	PBMC	peripheral blood cell
CXCR5	C-X-C chemokine receptor type 5	qPCR	quantitative polymerase chain reaction
DC	dendritic cell	PDPN	podoplanin
EAMG	experimental autoimmune myasthenia gravis mouse model	Poly(I:C)	polyinosinic-polycytidylic acid
eGC	ectopic Germinal center	ROR γ T	retinoic acid-related orphan receptor gamma T
FDA	food and drug administration	TEC	thymic epithelial cell
GM-CSF	Granulocyte-macrophage colony-stimulating factor	TGF	tumor growth factor
		Th	T-helper cell
		TNF α	tumor necrosis factor α
		Treg	regulatory T cell

described to be driven by the secretion of pro-inflammatory cytokines (Interleukin (IL)-6, Interferon γ (IFN- γ), tumor necrosis factor α (TNF α) and IL-17 [4,5]. An altered development and function of different subpopulations of T cells, such as regulatory T cells (Tregs) and T helper 17 cells (Th17), can be the result of the tissue microenvironment cytokines content. Accumulating data have illustrated that a disequilibrium between these two subpopulations of T cells is often involved in the pathogenesis of autoimmune diseases. In AChR⁺ MG patients, there is a strong defective function of Treg cells [6,7]. In addition, cytokines related to Th17 cells (IL-17 and IL-21) are increased in Treg cells and also in conventional T cells in the thymus and peripheral blood cells (PBMCs) of MG patients compared with controls [8,9].

AChR⁺ MG hyperplastic thymuses harbor ectopic germinal centers (eGCs), structures where B-cells are chemo-attracted and activated resulting in antibody production [10]. In various models of experimental autoimmune disease, IL-17 secreting CD4⁺ T cells participate to the loss of B-cell tolerance [11,12] and probably to eGC antibody secretion process [13] by expressing an anchoring protein, podoplanin (PDPN) [14] to stabilize their effects within the eGCs [15]. Previous studies, by using mice with spontaneous experimental autoimmune encephalomyelitis, have shown a correlation between PDPN expression levels and the formation of eGCs in the central nervous system. By blocking PDPN, the formation of eGCs is stopped, and disease progression and symptoms are partially prevented [16].

Therefore, we investigated the cellular components and the actors that may sustain IL-17 over-expression and its role in the thymic eGCs formation. Here, we quantified and located the Th17 cells inside the MG thymus. We then deciphered the level of expression of cytokines involved in the Th17-cell differentiation and activation processes, and the cellular mechanisms underlining their over-activation in MG thymuses. We identified an uncontrolled loop, involving IL-23, thymic epithelial cells (TECs), Th17 cells, PDPN and type 1 interferon (IFN-I) pathway, that may sustain the chronic thymic inflammation process and probably participate in antibody production.

2. Material and methods

2.1. Thymic biopsies

Control and MG thymic biopsies were obtained from patients undergoing respectively corrective cardiovascular surgery or thymectomy at Marie Lannelongue Chirurgical Center (Le Plessis-Robinson, France) and at the Strasbourg civil hospital (Strasbourg, France). 51 healthy controls (aged of two days to 45 years old) were used. 72 MG thymuses (aged of 12–53 years old) were included in the study (Table 1).

2.2. Human serum and blood cells

Human serum and peripheral blood mononuclear cells (PBMCs)

were obtained from whole blood of healthy patients collected by the Etablissement Français du Sang and AChR⁺ MG patients during their clinical follow up.

Clinical data of MG patients are summarized in Table 1. The study was under the French Bioethic Law that requires a written informed consent from the donors or the legal representant. In respect to this law, this study was approved by the local ethics committee (CPP, Kremlin-Bicêtre, France: agreement N°06-018; CCP Ile de France Paris 7, France agreement N°C09-36).

2.3. Primary cell cultures

Primary human thymic epithelial cell (TEC) cultures were established following the protocol previously described [17,18]. After 7 days of primary culture, cells were trypsinized, seeded in RPMI medium containing 5% of horse serum and then allowed to attach to the flask for 24 h before treatment. Cells were treated in RPMI medium containing 0.5% of horse serum for 24 h with Poly (I:C) (100 μ g/ml; InvivoGen, Toulouse, France), IL-17A (100 pg/ml or 10 ng/ml; R&D systems, Lille, France), anti-IL-17 receptor antibody (1 μ g/ml, R&D systems, Lille, France), IFN- γ (1000UI/ml; R&D systems, Lille, France), LPS (10 ng/ml, Enzo Life Sciences, Villeurbanne, France), IFN-type I (1000UI; R&D systems, Lille, France) and anti-IFN α / β receptor (10 μ g/ml; Enzo Life Sciences, Villeurbanne, France).

2.4. Co-culture assays

Freshly Ficoll isolated PBMCs were seeded into 12-well plates in an RPMI 1640 Glutamax I medium supplemented with 10% fetal calf serum, alone or with primary cultured TECs in a ratio of 2:1. A capture antibody anti-IL-23p19 (1 ng/ml; R&D systems, Lille, France) was added in the culture medium. TECs and PBMCs were incubated at 37 °C in contact for 24 h. For FACS analysis, to inhibit cytokine secretion by PBMCs, Brefeldin A (10 μ g/ml; Sigma-Aldrich, Lyon, France) was added to the culture medium for 4 h before the cells staining. For RNA analysis, PBMCs and TECs were washed twice with PBS 1X and quick frozen prior RNA extractions.

2.5. Quantitative real-time PCR

Gene expression was evaluated by quantitative real-time PCR performed using the Light-Cycler apparatus (Roche Diagnostics; Meylan, France) as previously described by Dragin et al. [19]. Arbitrary units were calculated as previously described [4,20]. Each PCR was performed using the Fast-start DNA Master SYBR Green I kit (Roche Diagnostics; Meylan, France) according to the manufacturer's instruction. Each cDNA sample was run in duplicate. mRNA expression was normalized to 28S for thymic global biopsies or GAPDH for purified thymocytes, PBMCs or cultured cells. The list of primers used is

Table 1

Clinical data of Myasthenia Gravis patients. The degree of thymic hyperplasia was determined by an anatomic-pathologist that quantified the number of germinal centers by thymic sections as followed: + + + + :very numerous; + + + : numerous; + + :Some, + :Rare; - :No clear germinal centers. NA: not available; NT: No treatment; IVIG: Intravenous Immunoglobulins.

	GENDER	AGE OF THYMECTOMY	DEGREE OF HYPERPLASIA	CHOLINESTERASE TREATMENT	CORTICOIDS & OTHER TREATMENTS	TECs	THYMOCYTES	THYMUS	SERUM
MG 1	F	14	++	NA	NA	X			
MG 2	F	27	++	mestinon	NT	X			
MG 3	F	28	-	mestinon	IVIG	X			
MG 4	F	37	++	mestinon	NT	X			
MG 5	F	19	+++	Yes	NT	X			
MG 6	F	40	+++	mestinon	NT	X			
MG 7	F	27	++	mestinon	NT	X			
MG 8	F	19	-	mestinon	NT	X	X		
MG 9	F	18	+++	mytelase	NT	X			
MG 10	M	21	-	mestinon	NT	X			
MG 11	F	32	+	mytelase	Mycophenolate mofetil	X			
MG 12	M	12	++	mytelase	IVIG	X			
MG 13	F	30	-	mestinon	IVIG	X			
MG 14	F	22	++	mytelase	IVIG	X			
MG 15	M	13	+++	NT	NT	X			
MG 16	M	41	+++	mytelase	NT	X			
MG 17	F	18	-	mestinon	Corticoids/IVIG	X			
MG 18	F	12	+++	mytelase	NT	X			
MG 19	F	14	++	mestinon	NT	X			
MG 20	F	38	++	mestinon	NT	X			
MG 21	F	29	++	mestinon	NT	X			
MG 22	F	36	+++	mestinon	NT		X		
MG 23	F	29	+	mestinon	Prednisone/IVIG	X			
MG 24	F	26	+++	mestinon	NT	X			
MG 25	F	25	+++	mytelase	NT	X			
MG 26	F	22	++++	mytelase	NT	X			
MG 27	F	22	++++	NA	NA	X			
MG 28	M	31	NA	NA	NA	X			
MG 29	F	29	++	mestinon	NT	X			
MG 30	M	19	+++	mestinon	NT	X			
MG 31	F	20	+++	mestinon	NT	X			
MG 32	F	24	++++	mytelase	NT	X			
MG 33	F	25	+++	mytelase	NT	X			
MG 34	M	25	++	mestinon	NT	X			
MG 35	F	16	++	mytelase	NT	X			
MG 36	F	16	+++	mytelase	NT	X			
MG 37	F	41	+++	mestinon	NT	X			
MG 38	F	32	+++	mytelase	NT	X			
MG 39	F	23	++	mestinon	NT	X			
MG 40	F	17	++	mestinon	NT	X			
MG 41	F	26	++	mestinon	NT	X			
MG 42	M	30	-	mytelase	NT	X			
MG 43	M	29	++	mytelase	NT	X			
MG 44	M	27	++	mytelase	NT	X			
MG 45	F	22	+++	mestinon	NT	X			
MG 46	F	22	+++	mestinon	NT	X			
MG 47	F	18	+++	mytelase	NT	X			
MG 48	M	53	++	mytelase	NT			X	
MG 49	F	44	++	mestinon	NT			X	
MG 50	M	38	-	mytelase	NT			X	
MG 51	F	16	-	mytelase	NT			X	
MG 52	F	50	++	mytelase	NT			X	
MG 53	F	18	++++	mestinon/mytelase	NT			X	
MG 54	F	14	+++	mestinon	IVIG			X	
MG 55	F	32	++	mestinon	NT			X	
MG 56	F	26	++	mytelase	NT			X	
MG 57	F	20	++	mestinon	NT			X	
MG 58	F	21	+++	mytelase	NT			X	
MG 59	F	34	++	mestinon	NT			X	
MG 60	F	15	++	mestinon	cortancyl			X	
MG 61	F	22	-	mestinon	cortancyl/IVIG			X	
MG 62	F	17	-	mytelase	NT			X	
MG 63	F	44	-	mestinon	cortancyl			X	
MG 64	F	32	++	mytelase	NT			X	
MG 65	F	33	-	mestinon/mytelase	cortancyl			X	
MG 66	F	35	-	NT	NT			X	
MG 67	F	24	+++	mestinon/mytelase	NT			X	
MG 68	F	35	+++	mestinon/mytelase	NT			X	
MG 69	F	29	+++	mytelase	NT			X	
MG 70	F	24	+++	mytelase	NT			X	
MG 71	F	25	+++	mytelase	NT			X	

(continued on next page)

Table 1 (continued)

	GENDER	AGE OF THYMECTOMY	DEGREE OF HYPERPLASIA	CHOLINESTERASE TREATMENT	CORTICOIDS & OTHER TREATMENTS	TECs	THYMOCYTES	THYMUS	SERUM
MG 72	F	33	–	mestinin/mytelase	cortancyl			X	
MG 73	F	38	+++	mytelase	NT				X
MG 74	F	19	+++	mestinin	NT				X
MG 75	F	32	++	mytelase	NT				X
MG 76	F	14	++	mytelase	NT				X
MG 77	F	40	+++	mytelase	NT				X
MG 78	F	29	+++	mestinin	NT				X
MG 79	F	31	++	mestinin	NT				X
MG 80	F	19	+++	mestinin	NT				X
MG 81	F	19	–	NT	cortancyl				X
MG 82	M	21	–	NT	cortancyl				X
MG 83	F	23	+++	mestinin	solupred				X
MG 84	F	26	+	imurel/mestinin	cortancyl				X
MG 85	M	28	–	mestinin	cortancyl				X
MG 86	F	29	–	mestinin	cortancyl				X
MG 87	M	31	++++	mestinin	cortancyl				X
MG 88	M	35	–	mytelase	cortancyl				X
MG 89	F	35	–	imurel/mestinin	cortancyl				X
MG 90	M	38	++++	mestinin	corticoids				X
MG 91	M	19	++++	mestinin	NT				X
MG 92	F	24	+++	mytelase	NT				X
MG 93	M	25	++	mestinin	NT				X
MG 94	F	26	++++	mestinin	NT				X
MG 95	F	29	++++	mytelase	NT				X
MG 96	M	29	++++	mestinin	NT				X
MG 97	F	35	++	mestinin	NT				X
MG 98	F	36	++	mestinin	NT				X
MG 99	F	39	+++	mytelase	NT				X
MG 100	M	27	++	mytelase	NT				X

summarized in Table 2.

2.6. ELISA

The levels of cytokines (IL-17, IL-23, IL-6, IL-1β, TGFβ3) were analyzed in serum and TEC supernatants using the DuoSet Elisa kit (R&D systems, Lille, France). For total thymic protein analysis, we followed the protocol previously described by Meraouna et al. [10]. Total thymic proteins were extracted in solution containing 5% Tris HCl 20 mM, 0.1% Triton X100, and one tablet of protease inhibitor cocktail (complete-mini; Roche-Diagnostics, Meylan, France) using the fast prep apparatus. Each ELISA was performed according to the manufacturer's instructions. ELISA reactions were read with a TECAN SPARK ELISA microplate reader.

2.7. Flow cytometry analyses

To analyze the secretion of IL-17 by thymocytes, frozen MG and control cells were unfrozen in fetal bovine serum. Cells were incubated in X-vivo medium and stimulated with Phorbol 12-Myristate 13-Acetate (100 ng/ml, Sigma-Aldrich, Lyon France), Ionomycin (1 μg/ml; Sigma-Aldrich, Lyon, France) and Brefeldin A (10 μg/ml; Sigma-Aldrich, Lyon, France) for 4 h. Activated thymocytes were then stained with fluorochrome-conjugated antibodies for 30 min at 4 °C before being permeabilized with the FoxP3 permeabilization kit (eBioscience, Paris, France) and labeled with anti-IL-17A antibody according to the manufacturer's instruction. All analyses were done with the cytometer FACS Canto II (BD Biosciences, Le Pont de Claix, France).

The same procedure was used for PBMCs recovered from co-cultured experiments.

2.8. Immunofluorescence microscopy

Cryostat sections (7 μm) of frozen human thymic tissues were fixed with acetone to glass superfrost slides and dried for 1 h. The human thymic sections were pre-incubated with a blocking buffer (PBS, 0.1%

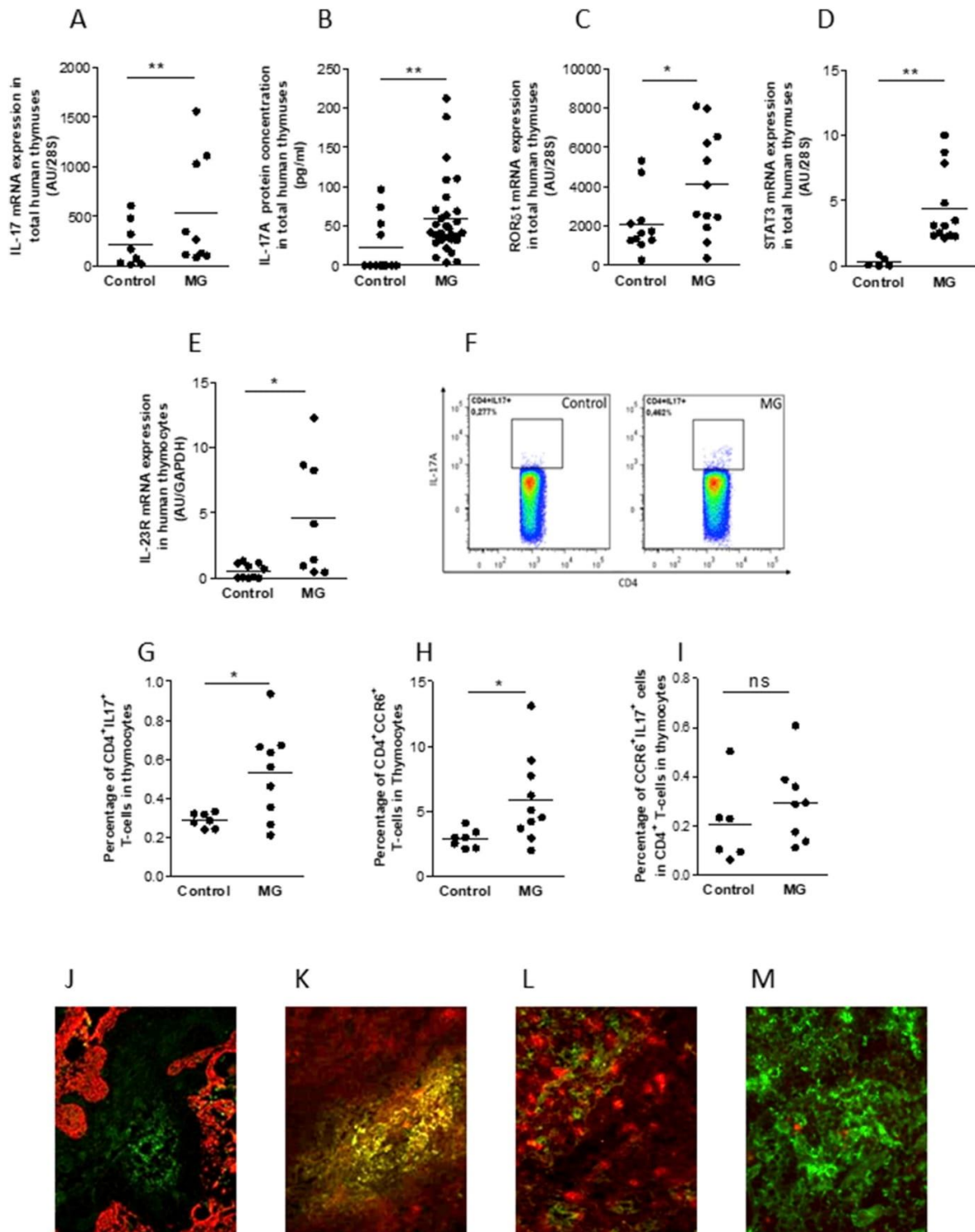
BSA, 10% FBS, 0.3 M Glycine, 1% Tween) for 1 h at room temperature and then, incubated overnight at 4 °C with antibodies raised against human antigens. The labeled cells were revealed with Alexa 488, Alexa 594, Alexa 350 and or Alexa 647 coupled secondary IgG raised in donkey, chicken or rat. Labelling were performed as previously described [18]. Images were acquired with a Zeiss Axio Observer Z1 Inverted Microscope using 20× magnification (Carl Zeiss, Le Pecq, France).

2.9. Statistical analysis

Non-parametric tests (Wilcoxon test for paired data or Mann-Whitney test for unpaired values) were used to compare groups as specified in each Fig. legend. Values were reported as Mean ± Sem. GraphPad Prism 5 software was used to generate the graphs and to perform the statistical analysis. Statistical significance was recognized at p < 0.05.

Table 2
List of primers used in the study.

Gene	Primer #1	Primer #2
28S	GGTAGGGACAGTGGGAATCT	CGGGTAAACGGCGGGAGTAA
AID	AAGGGCTGCATGAAAATTCAGT	CGTCTCGTAAGTCATCAACCTC
CD4	CCTGGTAGTAGCCCTCAGT	CTGGAAAGCTAGCACCACGA
GAPDH	CGACCACTTTGTCAAGTCA	AGGGGTCTACATGGAACCTG
IFN-γ	TCCCATGGGTTGTGTGTTTA	AAGCACCAGGCATGAAATCT
IL-1β	GGGCCTAAGGAAAAGAAATC	TTCTGCTTGAGAGGTGCTGA
IL-6	TGAGGTGCCCATGCTACATTT	TCTGCCGAGCTTTAAGGAGIT
IL-17A	CCCCTAGACTCAGGCTTCT	AGTTGATTCTGCCCATCAG
IL-17R	CACTAGCCTTTTGGGCTCAG	TACGAGGAAGAGTGATTG
IL-21	GGCAACATGGAGAGGATTGT	AAGCAGGAAAAGCTGACCA
IL-23	CAGCAACCTGAGTCCCTAA	CCAAATTTCCCTTCCCATC
IL-23R	TGCCTTGAATCTGAACCTG	GAGCTCCCGGAATTCCTAC
Podoplanin	TGTGGCGCTTGGACTTTGT	GTGTAACAGGCATTGGCATCG
RORC	CAAGAGAGGTTCTGGGCAAG	AGTGGGAAGGCAAGATCAGA
STAT3	CTGGCCTTTGGTGTGAAAT	AAGGCACCACAGAAACAAC
TGF-β3	AACGGTGATGCCACGTC	CCGACTCGGTGTTTCTCTGG



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Fig. 1. Expression of IL-17 in thymuses of AChR⁺ MG patients. Compared analyses in global thymic biopsies obtained from AChR⁺ MG patients and aged-matched controls of IL-17 expression **A)** at the mRNA and **B)** protein levels, **C)** of ROR γ T and **D)** STAT3 at the mRNA expression level. **E)** mRNA analysis of IL-23 receptor in purified human thymocytes from MG thymuses compared to aged matched controls. **F)** Representative image of Flow cytometry analysis of CD4⁺IL-17⁺ cells in the thymocytes from AChR⁺ MG patients and aged matched controls. **G)** Analysis by flow cytometry of the percentage of CD4⁺ cells expressing IL-17 in purified human thymocytes from AChR⁺ MG patients and aged matched controls. **H)** Analysis by flow cytometry of the percentage of CD4⁺CCR6⁺ cells in human purified thymocytes from AChR⁺ MG patients and aged matched controls. **I)** Analysis by flow cytometry of the percentage of CCR6⁺IL17⁺ cells in CD4⁺ cells in purified human thymocytes from AChR⁺ MG patients and aged matched controls. Representative images of AChR⁺ MG human thymic sections co-labeled with antibodies anti-IL-17 (green) and anti keratin 5/14 (red) **J)** or anti CD21 (red) **K)** or anti CD11c (red) **L)** or anti FoxP3 (red) **M)**. mRNA expression was analyzed by real-time PCR and is expressed as arbitrary unit (AU) normalized to 28S or GAPDH. Proteins were analyzed by ELISA. Each point represents the mean value of a duplicate analysis of each donor. Images were acquired with a Zeiss Axio Observer Z1 inverted microscope. N > 5 for control thymuses and n > 9 for AChR⁺ MG thymuses for mRNA and protein analyses. P values were obtained using the non-parametric Mann-Whitney test. Asterisks indicate significant differences (*p < 0.05; **p < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. IL-17⁺ cells in the thymus of AChR⁺ MG patients

Th17 cells and their signature cytokine, IL-17, are involved in pro-inflammatory mechanisms that sustain the development of autoimmune diseases. Gradolatto et al., by using microarray analysis, have reported the over-expression of IL-17, at the mRNA level, in purified thymocytes of AChR⁺ MG patients in both Treg and conventional CD4⁺ T cells [8].

Here, we validated this observation and demonstrated that the expression level of IL-17 in AChR⁺ MG thymus was increased at the mRNA level (Fig. 1A) as well as at the protein level (Fig. 1B). To prove that this IL-17 signature was linked to Th17 cells, we analyzed the mRNA expression of ROR γ T (the master transcription factor of Th17 cells), STAT3 (required for Th17 cell differentiation) and IL-23 receptor (IL-23R), a membrane protein classically expressed by Th17 cells. We observed a significant overexpression of ROR γ T (Fig. 1C), STAT3 (Fig. 1D) and of IL-23R (Fig. 1E) in AChR⁺ MG samples at the mRNA level. To further characterize the IL-17 producing cells in thymi of AChR⁺ MG patients, we analyzed by flow cytometry T cell sub-types using specific surface markers (CD4, CD8, IL23R, CCR6) in purified thymic cells from control and MG patients. We observed a higher percentage of CD4⁺ (CD4⁺CD8⁻) cells expressing IL-17 in AChR⁺ MG thymuses compared to controls (Fig. 1F and G). CCR6 is a chemokine receptor associated with Th17 cell phenotype. We observed a significant increased percentage of CD4⁺CCR6⁺ T cells (Fig. 1H). The percentage of CCR6⁺IL-17⁺ T cells (Fig. 1I), was also increased although not significant.

To understand the potential role of the IL-17 expressing cells, inside MG thymuses, we performed immunohistochemistry analyses of MG and control thymuses. Our analysis showed the presence of IL-17 producing cells within eGCs (Fig. 1J and K), suggesting an involvement of IL-17 in the eGC homeostasis. In addition, we co-stained AChR⁺ MG thymic sections with IL-17 and CD11c (marker for dendritic cells) (Fig. 1L) or FoxP3 (Treg cell marker) (Fig. 1M) and did not observe a co-localization, minimizing the contribution of these cells in the global production of IL-17. These data corroborate findings observed in other autoimmune diseases [21] suggesting an involvement of IL-17 producing cells in B-cell activation and stabilization inside the eGCs. Thus, in regards to what has already been shown in various autoimmune diseases, it is possible that in AChR⁺ MG thymus, IL-17 is involved in the formation of eGCs.

3.2. T cells expressing podoplanin are contained in MG thymic eGCs

Effector T cells and mainly Th17 cells that actively contribute to eGCs formation and homeostasis, express PDPN during autoimmune inflammation [15]. Therefore, we wondered whether PDPN was expressed in T cells surrounding the eGCs of AChR⁺ MG thymus. We first investigated the mRNA expression of PDPN in control and MG AChR⁺ thymuses. mRNA analysis showed a significant increase in PDPN in total thymus extracts of MG patients (Fig. 2A). This increase was due to the lymphocyte population (Fig. 2B) and not to the TECs that displayed

a decreased expression in PDPN in MG thymuses (Fig. 2C). We then corroborated the expression of PDPN by flow cytometry analysis of purified thymic lymphocytes. We observed a significant increase in CD4⁺ cells expressing PDPN (Fig. 2D) and CD4⁺IL23R⁺ PDPN⁺ cells subset (Th17 cells) (Fig. 2E and F). By performing IHC analyses, we observed that PDPN was mostly present in the interlobular zones in control thymic sections (Fig. 2G) while PDPN co-localized with CD4 positive, IL-23R positive cells found in MG thymic eGCs (Fig. 2H and J). eGCs are complex structures where both Th17 and Tfh cells collaborate to the development of B cells [22]. In order to clarify whether CD4⁺ PDPN⁺ cells or IL23R⁺ PDPN⁺ cells present in thymic MG eGCs, were not Tfh cells or B cells, we co-stained thymic MG sections with PDPN, CD20 and CXCR5 antibodies. Fig. 2K and L showed that cells expressing PDPN are not CXCR5⁺ cells (Tfh cells) neither CD20⁺ cells suggesting that cells expressing podoplanin in MG thymic eGCs are most likely to be Th17 cells. In addition, in the thymic biopsies of AChR⁺ MG patients, we observed an increased expression of activation-induced cytidine deaminase (AID) (Fig. 2M), a protein highly expressed in eGCs that contributes to B cells activation and clonal expansion, emphasizing the key role of Th17/PDPN cells in the GC formation and maintenance.

3.3. Th17 cell differentiation and activation in thymuses of AChR⁺ MG patients

Differentiation of naïve CD4⁺ T cells into Th17/IL-17 secreting cells is a complex process highly dependent on the presence of different cytokines including the transforming growth factor- β 1 and 3 (TGF β -1, TGF β -3), IL-6, IL-1 β , IL-21 and IL-23 [22,23]. In this context, we evaluated the thymic expression of these cytokines in AChR⁺ MG patients. The mRNA expression levels of IL-6, TGF- β 3, IL-1 β , IL-21 and IL-23 were significantly increased in AChR⁺ MG thymus compared to age-matched controls (Fig. 3A–E). In parallel, in the sera, we observed that among all these cytokines, the protein level of only IL-23 was significantly over-expressed in AChR⁺ MG patients (Fig. 3F and J). Since IL-23 is responsible for Th17-cell activation towards a pathogenic phenotype and also for stimulating IL-17 expression, these data suggest that in AChR⁺ MG thymuses an active process may sustain the CD4⁺ T cell differentiation into active and pathogenic Th17 cells through IL-23 pathway that is perpetuated in the periphery.

3.4. TECs are main IL-23 producer cells in MG thymuses

To identify and to decipher the mechanism underlining the over-production of IL-23, we evaluated the IL-23 expression level in the two main thymic cell subtypes (thymic lymphocytes and TECs). We found no significant difference of IL-23 expression in AChR⁺ MG thymic lymphocytes compared to control ones (Fig. 4A). However, TECs from AChR⁺ MG patients displayed an mRNA overexpression of IL-23 as compared to controls (Fig. 4B). In addition, protein analysis of TEC supernatants corroborated the overproduction of IL-23 by MG TECs (Fig. 4C).

In order to validate the overexpression of IL-23 by MG TECs, and since IL-23 is known to be expressed by activated dendritic cells (DCs)

Fig. 2. Podoplanin expression in AChR⁺ MG thymuses. Comparative analysis of podoplanin mRNA expression in **A)** global thymic biopsies, **B)** purified human thymocytes and **C)** primary cultured thymic epithelial cells obtained from AChR⁺ MG patients and controls individuals. Analysis by flow cytometry of the percentage **D)** of CD4⁺PDPN⁺ cells, **E)** of CD4⁺IL23R⁺ cells expressing Podoplanin in the purified thymocytes obtained from AChR⁺ MG patients and controls individuals. **F)** Representative graph of flow cytometry analysis of PDPN expression in CD4⁺IL-23R⁺ cells in purified thymocytes from AChR⁺ MG patients and controls. Representative thymic sections of control **G)** and AChR⁺ MG patient **H)** co labeled with antibodies anti Podoplanin (red), and anti Keratin 5/14 (blue). Thymic sections of AChR⁺ MG patient co-labeled with antibody anti Keratin 5/14 (blue), Podoplanin (red) and anti CD4 (green) **I)** or anti IL23R (green) **J)** or anti CXCR5 (green) **K)** or anti CD20 (green) **L)**. Comparative analysis of AID mRNA expression in global thymic biopsies from AChR⁺ MG patients and controls individuals **M)**. Images were acquired with a Zeiss Axio Observer Z1 inverted microscope using 20× magnification. For global thymus analyses, N > 4 for control and n > 4 for AChR⁺ MG thymuses. mRNA expression was analyzed by real-time PCR and is expressed as arbitrary unit (AU) normalized to 28S or GAPDH. Each point represents the mean value of duplicate analysis of each donor. P values were obtained using the non-parametric Mann-Whitney test. Asterisks indicate significant differences (*p < 0.05; **p < 0.005). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and phagocytic cells [24], we proceeded with in situ protein identification. As observed in Fig. 4D, immunohistochemical analysis of control thymus shows a subtle and focused IL-23 protein staining mainly in the Hassall's corpuscles while AChR⁺ MG thymuses display IL-23 staining in the interlobular zone, the germinal centers (Fig. 4E–H) as well as in keratin 5/14 positive cells (Fig. 4H). More, in agreement with previous studies reporting dendritic cell propensity to express IL-23 [24], we observed few dendritic cells (CD11c⁺) expressing IL-23 in AChR⁺ MG thymic biopsies (Fig. 4I), even-though their expression remained negligible as compared to the TEC levels. Altogether, these results demonstrated that in AChR⁺ MG thymuses, TECs were the main cells over-expressing IL-23, the Th17-cell activator. These data raise the question of the mechanism(s) underlining the over-expression of IL-23 in MG TECs.

3.5. Interferon type I pathway stimulates IL-23 expression in MG thymuses

We mimicked, in vitro, an inflamed environment by treating human control TECs with lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly (I:C)), a synthetic analog of double-stranded RNA to mimic viral infection). We observed that in control primary human TECs, after 24 h, LPS inhibited significantly IL-23 expression (Fig. 4J). While poly (I:C) stimulated it (Fig. 4J). Since Poly (I:C) activates the IFN-I pathway in human TECs [25], a pretreatment of human control TECs with an anti IFN α/β receptor blocking antibody (α -IFN $\alpha\beta$ receptor) inhibited Poly (I:C) effect (Fig. 4J), confirming that poly (I:C) activated IL-23 expression through IFN-I transduction pathway.

To precise this assumption, human control TECs were challenged with the two types of interferon (IFN-I and IFN- γ). Fig. 4K shows that IL-23 expression is induced by IFN-I pathway activation and not by IFN- γ . In addition, AChR⁺ MG TECs (cells that used to be in an inflammatory environment) remained responsive to Poly (I:C) (Fig. 4L) but displayed a lower ability to over-express IL-23 compared to controls, when stimulated with Poly (I:C), 2,6 Vs 5 fold change respectively (Fig. 4J and L). Fig. 4M illustrates the ratio of IL-23 expression in control versus MG TECs at steady state (Fig. 4B) and after Poly IC stimulation (Fig. 4J and L). Therefore, Fig. 4M demonstrates that whether MG TECs can overproduce IL-23, compared to control TECs, they are less inducible probably due to a constant in vivo stimulation that increased their steady state level of IL-23 production and consequently limit their capacity to be re-activated in vitro.

Altogether, these results demonstrate TECs participate in the amplification of the inflammation process in AChR⁺ MG thymus through their over-production of IL-23.

3.6. MG TEC secretion of IL-23 increases IL-17 production

Considering that MG TECs over-express IL-23, IL-1 β and IL-6 (cytokines involved in Th17 cell development), we suspected that crosstalk between TECs and T cells was important in the thymic inflammatory process in AChR⁺ MG patients.

To validate our hypothesis and as a proof of concept, we performed co-cultures of primary control or MG TECs with fresh purified PBMCs from healthy patients. We used PBMCs to analyze TECs effects on CD4⁺

mature cells and to avoid any confounding effects related to T cell maturation or developing process.

First, CD4 and IL23R expression levels were analyzed in PBMCs after co-culture. Results indicated that independently of the origin of the TECs (MG or control thymuses), when in contact with TECs, PBMCs harbored a decreased expression of CD4 and IL-23R, surface molecules (Fig. 5A–C), and no difference in CD8 (Fig. 5C). The decrease in IL-23R expression reinforces the anti-inflammatory effect of TECs described by Nazzari et al. [17]. More, previous reports have shown that a down-regulation of CD4 that occurs upon in vitro stimulation may lead to double negative T cells that exhibit an effector phenotype associated with an increased TCR dependent proliferation and increased production of IFN- γ and IL-17 [26].

Second the expressions of IL-17 and IFN- γ , cytokines expressed by Th17 and Th1 cells [27] respectively were assessed. When PBMCs were co-cultured with control TECs, no change was observed in IL-17 mRNA expression levels in PBMCs while a decrease in IFN- γ mRNA expression was observed (Fig. 5D and E). By contrast, PBMCs co-cultured with MG TECs displayed an increased mRNA expression of IL-17 but no change for IFN- γ (Fig. 5D and E). To determine the impact of IL-23 produced by MG TECs, we added a capture antibody anti-IL-23 in PBMCs co-cultured with MG TECs. The significant increase in the percentage of Th17 cells (CCR6⁺IL17⁺) within CD4⁺ cells (Fig. 5F and G) is reduced although not significantly, by the anti-IL-23 (Fig. 5G). These results show that MG TECs production of IL-23 is partially involved in the differentiation of T cells into IL-17⁺ T cells.

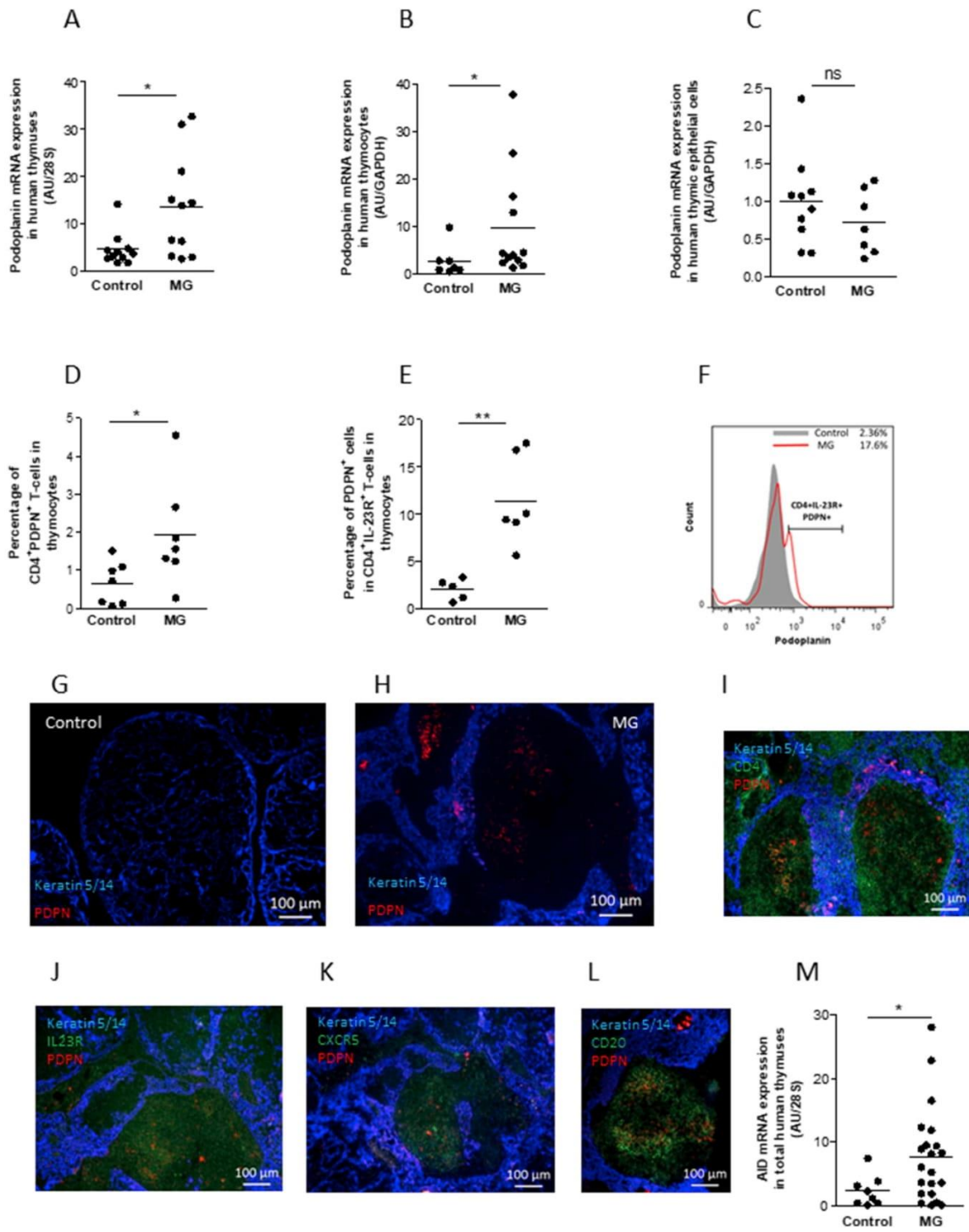
More, only PBMCs co-cultured with MG TECs over-expressed PDPN (Fig. 5H). The expression of PDPN in Th17 cells is known to be influenced by pro-inflammatory cytokines like IL-6 and IL-1 β [28], both cytokines being upregulated in MG TECs [29]. Altogether our results suggest that MG TECs stimulate differentiation and activation of T cells into a Th17 cell phenotype expressing podoplanin.

3.7. T cell secretion of IL-17 promotes a retro activation of TECs

Our results suggest that MG thymuses display an inflammatory chronicity and protective boundaries are ineffective to decrease/stop the “snowball” process. Therefore, we wondered whether a T cell retro-control on TECs may be also involved in this uncontrolled inflammation.

First, we observed no change in mRNA expression of IL-23 by control TECs in the presence of PBMCs (Fig. 6A) while MG TECs displayed an increased mRNA expression of IL-23 (Fig. 6A). To ensure the specificity of this effect, we analyzed TECs expression of IL-6, a cytokine known to be overexpressed by MG TECs. Similarly to IL-23, in co-culture with PBMCs, we did not observe any change in IL-6 mRNA expression by control TECs (Fig. 6B) while MG TECs displayed a significant over-expression of IL-6 (Fig. 6B).

Therefore, we hypothesized that PBMCs might produce factors that promote IL-23 production by MGs TECs among them IL-17. Hence, we treated control TECs with IL-17 at two different concentration levels found in AChR⁺ MG thymus (0.1 ng/ml and 10 ng/ml) (Fig. 1B). mRNA expression of IL-23 was only modified by the higher IL-17 concentration (10 ng/ml) (Fig. 6C), an effect blocked by adding an antibody against



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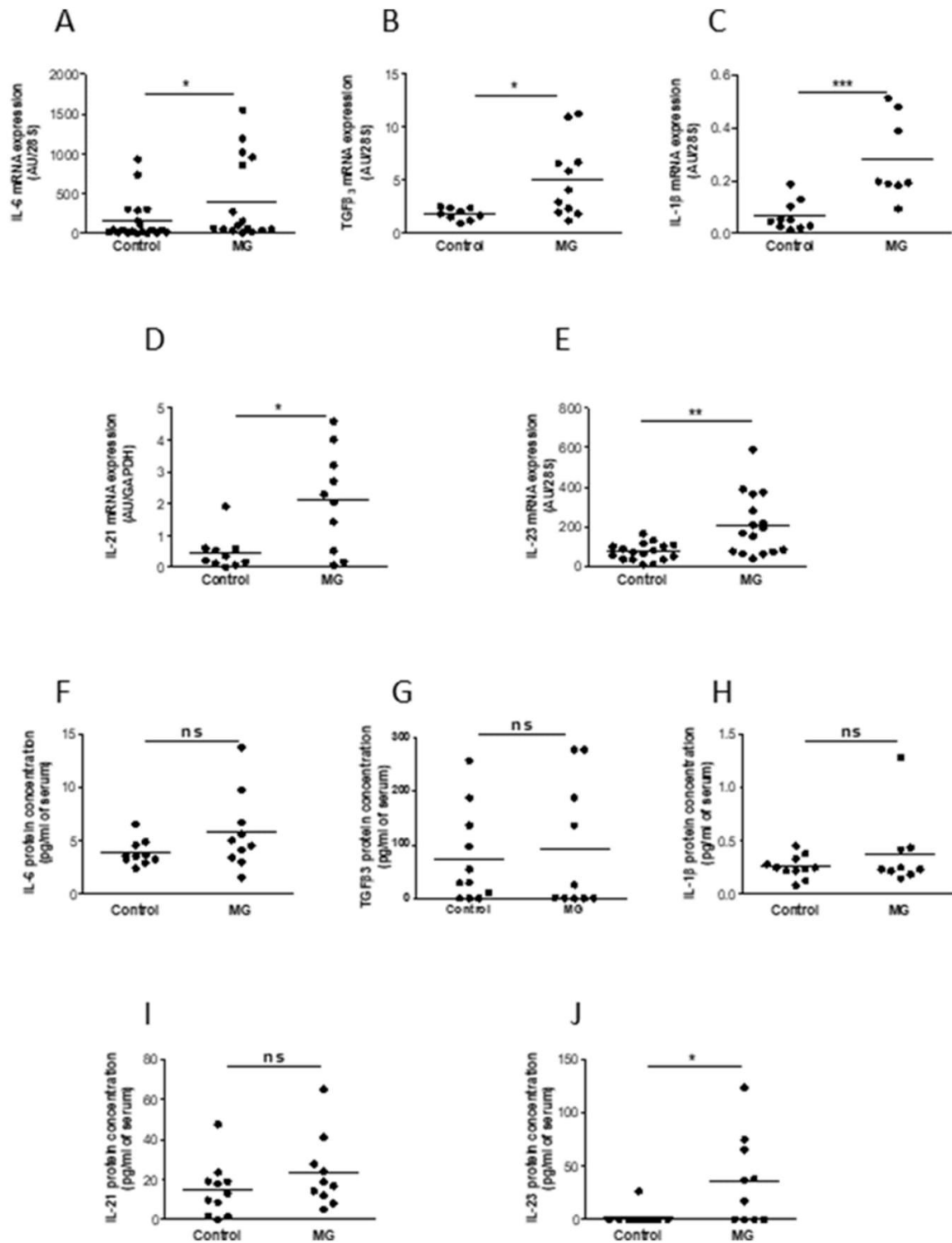
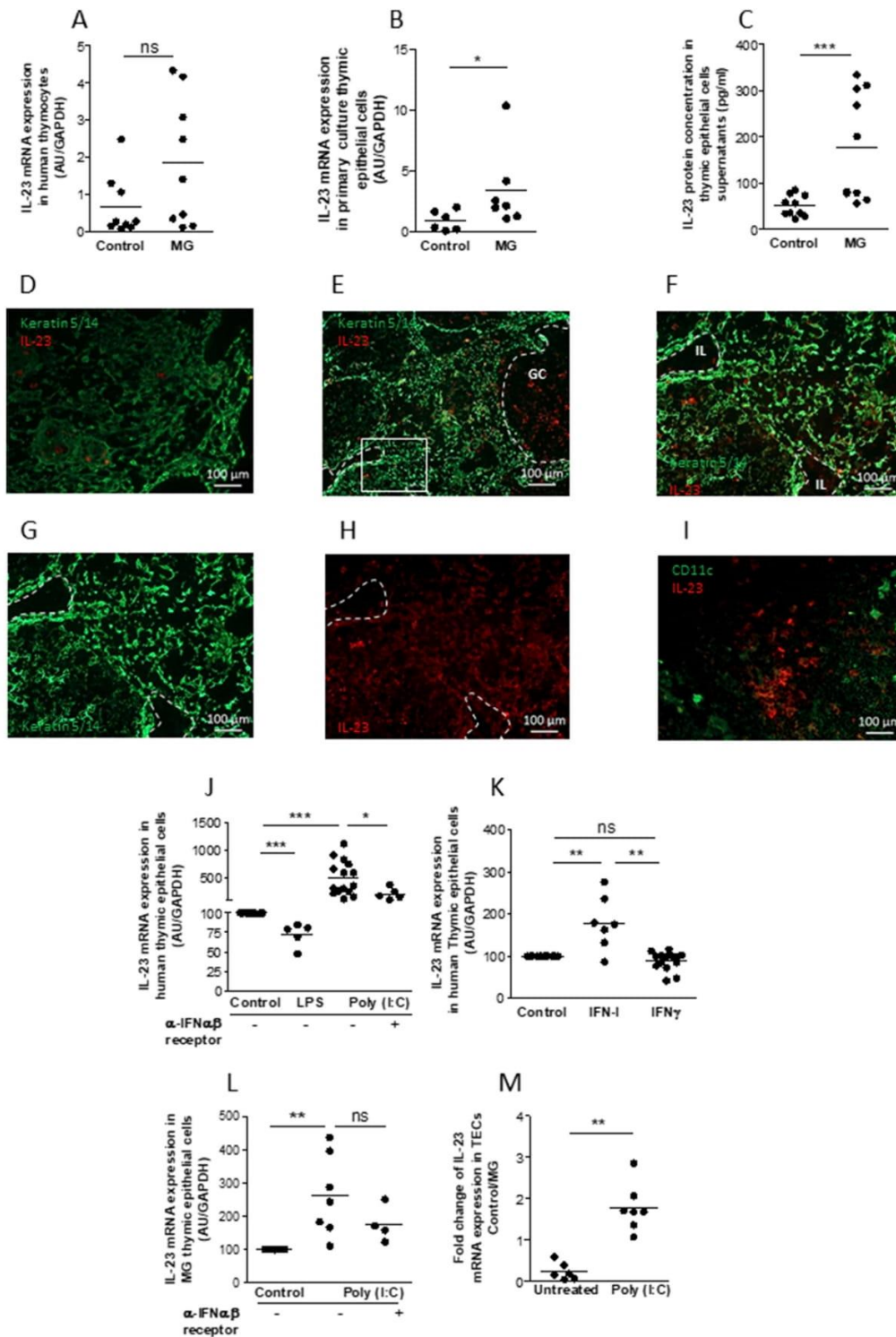


Fig. 3. Expression of cytokines involved in Th17 cells differentiation in thymuses of AChR⁺ MG patients. mRNA expression levels of A) IL-6, B) TGF-β₃, C) IL-1β, D) IL-21, and E) IL-23 in thymuses of AChR⁺ MG patients compared to aged matched control adults. Protein expression level of F) IL-6, G) TGF-β₃, H) IL-1β, I) IL-21 and J) IL-23 in sera of AChR⁺ MG patients compared to aged matched controls. mRNA expression was analyzed by real-time PCR and normalized to 28S or GAPDH. mRNA are expressed as arbitrary unit (AU). Proteins were analyzed by ELISA. Each point represents the mean value of duplicate analysis of each donor. N > 9 for control thymuses and n > 8 for AChR⁺ MG thymuses. P values were obtained using the non-parametric Mann-Whitney test. Asterisks indicate significant differences (*p < 0.05; **p < 0.01; ***p < 0.0005).



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Fig. 4. Overexpression of IL-23 is due to “inflamed” TECs in thymuses of MG patients. Analysis of mRNA expression level of IL-23 in **A)** thymocytes and **B)** primary cultured TECs of AChR⁺ MG patients compared to controls. **C)** Protein expression level of IL-23 in supernatants of primary cultured TECs obtained from controls and MG patients. Representative picture of human thymic sections **D)** from controls and **E)** from AChR⁺ MG patients co-labeled with an anti-IL-23 antibody (red) and antibody anti-keratin 5/14 (green) with a germinal center (GC). **(F–H)** Zoom of representative thymic human section of AChR⁺ MG patient co-labeled with anti-IL-23 antibody (red) and antibody anti-keratin 5/14 (green) with interlobular area (IL). **I)** Representative pictures of AChR⁺ MG patient thymic section co-labeled with an anti-IL-23 antibody (red) and an antibody anti CD11c (green). **J)** Effect of LPS (10 ng/ml) and Poly (I:C) (100 µg/ml) with or without an anti IFNα/β receptor (10 µg/ml), **K)** of type I IFN (1000UI/ml) or IFN-γ (1000UI/ml) on mRNA expression of IL-23 in human control TECs. **L)** Effect of Poly (I:C) (100 µg/ml) with or without an anti IFNα/β receptor (10 µg/ml) on IL-23 mRNA expression in AChR⁺ MG TECs. **M)** Relative increased of IL-23 mRNA expression induced by Poly (I:C) in primary control TECs compared to AChR⁺ MG. mRNA expression was analyzed by real-time PCR and normalized to GAPDH or Keratin 14. mRNA expression is expressed as arbitrary unit (AU). Proteins were analyzed by ELISA. Images were acquired with a Zeiss Axio Observer Z1 inverted microscope. Each IHC labelling was done and repeated on thymic biopsies of different individuals. For global thymus analyses n > 5 for controls and n > 9 for MG thymuses. For analyses in primary cultured human TECs, cells were obtained from n > 5 different donors. Each point represents the mean value of duplicate analysis of each donor. P values were obtained using the non-parametric Mann-Whitney test. Asterisks indicate significant differences (*p < 0.05; **p < 0.01; ***p < 0.0005). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

IL-17 receptor (α-IL-17R) (Fig. 6C). These data suggest that IL-23 overexpression by MG TECs could be reinforced by an IL-17 retro-control.

More, under IFN-I pathway activation through Poly (I:C), control TECs were able to produce IL-17 (Fig. 6D). Altogether, these data show that MG TECs chronic expression of IL-23 may be sustained by IL-17, a cytokine classically expressed by Th17 cells but also expressed by TECs following an IFN-I pathway activation. Therefore, an accumulation of IL-17 initiated by Th17 cells may help to develop a paracrine stimulation in TECs and possibly generating an unstoppable chronic inflammation (Fig. 6E).

4. Discussion

In autoimmune MG early-onset pathology, the thymus is an inflamed tissue that contains high levels of pro-inflammatory cytokines (IL-6, IFNγ, TGF-β, IL-1β) and chemokines (CXCL13, CCL21) that support the attraction of B-cells and the development of eGCs where production of pathogenic antibodies takes place.

We and others have previously analyzed the protein expression of different cytokines such as IL-6 [30–32], IL-1β [33] and TGF-β [34], and observed a higher expression of these cytokines in AChR⁺ MG patients as compared with controls thymuses.

Here, we showed, for the first time, that in hyperplastic AChR⁺ MG thymuses, the IL-23 pathway is implicated in a continuous loop of inflammatory events that sustains the development of Th17 cells and that may lead to eGC formation and probably sustain pathogenic antibody production.

4.1. MG TECs support thymic inflammation through Th17 cell activation

Th17 Cytokines are important controllers of the development of T cells. For instance, Th17 cell differentiation and activation require IL-6, IL-1β, IL-21, TGF-β1/3 and IL-23 [35]. IL-6 and TGF-β engage Naïve T cells to differentiate into the Th17 cells phenotype [36]. Then, IL-23 stabilizes the engaged Th17 cells into a pathological phenotype [36]. Here, we showed that AChR⁺ MG TECs displayed an increased expression of cytokines involved in the activation of the IL-23/Th17 pathway. These results are in line with various studies that have shown implication of pathogenic Th17 cells into inflammatory processes that occurred in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and Psoriasis [30,37]. Therefore, autoimmune MG shares with others autoimmune diseases the same IL-23/Th17 activation pathway, in its disease effector tissue, the thymus.

Of note, IL-23 is constituted of two subunits IL-23p19 and IL-12p40 [38]. IL-12p40 favors the differentiation of Th1 cells that produce IFN-γ [39] while IL-23p19 subunit is responsible for the Th17 cell pathogenicity through the stabilization of the phenotype and consequent IL-17 secretion [40]. Depletion of IL-12p40 and IFN-γ does not protect mice from the development of experimental autoimmune myasthenia gravis mouse model (EAMG) based on active immunization, a mouse model that does not involve thymic inflammation [41]. However, our

data demonstrate that AChR⁺ hyperplastic MG thymuses harbor an IL-23p19 over-expression. Interestingly, in experimental autoimmune encephalomyelitis, multiple sclerosis mouse model, stronger disease symptoms occur when Th17 cells are developed in presence of IL-23 [35]. An effect corroborated by the resistance to the disease induction in IL-23p19^{-/-} mice [42].

Numerous studies have highlighted the heterogeneity of Th17 cells [43,44]. Th17 differentiation into pathogenic and non-pathogenic cell subgroups have been shown to rely on the cytokine combination or the pathogens used for the differentiation [13]. More, pathogenic and non-pathogenic Th17 cells have been identified with specific gene signature (IL-10, IL-9, Aryl hydrocarbon receptor, Maf for non-pathogenic cells; IL17R, GMZB, IL-22, Stat4, IL-23R for pathogenic subsets [23]). Given the phenotypical gene signatures and component milieu identified to describe the complex Th17 cells, our study shows that in the AChR⁺ MG thymuses, TECs over-produce IL-23 and TGF-β3 in addition to IL-6, and IL-1β [30,37], a pro-inflammatory context that contributes to the differentiation and activation of pathogenic Th17 cells, emphasized by the IL-23R increased expression found in MG thymocytes.

4.2. Thymic extrinsic factors responsible for mTECs to sustain Th17 prone milieu

IL-23 expression is induced by various factors such as TNF-α, IL-1β and IFN-γ through the NF-κB pathway in dendritic cells, macrophages and keratinocytes [21,45]. In MG thymus, inflammatory factors such as IFN-γ and TNF-α are known to be overexpressed [46,47]. Poly (I:C) activated IFN-I pathway in TECs induced an overexpression of the α-AChR subunit (normally expressed in the thymus as a tissue specific antigen), the IFN-I production [25] and stimulates respectively in TECs and lymphatic endothelial cells, CXCL13 and CCL21 expression, two B-cell chemoattractant proteins involved in eGC development in MG thymuses [48]. Here, we have added IL-23, as another target of IFN-I transduction pathway in MG TECs. Deregulation in the IFN-I pathway is possibly a critical factor that activates inflammatory signaling in MG TECs and induces the expression of IL-23 among other cytokines.

AChR⁺ MG with hyperplastic thymus is a “female” pathology [1]. Estrogens may then play different roles in this disease, by facilitating the autoimmune reaction though a defective tolerance process [19] and by modulating the basal level of IFN-I [48]. In AChR⁺ MG thymus, an activation of IFN-I may be potentialized by estrogen receptor-α signaling, contributing then to induce the production of IL-23 in TECs. Estrogens can also affect IL23R expression, the production of IL-17 and the percentage of Th17 cells [49]. Therefore, AChR⁺ MG female may challenge a synergy of an activated pathway that shifts towards a Th17 inflammatory status in the thymus.

4.3. MG TECs contribution in eGC formation

A common characteristic of autoimmune MG with other organ-specific autoimmune diseases is immune cells infiltrations (i.e. B cells)

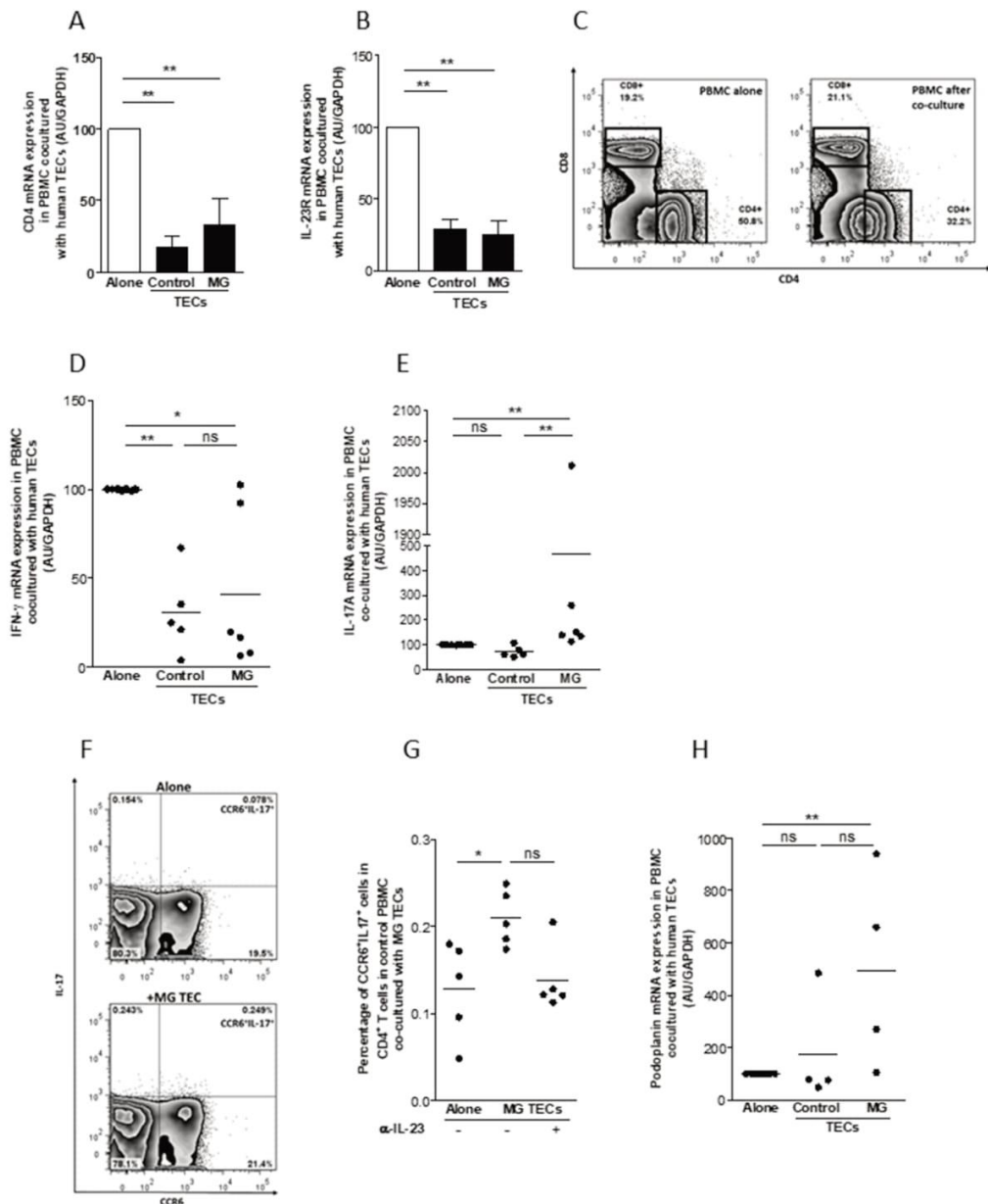


Fig. 5. MG TEC secretion of IL-23 stimulates IL-17 production by PBMCs. Analysis of the mRNA expression of A) CD4 and B) IL-23 receptor in PBMCs co-cultured for 24 h with controls or AChR⁺ MG primary TECs. C) Representative images of flow cytometry analysis of CD4 and CD8 labelling of PBMCs cultured with or without control TECs. mRNA expression levels of D) IFN- γ and E) IL-17A in PBMCs co-cultured for 24 h with control and AChR⁺ MG TECs. F) Representative image of flow cytometry analysis of CCR6⁺ IL-17⁺ cells in control PBMCs cultured with or without AChR⁺ MG TECs. G) Flow cytometry analysis of the percentage of CCR6⁺ IL-17⁺ cells among the CD4⁺ cells in PBMCs co-cultured with AChR⁺ MG TECs and an antibody anti-IL-23. H) mRNA expression level of Podoplanin in PBMCs co-cultured with control or AChR⁺ MG TECs. mRNA expression was analyzed by real-time PCR and normalized to GAPDH. mRNA expression is expressed as arbitrary unit (AU). Each point represents the mean value of duplicate analysis of different PBMC donor. n > 4 PBMCs from different controls. Primary cultured human TECs were obtained from N > 3 different individuals for controls and AChR⁺ MG patients. P values were obtained using ANOVA analysis or non-parametric t-test (*p < 0.05; **p < 0.01; ***p < 0.0005).

in the inflamed tissues [50]. In MG thymuses, TECs attract B cells via CXCL13 expression [10] that could contribute to eGC development. In addition here, we showed that in thymic eGCs, CD4⁺ cells expressed IL-17 and PDPN. Then our observations may suggest that Th17 cells that develop in the presence of IL-23 participate in the maintenance of eGCs

in MG thymuses through PDPN as shown in other pathologies [14,51]. More, the overexpression of IL-21 in MG thymus, a cytokine expressed by Th17 cells (among other cells) and involved in the differentiation of B cells into plasma cells reinforces the potential role of the IL-23/Th17 pathway in MG thymic eGC homeostasis.

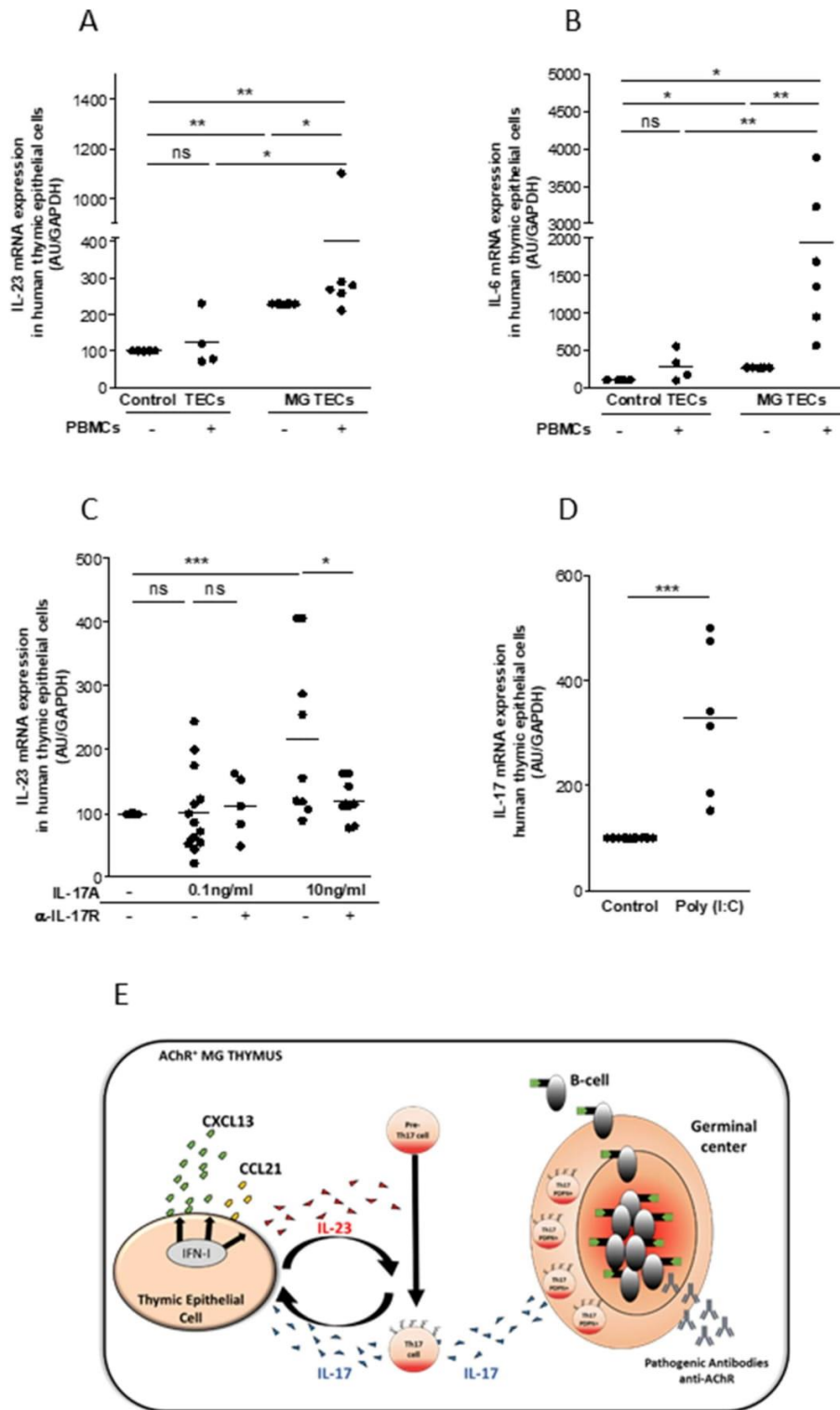


Fig. 6. PBMCs co-cultured with TECs influence the TEC expression of cytokines. mRNA expression level of **A)** IL-23 and **B)** IL-6 in control and MG AChR⁺ TECs co-cultured with control PBMCs. **C)** Effect of IL-17 (0.1 ng/ml and 10 ng/ml) and anti-IL-17 receptor (α-IL-17R) (1 μg/ml) on the mRNA expression level of IL-23 by control TECs. **D)** Effect of Poly (I:C) on the mRNA expression level of IL-17 by control TECs. **E)** Schematic representation of IL-23/IL-17 loop of regulation in AChR⁺ MG thymuses. mRNA expression was analyzed by real-time PCR and normalized to GAPDH. mRNA expression is expressed as arbitrary unit (AU). Each point represents a different donor. N > 4. Primary cultured human TECs were obtained from n > 5 different individual controls. P values were obtained using ANOVA analysis or non-parametric t-test (*p < 0.05; **p < 0.01; ***p < 0.0005).

4.4. Why does the inflammatory process do not stop?

The immune system relies on the capacity of cells to induce, when needed, inflammation and to resolve it. Similarly, to other autoimmune diseases, AChR⁺ MG patients face a chronic inflammation. One can wonder why the immune system is powerless to resolve the intra thymic inflammation. Some clues have emerged from our data and previous studies.

First of all, AChR⁺ MG thymic Treg cells display a defective capacity to suppress effector T cell proliferation [6]. In parallel, thymic effector CD4⁺ T cells are not responsive to suppression. The increased percentage of activated IL-17 expressing cells in MG thymus may be a consequence of the impaired ability of Treg cells to suppress effector T cells and to resolve inflammation. Moreover, Treg cells can mimic Th17 cells [52] in the presence of increased IL-23 medium level. Therefore, plastic Treg cells in AChR⁺ MG tend to become Th17-like ex-Treg cells [8] and contribute to rise IL-17 concentration in the thymus.

IL-17 activates in stromal cells the NF- κ B pathway and induces the expression of pro-inflammatory molecules like IL-6, IL-8, GM-CSF and CCL20 [53,54]. As our results show, MG TECs are also responsive to IL-17. Therefore, we can envisage that MG TECs have a double side participation in the inflammatory process. One side by inducing the production of IL-17 by effector and regulatory T cells while on another side by sensing IL-17, amplifying the inflammatory signal and starting an unstoppable inflammatory process.

5. Conclusions

Within these settings, we propose that activation of the IL-23/Th17 pathway engenders an endless cascade of signals that might be triggered by a deregulation of the IFN-I pathway (Fig. 6E). In addition, the IFN-I pathway induces expression of chemokines promoting B-cell infiltration as well as of pro-inflammatory cytokines involved in the differentiation of pathogenic Th17 cells expressing PDPN. These pathogenic cells might collaborate in the inflammatory process by sustaining the formation of eGCs through IL-21 signaling, and also by stimulating and amplifying IL-23 expression by TECs through IL-17 signaling retro positive control.

Altogether, our study reveals a new promising therapeutic target for AChR⁺ MG patients, the IL-23/Th17 pathway. To date, monoclonal antibodies anti-IL-23 (such as Ustekinumab) are already approved by FDA and are used to treat patients affected with Crohn's disease and psoriatic arthritis [55]. Therefore, we consider that targeting IL-23 in AChR⁺ MG could decrease concomitantly thymic and peripheral inflammation and antibody production. Therefore, IL-23 should be proposed as a new promising therapeutic target in MG.

Authors contributions

J.V., J.B. and N.D. performed the experiments, analyzed the data and interpreted the results. F.T. provided help to obtain samples. I.K. and A.C.B. performed the podoplanin experiments. R.R. and N.S. provided human thymic tissues. R.L.P. provided samples and helpful suggestions to design experiments. S.B.-A. initiated the study. N.D. and S.B.-A. were involved in all aspects of the study including: design, data analysis and interpretation of the results. N.D. and J.V. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Declaration of interest

The authors declare that they have no relevant conflicts of interest.

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The Muscle Is Not a Passive Target in Myasthenia Gravis

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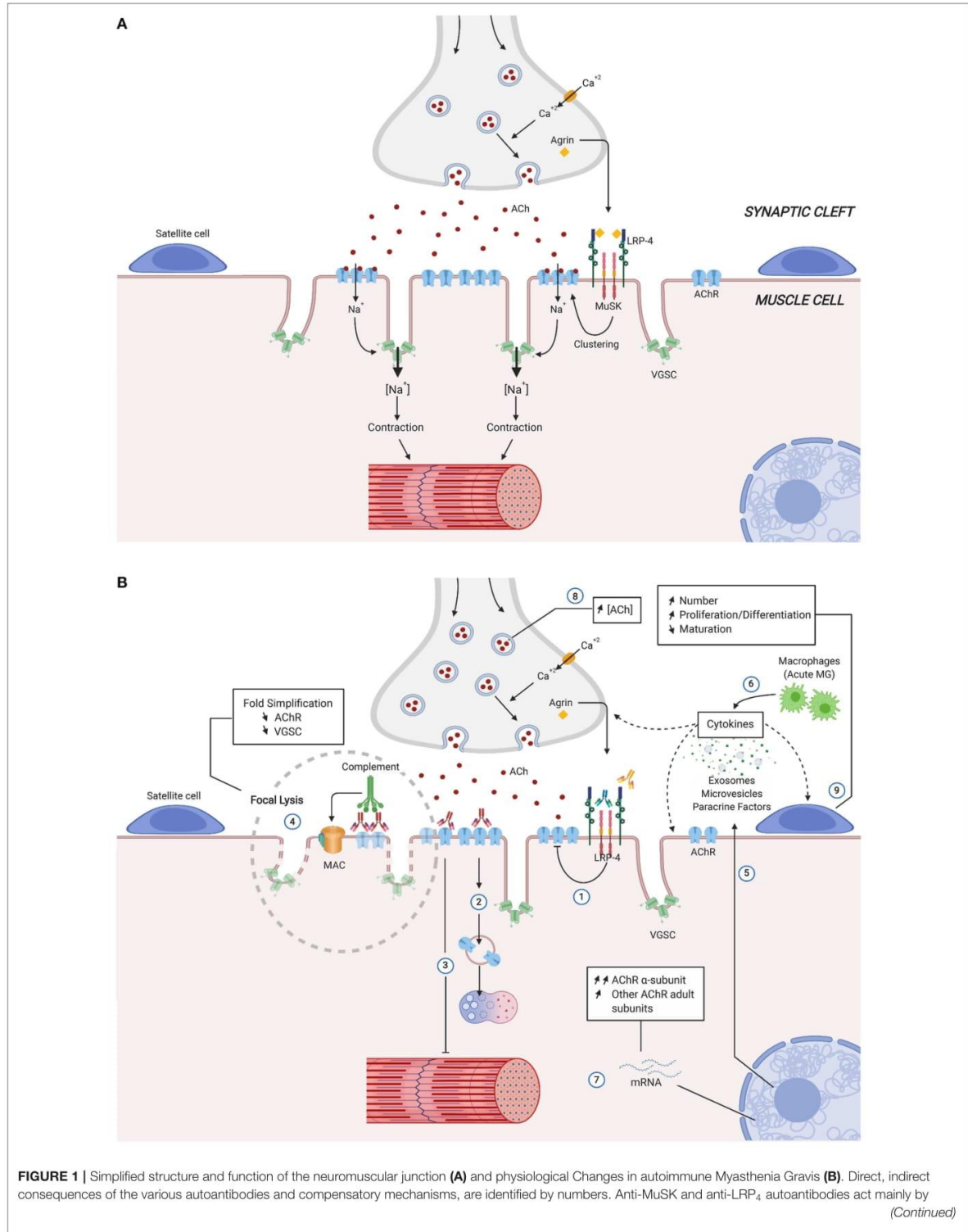
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Myasthenia gravis (MG) is a rare autoimmune disease mediated by pathogenic antibodies (Ab) directed against components of the neuromuscular junction (NMJ), mainly the acetylcholine receptor (AChR). The etiological mechanisms are not totally elucidated, but they include a combination of genetic predisposition, triggering event(s), and hormonal components. MG disease is associated with defective immune regulation, chronic cell activation, inflammation, and the thymus is frequently abnormal. MG is characterized by muscle fatigability that is very invalidating and can be life-threatening when respiratory muscles are affected. MG is not cured, and symptomatic treatments with acetylcholinesterase inhibitors and immunosuppressors are life-long medications associated with severe side effects (especially glucocorticoids). While the muscle is the ultimate target of the autoimmune attack, its place and role are not thoroughly described, and this mini-review will focus on the cascade of pathophysiologic mechanisms taking place at the NMJ and its consequences on the muscle biology, function, and regeneration in myasthenic patients, at the histological, cellular, and molecular levels. The fine structure of the synaptic cleft is damaged by the Ab binding that is coupled to focal complement-dependent lysis in the case of MG with anti-AChR antibodies. Cellular and molecular reactions taking place in the muscle involve several cell types as well as soluble factors. Finally, the regenerative capacities of the MG muscle tissue may be altered. Altogether, the studies reported in this review demonstrate that the muscle is not a passive target in MG, but interacts dynamically with its environment in several ways, activating mechanisms of compensation that limit the pathogenic mechanisms of the autoantibodies.

Keywords: myasthenia, muscle, neuromuscular junction, acetylcholine receptor, autoimmunity, cytokines, transcriptome

INTRODUCTION

Autoimmune Myasthenia Gravis (MG) is characterized by muscular weakness aggravated by exercise and improved by rest. The symptoms fluctuate, which makes the clinical diagnosis difficult. MG is mediated by antibodies (Ab) to components of the neuromuscular junction (NMJ), the muscle is thus the target of the autoimmune attack. About 85% of MG patients present Ab against the acetylcholine receptor (AChR) (1). In about 5% of MG patients, the autoreactive Ab target the muscle-specific kinase (MuSK) protein (2), which is involved in the clustering of AChRs (3). More recently, the agrin receptor LRP₄ (low-density lipoprotein receptor-related protein 4), which forms a complex with MuSK, has been recognized as a novel autoantigen in a small proportion of MG patients without anti-AChR or -MuSK Ab (4). Antibodies to cortactin and agrin (5, 6) have been described, but their presence is most often concomitant to one of the other types of Ab.



MG is a complex disease to which genetic predispositions and defects of the immune system contribute (7–9). Thymic abnormalities are frequently found in the subgroup of MG with anti-AChR Ab but not in that with anti-MuSK Ab (10), and thymectomy has clinically favorable effects in AChR-MG (11), but not in MuSK-MG (12). MG patients with anti-AChR Ab can be classified in several subgroups according to the age of onset, the gender, thymic pathology, and anti-AChR antibodies [Reviewed in (13)]. While the defects of the immune system are richly described (7, 14, 15), reviews on the mechanisms taking place at the level of the muscle tissue are more sporadic (16–18), therefore we will focus on this aspect.

ULTRASTRUCTURAL AND PHYSIOLOGICAL CHANGES OF THE NMJ IN MG

The development and maintenance of the NMJ are primarily dependent on the agrin-MuSK-LRP₄ signaling system (19, 20). LRP₄ and MuSK are anchored in the post-synaptic membrane. Agrin, secreted by the nerve terminal, binds to LRP₄, which then binds to the extracellular domain of MuSK, resulting in phosphorylation and activation of MuSK (19). Phosphorylated MuSK recruits then Dok-7, an adaptor protein that becomes phosphorylated and recruits additional signaling molecules essential for synapse formation and AChR clustering (21).

Detailed structure and mechanism of the NMJ have been described in several reviews (22–25). Briefly, the post-synaptic membrane is characterized by deep junctional folds, the top of which are rich in AChRs, while voltage-gated Na⁺ channel (VGSCs) are concentrated in the depths [Review in (22, 24)]. There are ~10,000 AChR per square micrometer on the muscle surface in the motor plate, whereas the concentration is negligible outside the synaptic area. At the presynaptic side, 150,000–300,000 vesicles contain a quantum of acetylcholine (ACh) each (~10 000 molecules). Upon local depolarization, one quantal content (about 20 vesicles) is released in the synaptic cleft. The binding of ACh to AChRs induces an entry of Na⁺ into the muscle fibers, causing the local depolarization of the membrane and forming the endplate potential (EPP). The EPP stimulates the opening of the VGSCs, and upon reaching the firing threshold, a further influx of Na⁺ ions ensues, and the action potential spreads along the muscle fiber. It reaches and opens the stocks of intracellular calcium that finally trigger the muscle contraction (**Figure 1A**). In the healthy NMJ, the amplitude of EPP exceeds the threshold necessary to produce an action potential in the muscle. The ratio between the actual EPP and the threshold required to generate an action potential represents the safety factor of neuromuscular transmission, which is especially important during intense activation of the NMJ (26). In humans, the safety factor is about two, whereas it is higher in rodents or feline (27).

In AChR-MG disease, morphometric analysis reveals degenerative changes of the postsynaptic regions with widening and simplification of synaptic clefts and accumulation of debris in the synaptic zone (28, 29) (**Figure 1B**). In addition, nerve

terminals are often smaller than normal size, and their sprouting may be observed (28). The degradation of the post-synaptic membrane results in a decrease in the expression of the AChR and the VGSCs channels, both contributing to the significant reduction of the safety factor: (1) EPP is lowered by the partial loss of functional AChRs and (2) the firing threshold is raised due to the reduction in the density of the sodium channels (30). During prolonged synaptic activity, as the quantal content of ACh normally runs down, the summation of EPP falls below the threshold, and they can no longer trigger the action potential of the muscle fibers (**Figure 1B**, numbers 1, 2, 3, 4). Then, several NMJ will present perithreshold EPP and intermittent transmission failures concomitantly, and the summation of several progressive blocks of NMJ transmission will lead to the MG symptoms (31).

Interestingly, the extraocular muscles (EOM) have physiologically less developed post-synaptic folding, hence a lower baseline safety factor, which could explain their high predisposition to dysfunction in MG (32). Furthermore, in ocular MG, these muscles are susceptible to complement-mediated attack due to a deficiency in complement-inhibitory proteins of the EOM and orbital tissue (33).

MECHANISMS OF ACTION OF THE Ab

Anti-AChR Ab

The pathogenicity of anti-AChR Ab has been shown by their ability to transfer the disease to control animals (34) and to reduce the number of α -bungarotoxin binding sites in myotube cultures (35). There is no correlation between the clinical severity of the disease and the Ab titer, but there is a correlation between the Ab titer and the ability of the sera to degrade AChR *in vitro* (36). However, in patients with immunosuppressive treatment, the changes in the level of anti-AChR antibodies is correlated with the clinical score (37).

Anti-AChR Ab can reduce the expression of muscle AChR by several mechanisms (**Figure 1B**): (1) removal of AChRs due to cross-linking and subsequent internalization (number 2); (2) functional AChR block (number 3), and (3) activation of complement with formation of membrane-attack complexes (MAC) that cause focal lysis (number 4) [Review in (38)]. Anti-AChR Ab are mainly IgG1 and IgG3 isotypes that bind the complement. This mechanism is likely the most pathogenic one: (a) there is an inverse relationship between the integrity of junctional folds and the abundance of C9, one molecule of the MAC (39); (b) mice mutated for complement factors (C3, C4, C5, C6) develop a lower incidence of MG upon active immunization, and their NMJ does not harbor the MAC [Review in (38)]; (c) Some patients with refractory MG have significant, often rapid, improvement in symptoms when treated with eculizumab, that inhibits the cleavage of C5 (40); (d) NMJ degradation decreases the safety factor and the efficacy of the transmission (41).

Anti-MuSK Ab

As a receptor tyrosine kinase, MuSK interacts with a plethora of proteins and downstream pathways, some of which involved in nuclear anchoring, gene transcription, Wnt interactions,

FIGURE 1 | Inhibiting AChR clustering (1). Anti-AChR antibodies reduce the expression of muscle AChR by removal of AChRs due to cross-linking, internalization, and degradation (2), functional AChR block (3), and activation of complement with formation of membrane-attack complexes that cause focal lysis (4). Blinding of anti-AChR antibodies also include muscle production of paracrine factors, microvesicles and exosomes, as well as cytokines (5) with potential effects over neighboring structures (satellite cells, muscle cells and nerve terminal). Pro-inflammatory environment can be enhanced during MG acute phase by infiltrating macrophages release of cytokines (6). Compensatory mechanism at molecular (7,8) and cellular levels (9) preserve MG muscle fibers from the AChR autoantibodies induced damage. Ach, Acetylcholine; AChR, Acetylcholine receptor; LRP-4, low-density lipoprotein receptor-related protein; MAC, membrane attack complex; MuSK, muscle specific kinase; VGSC, voltage-gated sodium channel.

scaffolding, and AChR stabilization (20). MuSK-MG is often characterized by muscle atrophy and excellent response to plasma exchanges. Experimentally, animals that received repeated daily injections of patient IgG (42) or actively immunized with MuSK (43) show impaired neuromuscular transmission, with reductions in endplate AChR and EPP amplitudes [Review in (44)]. *In vitro*, anti-MuSK Ab induce inhibition of proliferation of a cell line, an effect correlated with disease severity and anti-MuSK Ab titer, that could explain the muscle atrophy in MuSK+MG patients (45). The isotype of anti-MuSK Ab is generally IgG4 that lacks complement-activating properties and is considered functionally monovalent and is thus unable to induce antigenic modulation (46). Anti-MuSK Ab bind to a structural epitope in the first Ig-like domain of MuSK, prevent binding between MuSK and LRP₄ and inhibit agrin-stimulated MuSK phosphorylation resulting in defects of AChR clustering (Figure 1B, number 1) (47). In addition, anti-MuSK Ab block binding of ColQ to the NMJ, that may lead to compromised agrin-mediated AChR clustering and AChR deficiency in MuSK-MG patients (48). Finally, some anti-MuSK Ab are directed against the Cysteine-rich domain of MuSK that mediates the Wnt-MuSK interactions (49). In summary, by contrast with anti-AChR Ab, anti-MuSK Ab induce a functional effect by interfering with MuSK signaling and AChR clustering.

Anti-LRP₄ Ab

Mice immunized with the extracellular domain of LRP₄ exhibit MG-associated symptoms, including muscle weakness, reduced compound muscle action potentials, and compromised neuromuscular transmission (50, 51). Additionally, fragmented and distorted NMJs are evident at both the light and electron microscopic levels suggesting that LRP₄ contributes to NMJ maintenance in adulthood. In nerve terminals, a reduction in synaptic vesicle density and ACh release is observed, while on the postsynaptic side, AChR density is significantly reduced, with flattened junctional folds (50). Interestingly, injection in mice of neural agrin (N-agrin) that binds to LRP₄ leads to MG-associated symptoms, suggesting that agrin Ab may also play a role in MG pathogenesis (52).

MOLECULAR AND CELLULAR CHANGES IN MG MUSCLE

Several changes have been described inside and outside the giant syncytial muscle cell, and the importance of the local environment is increasingly considered (Figure 1B).

Inflammation and Cytokines

It is generally admitted that diffuse signs of inflammation are not evident in the muscle of MG patients. First of all, immune cells are scarcely found (29) [Review in (53)]. Second, the transcriptome analysis did not reveal an inflammatory signature (54).

However, increased expression of cytokines (TNF- α , IL-1, and IL-6) due to infiltrating macrophages has been described in the muscle of models of experimental autoimmune MG (EAMG), during the early phase of the disease (55) (Figure 1B, number 6). In addition, muscle tissues can also produce immunologically relevant factors. Rat skeletal muscle exposed to anti-AChR Ab synthesizes MCP-1, IL-15, and NO, that promote the generation of disease symptoms (56–58) (Figure 1B, number 5). Besides, myotubes in MG and EAMG overexpress IP-10 and CXCR3, two molecules regulated by interferon- γ (59). Interestingly, the skeletal muscle also upregulates the PD-L1 in MG, which may participate in the control of the local immune-mediated damage through the function of a checkpoint inhibitor (60).

Some cytokines and inflammatory proteins are increased in the sera of MG patients and constitute an inflammatory environment (61–64), then direct effects of these molecules on muscles could be suspected. As a proof of concept, muscle cells are responsive to IL-4, IL-6, IFN- γ , and LPS, by producing immunologically relevant molecules and may become antigen-presenting cells (65, 66). The expression of Toll-like receptors by the skeletal muscle could favor the sensitization of the muscle to the environment [reviewed in (67)].

Molecular Changes and Mechanisms of Compensation

Whether the molecular and cellular changes observed in and around the NMJ participate in the pathogenesis of MG disease or provide a mechanism of compensation are still an open question. Here, we will focus on two of these compensatory mechanisms.

First, the decreased expression in AChR is compensated by the release of an increased number of vesicles containing ACh, that has been shown in both muscles of MG patients and experimental rat models (Figure 1B, number 8) (27, 31, 68). The mechanism of this compensation may reside in several elements of the NMJ [Review in (27)]. At the presynaptic level, Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) act through activation by Ca²⁺ (69), and this mechanism has been shown to be involved in the model of rats treated with alpha-bungarotoxin (70). Although not directly demonstrated in MG models, neuroligin (71), and Munc18 would act through the modulation of the number of docked release-ready vesicles (72). From the post-synaptic side,

LRRK2 would trigger the increase of the size of the release-ready pool of vesicles (73). It has also been suggested that a specific pool of ACh vesicles, with a slower turn-over, would be used for transient increase of quantal content (74). LRP₄ may be considered as a retrograde factor acting from muscle toward the presynaptic side (75). Clinically, the compensatory mechanism mediated by increased quantal content would be especially important during phases of intense, repetitive stimulation of the NMJ as it would counterbalance the natural rundown of quantal contents partially. Importantly, it should be noted that in MuSK-MG, this compensatory mechanism is not present, or it is blocked by the Ab, and these patients develop more severe disease (25, 27, 76).

Second, as a consequence of the attack of the AChR by Ab (Figure 1B, number 7), the degradation of the AChR is followed up by increased mRNA level expression of AChR subunits in muscles of myasthenic rats, rabbits, and mice compared with control animals (77–79). In MG patient muscle, the increase in AChR subunit transcripts correlates with the severity of the disease, indicating that this mechanism takes place only when the expression of AChR is significantly altered (80); *in vitro* studies show that the increase in AChR mRNA appears after a certain threshold loss of AChR (induced by monoclonal anti-AChR Ab) (80, 81). The expression of AChR is the resultant of loss and re-expression. Without such a mechanism of compensation, the AChR expression could be dramatically reduced, resulting in a fatal disease. Thus, this compensatory mechanism aims to balance the loss of AChR in human MG and is triggered above a certain degree of AChR loss (80).

Upregulation of AChR expression could also result from activation of neuregulin1/ErbB signaling pathway through overexpression of MuSK and rapsyn (82). Whether this pathway is implicated in MG has not been documented.

Other molecular alterations have been described in EAMG models and are likely to be secondary to the cross-reactive immune response. Notably, caveolin-3 shows aberrant overexpression. This muscle-specific membrane protein localized to the sarcolemma and T-tubule system is usually needed for muscle repair and skeletal muscle development (83). Also, the glucose-regulated protein 78 (GRP78) mRNA that is activated by ER stress is increased, suggesting that muscle weakness in MG might be caused by both NMJ disruption and ER stress (84). Another intriguing observation relates to the bone mineral density at skeletal sites, which is significantly decreased in the femur of EAMG mice compared to control animals, in parallel with the severity of the disease (85).

TRANSCRIPTOMIC ANALYSIS

A transcriptomics study was performed in 3 different muscles [EOM, diaphragm, and extensor digitorum longus (EDL)] in rats passively receiving anti-AChR Ab. Changes in 62 genes common among all muscle groups fall into four major categories (stress response, immune response, metabolism, and transcription factors). Interestingly, the EOM demonstrated a distinct RNA expression signature from EDL and diaphragm (86).

Transcriptome analyses were also performed on muscle biopsies from MG patients (compared with healthy controls) and on models of active EAMG in rats (compared with control rats). Similar changes in human and rat myasthenic muscles were found, highlighting the deregulation of genes included in the muscle fiber category. Also, genes related to cell metabolism and immune response were deregulated: Insulin-Like Growth Factor 1 (IGF-1) and Interleukin-6 (IL-6) pathways were identified. Indeed, increased IL-6 production was observed in human muscle cell cultures treated with MG sera or anti-AChR Ab. Besides, monoclonal anti-AChR Ab decrease Akt phosphorylation in response to insulin, indicating an effect of the Ab on cell metabolism (54). Since Akt plays a key role in multiple cellular processes such as growth and glucose metabolism, this reduced phosphorylation of Akt may have a significant impact on the muscle homeostasis, and fatigability observed in MG patients.

EFFECTS ON SATELLITE CELLS

Satellite cells (SCs) are quiescent muscular stem cells (Figure 1). After an injury, a process of muscle degeneration occurs, followed by the activation of the SCs that proliferate, become so-called myoblasts, differentiate, and fuse to give rise to new fibers (87).

Recently, the article by Attia et al. (88) unveiled an unexpected action of the anti-AChR Ab on these SCs. First, muscle sections from MG and EAMG contain an increased number of SCs identified by the Pax7 marker. Besides, SCs isolated from MG muscles proliferate as myoblasts and differentiate more actively than cells from control muscles. In addition, after a muscle injury induced in the EAMG mouse model, several changes were observed: a decrease in fiber size and MyoG mRNA expression and an increase in the number of fibers and embryonic myosin heavy-chain mRNA expression. These alterations suggest that as a result of the autoimmune attack, there is a delay in maturation of the muscle fibers.

A direct effect of the anti-AChR Ab on SC is unlikely since SCs do not express AChR. More likely, the binding of anti-AChR Ab to their antigens impairs the NMJ (see the mechanisms above) and alters the production of several paracrine factors, micro-vesicles, or exosomes by the muscle. These factors could then induce paracrine effects on the neighboring SCs associated with subtle modifications of the epigenetic signatures (Figure 1B, Number 9). This leads to the expression of MyoD and MyoG in MG SCs that will proliferate and differentiate more than in healthy ones.

Together, these data propose that MG muscles from EAMG mice regenerate worse than control ones. From a clinical perspective, symptom exacerbation upon sports practice or after a muscle injury could also be due to difficulties for MG patients to regenerate their muscle.

CONCLUSION AND PERSPECTIVES

In MG disease, the Ab to the different components of the NMJ have pathogenic consequences that are more extended than a focused effect on the target antigens. In other autoimmune

diseases, the attack by the Ab and by the MAC would have induced the death of the target cells. In the case of the muscle, this does not occur, but activation of molecular transcription and signaling pathways, mechanisms of compensation, and biological effects on local cell types such as satellite cells demonstrate that the muscle responds actively. Thus, the muscle is not a passive target in MG but interacts dynamically with its environment in several ways. However, the number of studies examining these processes is still quite limited. A better appraisal of these processes would allow identifying new mechanisms and pathways, and new levels for symptomatic medical interventions. New approaches are rapidly developing to model MG and facilitate such studies. Indeed, with the advent of pluripotent stem cells differentiation, and the growth of bioengineering, cocultures of human myogenic and neurogenic

cells are possible in two (89) or three dimensions (90, 91), so as to study the effect of MG Ab, and/or to provide organoid-like platforms for the study of pathologies and their drug design.

AUTHOR CONTRIBUTIONS

SB-A and J-TV wrote the manuscript with support from RL. AB conceived and designed the figure.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Évidence préclinique de l'effet thérapeutique de l'efgartigimod dans un modèle de myasthénie anti-MuSK

Alexandra Clarissa Bayer Wildberger, Jean-Thomas Vilquin

Résumé

L'injection d'efgartigimod dans un modèle murin expérimental de myasthénie (anti-MuSK) permet une réduction importante du taux d'autoanticorps circulants avec comme corollaire une amélioration des signes cliniques et une augmentation des performances physiques chez les souris traitées par rapport aux souris non-traitées [1].

La Myasthenia Gravis (myasthénie, MG) est une maladie neuromusculaire auto-immune liée à une réaction immunitaire aberrante dirigée contre des constituants de la jonction neuromusculaire. Cette attaque est médiée par des auto-anticorps (Ac) ciblant des récepteurs post-synaptiques : le récepteur de l'acétylcholine (AChR) en premier lieu, le récepteur tyrosine-kinase spécifique du muscle (MuSK), la lipoprotéine-related protein 4 (LRP4), ou l'agrine. Ce processus diminue l'efficacité de la dépolarisation musculaire, ce qui se traduit par une faiblesse musculaire fluctuante et une fatigabilité. Les patients atteints de MG avec Ac anti-MuSK (appelée aussi MuSK-MG, soit environ 5 % des cas de MG) présentent souvent des signes respiratoires. La sévérité de la maladie est d'ailleurs liée au taux d'Ac circulants. Un modèle animal de MuSK-MG a pu être obtenu par transfert passif des Ac de patients à des souris immunodéficientes, une MG se développant chez elles en quelques jours.

La diminution du titre des Ac circulants pathogènes est l'une des stratégies thérapeutiques mises au point dans la MG. Les séances de plasmaphérèse lors d'une poussée de la maladie (crise myasthénique) permettent de l'obtenir. Une autre approche innovante, cette fois-ci sous la forme d'une immunothérapie vise à augmenter le catabolisme naturel de ces Ac. Ainsi, le récepteur néonatal Fc (FcRn) régule le catabolisme des immunoglobulines de type G (IgG) en diminuant leur dégradation lysosomale. L'utilisation d'antagonistes de ce FcRn empêche le recyclage des IgG et favorise leur dégradation. Dans le contexte de la MG, ceci favoriserait la clairance des IgG pathogènes et réduirait leur effet délétère au niveau clinique. L'efgartigimod (ou ARGX-113) est le fragment Fc d'un anticorps monoclonal de type IgG1 humain amputé du domaine variable (Fab). Ce fragment Fc a été modifié pour assurer une liaison de forte affinité aux FcRn, en particulier dans un environnement lysosomal acide.

Dans cette étude, les souris ont reçu des Ac de patients Musk-MG d'abord quotidiennement, puis une combinaison d'Ac et d'efgartigimod *versus* placebo, pendant les onze jours suivants. Alors que les souris non traitées ou traitées par placebo ont vu leur état clinique se dégrader, les souris traitées par efgartigimod ont montré des signes d'amélioration. Sur le plan humoral, une réduction d'environ 80 % du taux d'Ac humain anti-MuSK circulants a été observée. Sur le plan général, l'efgartigimod empêche la perte progressive de poids habituellement observée chez les souris malades. Au niveau fonctionnel, l'efgartigimod évite la perte de force détectée aux tests d'agrippement. Il réduit aussi la perte de force musculaire et la fatigabilité. En électrophysiologie, on observe un décrétement moindre lors de la stimulation nerveuse répétitive. En revanche, aucune différence n'a été notée entre les deux groupes concernant les aspects ultrastructuraux de la jonction neuromusculaire. Ces résultats soulignent le potentiel thérapeutique intéressant de cette nouvelle molécule dans cette forme spécifique de MG.

Commentaire

Cette étude est extrêmement prometteuse, mais a des limites. Le nombre d'animaux (n=16) est réduit, le bénéfice et la toxicité nécessitent d'être étudiés à plus long terme, et les Ac anti-MuSK de plusieurs patients devraient pouvoir être comparés. L'utilisation de l'efgartigimod pourrait s'apparenter à une « plasmaphérèse pharmacologique », visant à réduire le taux d'Ac pathogènes, particulièrement en situation d'aggravation aiguë. Cette molécule a traversé avec succès les étapes de validation préclinique chez le singe. Une étude de phase 1 a montré sa tolérance, défini les doses et les protocoles d'administration optimaux. Un essai de phase 2 randomisé contre placebo a été mené chez 24 patients atteints de la forme de MG associée aux Ac anti-AChR. Cet essai, bien qu'encore limité par le nombre de patients, a montré la bonne tolérance, et l'efficacité clinique de l'efgartigimod [2]. L'utilisation de l'efgartigimod dans les MG MuSK pourrait être encore plus intéressante puisque dans ce cas, la gravité de la maladie est liée à la quantité d'Ac, ce qui n'est pas vrai dans la forme avec Ac

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anti-AChR. Cependant, l'approche n'est pas spécifique des Ac pathogènes et entraîne la déplétion de l'ensemble des IgG, ce qui peut prédisposer certains patients aux infections. Le traitement ne cible pas les acteurs cellulaires de la réponse immunitaire (plasmocytes, cellules T et B auto-réactives) et sa durée d'action concerne le moyen terme (quelques semaines). Traitement d'attaque, traitement de fond, en relais ou substitution des immunoglobulines polyvalentes ou de la plasmaphérèse, la place de l'éfgartigimod dans l'arsenal thérapeutique reste

encore à définir. Dans tous les cas, cette molécule représente une nouvelle classe thérapeutique non seulement dans la MG mais aussi pour les maladies auto-immunes médiées par des IgGs en général.

Preclinical evidence of the therapeutic benefit of Efgartigimod in a model of myasthenia gravis mediated by anti- MuSK antibodies

LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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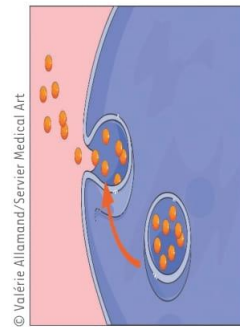
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Lu pour Vous Pré-Clinique

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Les exosomes, des messagers intercellulaires naturels aux mécanismes polyvalents pour le traitement des myopathies ?



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Résumé

Dans la souris *mdx*, l'injection d'exosomes permet d'améliorer les phénotypes pathologiques musculaires, cardiaques et squelettiques, et ce grâce à plusieurs mécanismes non exclusifs. Les exosomes, qui font partie des vésicules extracellulaires, mesurent une centaine de nanomètres et sont produits par de très nombreux types cellulaires. Leurs marqueurs de surface et leurs contenus sont variables en fonction des cellules productrices et des conditions environnementales. Libérés par exocytose, les exosomes sont ensuite internalisés par endocytose ou fusion membranaire. Ils présentent une action pléiotropique et jouent un rôle primordial de messagers intercellulaires en conditions normales et pathologiques. Dans la souris *mdx*, l'absence de dystrophine entraîne une perte de l'intégrité membranaire, un déséquilibre de l'homéostasie, une dégénérescence progressive et une inflammation musculaire, le tout se traduisant par une baisse de force, une fatigabilité, et le développement d'une insuffisance cardiaque.

Dans une première étude [1], l'injection intrapéritonéale d'exosomes produits à partir de cellules souches mésenchymateuses humaines et murines, de cellules dendritiques immortalisées murines, de myotubes murins, ou d'extraits de sérum murin, permet de limiter l'afflux de calcium intracellulaire, de diminuer l'activation de protéases, et de stabiliser les complexes existants de protéines associées à la dystrophine (sarcoglycans, dystroglycans). En restaurant partiellement la myo-architecture et l'intégrité membranaire des cel-

lules musculaires squelettiques des souris *mdx*, les exosomes bloquent l'influx de marqueurs exogènes (tel le colorant bleu Evans) et la fuite de protéines endogènes (comme les CPK et la LDH). Leur activité immunomodulatrice diminue l'infiltration leucocytaire et l'inflammation, et réduit l'expansion ultérieure de la fibrose. Ces effets sur des cibles multiples se traduisent par une augmentation de la force et de l'endurance des souris.

Dans une autre étude sur le même sujet [2], une injection intraveineuse unique d'exosomes produits par des cellules dérivées de progéniteurs cardiaques (cardiosphères) améliore les fonctions musculaires cardiaques et squelettiques des souris *mdx*. Les exosomes font régresser les lésions cardiaques, augmentent la fraction d'éjection myocardique, améliorant la capacité maximale d'exercice et diminuant la fibrose myocardique. Ils stimulent la régénération musculaire squelettique, augmentent la prolifération des cellules satellites, diminuent les réponses inflammatoires, et augmentent *in fine* la force isométrique des muscles squelettiques. Les bénéfices sont liés aux modifications transcriptomiques exercées par les micro-ARN (miR) contenus dans les exosomes, en particulier miR148a, ainsi qu'aux mécanismes d'immunomodulation. Les exosomes sont aussi efficaces que les cellules productrices elles-mêmes et constituent probablement leur principal mécanisme d'action.

Commentaire

Les exosomes sont des structures sub-cellulaires dont la production à large échelle, d'une qualité utilisable en clinique, devient possible. Ces nanoparticules peuvent être caractérisées de plus en plus finement concernant leurs marqueurs phénotypiques, leurs contenus en protéines (facteurs de croissance, de différenciation, de transcription), en acides nucléiques (ADN, ARN régulateurs...), en lipides, et leurs

fonctionnalités biologiques. Ils présentent peu, voire pas d'immuno-génicité, une biodistribution large et durable, et peuvent donc être administrés de manière répétée par voie systémique sans présenter de toxicité.

Les exosomes permettent ici de ralentir la progression de la maladie dans la souris *mdx*, voire d'observer un rétablissement phénotypique. Si la restauration de l'intégrité membranaire peut être considérée comme une cible primaire, des mécanismes complémentaires ou supplémentaires sont à l'œuvre : effets trophiques médiés par des facteurs de croissance, modulations géniques par des miR, effets indirects médiés par l'immunomodulation... Tous ces mécanismes ne sont pas exclusifs ; ils perdurent et dépendent de la nature, de l'origine, de la stimulation et de l'environnement de la cellule productrice. Les exosomes pourraient finalement représenter le chaînon mécanistique manquant pour expliquer le bénéfice observé de certaines approches de thérapie cellulaire, dans lesquelles on constate une disparition rapide des cellules après leur injection : les effets seraient médiés par les exosomes libérés durant la présence transitoire des cellules.

Ces traitements à partir d'exosomes ne peuvent remplacer la dystrophine mais limitent les conséquences de son absence. Ils peuvent

représenter des approches adjuvantes ou complémentaires permettant de ralentir la progression de la maladie. Ni tout à fait une thérapie cellulaire, ni même une forme de thérapie génique contrôlée, l'utilisation des exosomes pourrait ainsi représenter un nouveau paradigme thérapeutique, une nouvelle génération de candidats médicinaux biologiques. ♦

Exosomes: Multi-faceted natural intercellular messengers as new candidates for the treatment of myopathies?

LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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Aldehyde dehydrogenases contribute to skeletal muscle homeostasis in healthy, aging, and Duchenne muscular dystrophy patients

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Abstract

Background Aldehyde dehydrogenases (ALDHs) are key players in cell survival, protection, and differentiation via the metabolism and detoxification of aldehydes. ALDH activity is also a marker of stem cells. The skeletal muscle contains populations of ALDH-positive cells amenable to use in cell therapy, whose distribution, persistence in aging, and modifications in myopathic context have not been investigated yet.

Methods The Aldefluor® (ALDEF) reagent was used to assess the ALDH activity of muscle cell populations, whose phenotypic characterizations were deepened by flow cytometry. The nature of ALDH isoenzymes expressed by the muscle cell populations was identified in complementary ways by flow cytometry, immunohistology, and real-time PCR *ex vivo* and *in vitro*. These populations were compared in healthy, aging, or Duchenne muscular dystrophy (DMD) patients, healthy non-human primates, and Golden Retriever dogs (healthy vs. muscular dystrophic model, Golden retriever muscular dystrophy [GRMD]).

Results ALDEF⁺ cells persisted through muscle aging in humans and were equally represented in several anatomical localizations in healthy non-human primates. ALDEF⁺ cells were increased in dystrophic individuals in humans (nine patients with DMD vs. five controls: 14.9 ± 1.63% vs. 3.6 ± 0.39%, *P* = 0.0002) and dogs (three GRMD dogs vs. three controls: 10.9 ± 2.54% vs. 3.7 ± 0.45%, *P* = 0.049). In DMD patients, such increase was due to the adipogenic ALDEF⁺/CD34⁺ populations (11.74 ± 1.5 vs. 2.8 ± 0.4, *P* = 0.0003), while in GRMD dogs, it was due to the myogenic ALDEF⁺/CD34⁻ cells (3.6 ± 0.6% vs. 1.03 ± 0.23%, *P* = 0.0165). Phenotypic characterization associated the ALDEF⁺/CD34⁻ cells with CD9, CD36, CD49a, CD49c, CD49f, CD106, CD146, and CD184, some being associated with myogenic capacities. Cytological and histological analyses distinguished several ALDH isoenzymes (ALDH1A1, 1A2, 1A3, 1B1, 1L1, 2, 3A1, 3A2, 3B1, 3B2, 4A1, 7A1, 8A1, and 9A1) expressed by different cell populations in the skeletal muscle tissue belonging to multinucleated fibres, or myogenic, endothelial, interstitial, and neural lineages, designing them as potential new markers of cell type or of metabolic activity. Important modifications were noted in isoenzyme expression between healthy and DMD muscle tissues. The level of gene expression of some isoenzymes (ALDH1A1, 1A3, 1B1, 2, 3A2, 7A1, 8A1, and 9A1) suggested their specific involvement in muscle stability or regeneration *in situ* or *in vitro*.

Conclusions This study unveils the importance of the ALDH family of isoenzymes in the skeletal muscle physiology and homeostasis, suggesting their roles in tissue remodelling in the context of muscular dystrophies.

Keywords Aging; Aldehyde dehydrogenase; Dog model; Duchenne muscular dystrophy; Human; Skeletal muscle; Myogenic; Non-human primate

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Introduction

Aldehyde dehydrogenases (ALDHs) constitute a large family of isoenzymes that forged their diversity throughout evolution.¹ In human, 19 isoenzymes harbour different functions involved in ontogenesis, development, regeneration, and homeostasis.^{1–7} Most ALDHs detoxify cells from endogenous or xenogeneous aldehydes that result from catabolic reactions or oxidative stress by catalysation into corresponding carboxylic acids.^{4,7} Strong cellular protections against oxidative stress are conferred directly by these activities or, indirectly, through the production of secondary messengers able to activate other detoxifying enzymes (e.g. activation of glutathione peroxidase by retinoic acid).⁸ Such functions are responsible for resistance against cytotoxicity, mutagenicity, genotoxicity, and carcinogenesis. They also protect against toxic accumulation of reactive aldehydes produced by lipid peroxidation, such as malonaldehyde and 4-hydroxynonenal, and the aldehyde-protein adducts, which are especially involved in sarcopenia and the general process of aging.^{9,10} These catalytic functions are also responsible for the resistance against some chemotherapeutic agents and participate in the status of cancer stem cells ascribed to some ALDH-positive cell populations.^{11–17}

Some ALDH isoenzymes provide retinoic acid (ALDH1A1, 1A2, 1A3, 1B1, 3B1, and 8A1),⁷ and their coordinated expression is finely tuned during ontogenesis of several tissues,¹⁸ such as the skeletal muscles^{19–22} and heart.^{23,24} ALDH1A1 is involved in myogenesis,^{25–28} maintaining myogenic progenitors in an undifferentiated stage *in vitro*.²⁹ ALDH1A2 and ALDH1A3 are involved in cardiogenesis,³⁰ and ALDH1B1 is involved in the formation of pancreatic progenitors.^{31–33} ALDH3B1 is involved in diethylamino-benzaldehyde (DEAB)-insensitive retinoic acid synthesis in some cell types.³⁴ ALDH8A1 is involved in retinaldehyde metabolism, specifically the 9-*cis* retinal, and in oxidation of aliphatic aldehydes and glutaraldehyde. ALDH1A2, 1A3, 3B1, and 8A1 especially metabolize aldehydes derived from lipid peroxidation.^{2,35}

Several isoenzymes are involved in other metabolic pathways. ALDH1L1 encodes the formyltetrahydrofolate dehydrogenase and is involved in neurulation and in neural and glial stem cells.^{36,37} ALDH2 metabolizes acetaldehyde, and several mutations trigger intolerance to alcohol.³⁸ ALDH2 detoxifies aldehydes derived from lipid peroxidation.² ALDH2 is also involved in the metabolism of nitric oxide and plays roles in vascular adaptation, reactivity, and protection against ischaemia.³⁸ ALDH3A2 is involved in the oxidation of fatty aldehydes and in stabilization of cellular lipid membranes. ALDH5A1 is involved in catabolism of gamma-aminobutyric acid.² ALDH7A1 is involved in the formation of zebra fish eyes and fins³⁹ and scavenges peroxidized lipids,⁴⁰ semialdehydes, acetaldehyde, and benzaldehyde. ALDH9A1 catalyses the oxidation of betaine and the synthesis of gamma-aminobutyric

acid.^{2,11} ALDH isoenzymes, either alone or as a family of complementary agents, are therefore important regulators of several cell functions.

The fluorescent Aldefluor® (ALDEF) reagent identifies cell populations displaying ALDH activity, and it is widely used to identify stem cell populations from various tissues,^{41–47} including the skeletal muscle.^{27–29,48} Upon oxidation, ALDEF becomes hydrophilic and is trapped within cells, which can be discriminated using flow cytometry or fluorescence microscopy. Previously, we described SSC^{lo}/ALDEF^{br} cells extracted from dissociated biopsies of human skeletal muscles,⁴⁸ and we distinguished two main sub-populations according to the co-expression of CD34 marker. ALDEF⁺/CD34⁻ cells developed *in vitro* as a population of CD56⁺ myoblasts were able to form myotubes and participated efficiently in muscle regeneration *in vivo* in immunodeficient mice, while ALDEF⁺/CD34⁺ cells harboured adipogenic and osteogenic capacities suggestive of a fibro-adipogenic nature.^{48–50} The myogenic capacities of ALDEF⁺/CD34⁻ cells, together with the documented resistance of ALDH⁺ cells to oxidative stress, make them attractive candidates for cell therapy attempts to regenerate muscle tissues, especially in pathological contexts such as Duchenne muscular dystrophy (DMD).^{27–29,48,51–53} However, the persistence of ALDEF⁺ cell populations with aging, or their modulations in DMD, remains to be addressed, as several progenitors are reputed to decrease under these conditions.^{54–56} The exact nature of isoenzymes able to metabolize ALDEF is partly unknown, and most studies of muscle tissue focused on ALDH1A1 leaving unexplored the whole panel of ALDH isoenzymes expressed in parallel by muscle cells *in vivo* and *in vitro*.

In this study, we first assessed the persistence of ALDEF-positive cell populations harvested from healthy patients of different ages. Then, to study a potential role in pathological context, we investigated the proportions of ALDEF⁺ cells in muscle samples of DMD patients and healthy donors, and of the suitable preclinical model, the Golden Retriever dog (Golden retriever muscular dystrophy [GRMD]).⁵⁷ While no significant variation was observed with aging, important qualitative and quantitative changes were observed in those dystrophic tissues. We also associated several extracellular markers with ALDEF-positive sub-populations in these physiological contexts. In a second step, we identified the isoenzymes expressed *ex vivo* upon dissociation of muscle tissues and finally *in vitro* in both proliferation and differentiation, using flow cytometry, immunohistology, and semi-quantitative PCR. Several isoenzymes were found associated with distinct cell types in the muscle tissue and may constitute potential new cellular markers. Taken together, our results suggest that several ALDH isoenzymes are expressed by myogenic and non-myogenic cells, constituting new phenotypic or metabolic markers, and they underline quantitative and qualitative variations in dystrophic condition.

Materials and Methods

Biological samples

Human skeletal muscle samples were obtained as post-surgical *res nullius* via the Tissue Bank for Research (Myobank-AFM of Myology Institute, authorization no. AC-2013-1868), in agreement with the French bioethical law (Law no. 94-654 of 29 July 1994, modified 22 January 2002; Ethics Committee number BB-0033-00012, norma NF S-96-900) upon informed and signed consent of the donors. The healthy donors were adults, they had no clinical signs of muscular disease, and they underwent uneventful hip surgery allowing harvesting *tensor fasciae latae* (TFL) samples. Men and women were equally represented. Hip surgery is typically performed as a consequence of hip aging; therefore, the number of biopsies from young donors was lower than that from older patients. Samples were provided in transport medium (Dulbecco's modified Eagle medium [DMEM] supplemented with gentamycin) for use as fresh tissue for dissociation and culture and used within 24 h after extraction. Samples were also snap frozen in isopentane cooled in liquid nitrogen for immunohistochemical analysis or snap frozen in liquid nitrogen for analysis of RNA expression. Pathological paravertebral samples were obtained from young boys presenting with DMD, aged 13–16 years and undergoing spinal orthosis surgery. Healthy controls for this group were young patients of both genders presenting with idiopathic scoliosis. Human liver and kidney samples were provided snap frozen in liquid nitrogen upon necropsia by the Department of Neuropathology R. Escourrolle.

Samples from GRMD and healthy dogs (*biceps femoris*) were provided by École Nationale Vétérinaire (Alfort). Samples from non-human primate (NHP) *Macaca fascicularis* were obtained from Institut du Cerveau et de la Moelle (Paris) and harvested from several distinct anatomical localizations. Dogs and NHP were obtained at the time of sacrifice of these animals involved in unrelated protocols, and none of these animals were sacrificed for the purpose of our study.

Muscle dissociations

The human muscle biopsies were processed within 24 h after collection.⁵⁸ They were sliced, finely minced, and digested for 1 h at 37°C using 0.2% type II collagenase (Worthington) in DMEM (Invitrogen) supplemented with 10% defined foetal bovine serum (DFBS; HyClone). Mechanical dissociation was completed by passage of the cell dissociate through a 10 mL pipette, and an 18G needle. The suspension was filtrated through 100 then 40 µm cell strainers (Becton-Dickinson, BD). The resulting cell suspensions were centrifuged, washed, and

frozen in a medium containing 10% dimethyl sulfoxide, 20% DFBS, and 70% DMEM for later use. NHP and dog muscle biopsies were dissociated within 12 h after collection.

Cell cultures and expansion

Upon being thawed, the dissociated cells were washed once in phosphate-buffered saline (PBS) supplemented with 2% DFBS and then seeded in 25 cm² flasks (100–200 mg of the initial suspension) in the proliferation medium containing: 80% of modified MCDB120 (Molecular, Cellular and Development Biology) medium (custom-made by HyClone-Perbio), 20% DFBS, 25 µg/mL of gentamycin (Gibco), 10 ng/mL of human recombinant basic fibroblast growth factor (R&D Systems), and 1 µM of dexamethasone (Merck).⁵⁸ After medium change on the following day, cultures were grown for 10 days to reach 60% confluence, and then cells were harvested by trypsinization and further expanded before reaching 80% confluence for three passages. Aliquots were analysed for ALDEF activity, CD56 expression, or differentiation studies.

ALDEF and extracellular markers characterization by flow cytometry

Following being thawed and washed, cell suspensions were incubated with the ALDEF substrate (Aldefluor®, 1 µM, Stemcell Technologies) for 20 min (human and NHP cells) or 45 min (dog cells), at 37°C. Controls were obtained by prior incubation of cells with 50 mM of the specific ALDEF inhibitor DEAB.⁴⁸ Cells were centrifuged and kept on ice; surface antigens were detected by incubation with allophycocyanin-labelled CD34 (BD) or phycoerythrin (PE)-labelled CD9, CD10, CD29, CD31, CD36, CD44, CD47, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD56, CD61, CD71, CD105, CD106, CD140a, CD140b, CD143, CD146, CD184, and CD309 (3/100, 15 min, 4°C, BD). Cells were analysed using FACSCalibur (BD) and the CellQuest Software (2.10³ to 10⁴ events analysed, owing to the small size of the biopsies). Non-specific fluorescence was determined using negative isotype controls (BD).⁴⁷ Data were analysed and plotted using GraphPad Prism.

Cell sorting and myogenesis differentiation assay

Aldefluor® labelling was used for cell sorting in combination with labelling of CD34. Populations were sorted using a FACSAria cell sorter (BD) on the basis of ALDEF activity, APC-labelled CD34, and PE-labelled CD9, CD10, CD49e, or CD56. Cells were seeded into 12-well plates, grown for 1 week in proliferation medium, and expanded. When confluence was reached, differentiation was induced using DMEM

supplemented with 10% horse serum (AbCys). Five days later, cells were fixed and permeabilized using methanol (10 min, -20°C). The expression of desmin in myoblasts and myotubes was assessed using anti-desmin mAb (1/300, 1 h, room temperature [RT], Dako) followed by goat anti-mouse IgG1 Ab (Alexa Fluor 568, 1/1000, 1 h, RT). Nuclei were labelled with DAPI in mounting medium (Vectashield + DAPI, AbCys). Images were observed using a Zeiss epifluorescence microscope, images were captured using a Sony CCD cooled camera and the Metaview[®] software, and final figures were made using Adobe Photoshop[®].

Muscle subcellular localization of aldehyde dehydrogenase isoenzymes by immunohistofluorescence

Six-micrometre transverse cryostat sections were prepared from samples frozen in isopentane and fixed and permeabilized in acetone (10 min, -20°C); and non-specific labelling was blocked using 10% DFBS in PBS (30 min, RT). Human ALDH1A1, 1A2, 1A3, 1B1, 1L1, 2, 3A1, 3A2, 3B1, 3B2, 4A1, 5A1, 6A1, 7A1, 8A1, 9A1, and 18A1 were labelled using rabbit, goat, or mouse Ab (1/100–1/500, 2 h, RT, Table 1) followed by the secondary Ab linked to a fluorophore (Alexa Fluor 568, 1/300 in PBS, 30 min at 4°C). Then sections were incubated with a rabbit polyclonal anti-laminin Ab (1/200, 1 h, RT, Dako), followed by goat anti-rabbit Ab (Alexa Fluor 488, 1/400, 1 h, RT) to delineate skeletal muscle fibres. In situations where the first Ab was already produced in the rabbit Ab, we first coupled the anti-laminin Ab using the Mix-n-Stain CF488A antibody labelling kit according to supplier instructions (Sigma) and incubated this stained product (1/300, 30 min, RT). Nuclei were labelled with DAPI in mounting medium

(Vectashield, AbCys). Negative controls were obtained by omitting primary Abs. Sections were observed using a Zeiss fluorescence microscope, images were captured using a Sony CCD cooled camera, and the Metaview[®] software and final figures were made using Adobe Photoshop[®].

Localization of aldehyde dehydrogenase isoenzymes in primary cell cultures

The cell layers were washed in PBS, fixed in paraformaldehyde (PFA) (4% in PBS, 10 min, RT), permeabilized using saponin (0.2% in PBS, 10 min, RT), rinsed in PBS, and incubated using the rabbit Ab directed against isoenzymes (Table 1) followed by the secondary goat anti-rabbit Ab linked to a fluorophore (Alexa 568, 1/300 in PBS, 30 min, RT). Simultaneously, cells were incubated with mouse anti-desmin Ab (1/300, 1 h, RT, Dako), followed by goat anti-mouse IgG1 Ab (Alexa Fluor 488, 1/400, 1 h, RT) to define the myogenic structures. Nuclei were labelled using DAPI. Images were captured using a Sony CCD cooled camera and the Metaview[®] software, and final figures were made using Adobe Photoshop[®].

Characterization of isoenzymes by flow cytometry

After being thawed and washed, cell suspensions were fixed in PFA (4% PBS, 10 min, RT), permeabilized in saponin (0.2% in PBS, 10 min, RT), then rinsed in PBS, and incubated with ALDH isoenzymes, followed by the corresponding secondary Ab linked to fluorescein isothiocyanate (FITC) (1/300 in PBS, 30 min, RT). Some Abs were not available at the time of these experiments or not indicative under these conditions. Cells were centrifuged and analysed by flow cytometry (FACSCalibur, BD) using the Cell Quest Software (1.10^3 to

Table 1 Antibodies used in this study

Isoenzyme	Histology: species, provider, reference	Flow cytometry, cytofluorescence: species, provider, reference
ALDH-1A1	Goat, Everest EB05049; goat Abcam 9883	Rabbit, Abcam 23375
ALDH-1A2	Goat, SC 22591	Rabbit, SC 367527
ALDH-1A3	Rabbit, Abgent AP7847a	Rabbit, Abgent AP7847a
ALDH-1B1	Goat, Abcam 103896	NT
ALDH-1L1	Rabbit, Abcam 190298	NT
ALDH-2	Rabbit, Abcam 108306	Rabbit, Abcam 108306
ALDH-3A1	Mouse, SC 137168	Rabbit, SC 67309
ALDH-3A2	Rabbit, Abcam 113111	Rabbit, Abcam 113111
ALDH-3B1	Goat, SC 109191	Rabbit, Abgent AP8706c
ALDH-3B2	Rabbit, Abcam 112527	Rabbit, Abcam 112527
ALDH-4A1	Rabbit, Abcam 59011, 185208	Rabbit, Abcam 59011, 185208
ALDH-5A1	Goat, SC 70004; Mouse SC 515022	Goat, SC 70004; Mouse SC 515022
ALDH-6A1	Mouse, SC 271582	Mouse, SC 271582
ALDH-7A1	Rabbit, Epitomics 2300-S	Rabbit, Epitomics 2300-S; Abcam 68192
ALDH-8A1	Rabbit, SC 130686; FS PA5-63125	Rabbit, SC 130686
ALDH-9A1	Rabbit, Abgent AP7850a	Rabbit, Abgent AP7850a
ALDH-18A1	Rabbit, FS PA5-52955	NT

FS, Fisher Scientific; NT, not tested; SC, Santa Cruz.

Table 2 List of human primers used in the study

	Primers
h/ALDH1A1	F: GGCCTCAGATTGACAAGGA R: ATGATTTGCTGCACTGGTCC
h/ALDH1A2	F: AAGCTGGGACTGTTGGATCA R: TACTCCCCAAGCCAAATTC
h/ALDH1A3	F: ACGGTCTGGATCAACTGCTA R: CCGTCCGATGTTGAGGAAG
h/ALDH1B1	F: AGACGGTCACCATCAAGGTT R: AGCATTCTGCAAGTGGTTG
h/ALDH1L1	F: AGACCTTCCGCTACTTTGCT R: ATGATGCCACAAACCCCAAC
h/ALDH1L2	F: GCTTTCCAAAGGGGGTCCATC R: GCTAACAGCACAGCTCTTCAT
h/ALDH2	F: GGGAGAGCCAACAATCCAC R: CCACTCCCCGACATCTTGTA
h/ALDH3A1	F: ATCGCCTGGGGAAATTCAT R: AGTCCCGGGATTTCTAGCA
h/ALDH3A2	F: TTGGTACTCCAGGGGTAC R: GGTCAAGTCTTGAGTCCCA
h/ALDH3B1	F: CTTTTGGAGGAGTGGGTGC R: GCGTTGAGCTTCTCCATCC
h/ALDH3B2	F: CCACTACCCACCCTATACCG R: GTGAGTTGGGAGCATAAGCC
h/ALDH4A1	F: AGCCTCTGGAACCAATGACA R: CACCTGGACGGACAGACAG
h/ALDH5A1	F: GACGAAGCACCTTCTTTCC R: ATAGCTTCCCAGTGGCTCAA
h/ALDH6A1	F: TCACCGCTTTTGGTTGATCC R: TGTGGGATAAAGAGGGGCT
h/ALDH7A1	F: GGTTGCCCTTGGATCTGTTC R: TGAACCTTGCCAGCTCTCT
h/ALDH8A1	F: GCAGGGAACACTGTGATAGC R: GGTGGAACACTGCTTTATCC
h/ALDH9A1	F: AGACGACATGACCTGTGTGA R: CCGTTGGATGTCCCTGGTAA
h/ALDH16A1	F: TTCGGATCAGCCCAGGGTTC R: TCAGGCATCAGTCCCCATA
h/ALDH18A1	F: CCTGCAGGGGGTAAATGTTATT R: TCACGACTGCTGATCTCCG
h/SRP72	F: TGCTGCTGTGTTGACTCTG R: GCAGCACCCCATTTCTTCT

10⁴ events analysed); results were plotted and analysed using GraphPad Prism.

Messenger RNA expression analysis by quantitative PCR

The primers were designed using the OLIGO Primer Analysis Version 7, with a size between 18 and 23 nucleotide and annealing temperature of 60°C (Table 2). Primers were designed containing an intron sequence for specific cDNA amplification, and reactions were performed with appropriate negative template-free controls.

Total mRNA was isolated from snap-frozen tissue biopsies (100 mg) or cell pellets (100 000 cells at least) using TRIzol. Concentration and purity were evaluated with NanoDrop® ND-1000 Spectrophotometer (Labtech).

First-strand cDNA was synthesized from 250 ng of total mRNA with random hexamer primers using the RevertAid first-strand cDNA synthesis kit (Thermo Scientific).

Quantitative PCR was carried out in 384-well plates on cDNA products diluted to 1/25 in duplicate using SYBR Green (Roche) on a LightCycler® 480 (Roche) with the following parameters: initial denaturation step (95°C, 5 min), then 40 cycles composed of denaturation step (95°C, 30 s), and annealing/extension steps (60°C for 15 s, 72°C for 15 s). Srp72 was used as the reference gene. The ratio between the amounts of a target gene and the endogenous reference gene was determined. The amplification efficiencies between genes were compared by preparing a dilution series for genes from cDNA samples. Each dilution series was amplified in RT-Q-PCR, and the C_T values obtained were used to construct standard curves for targets. The amplification efficiency (*E*) for each target was calculated according to the following equation: $E = 10^{(-1/S)} - 1$ (where *S* = slope of the standard curve). Results were plotted, analysed, and compared using GraphPad Prism.

Statistical methods

All plotted dots are independent biological replicates (individual human or non-human primates), and all statistical tests were performed on GraphPad Prism software. Single comparisons were made using unpaired *t*-test (Figures 1G and 2B) or unpaired *t*-test with Welch's correction (Figure 2A), while multiple comparisons were performed using two-way analysis of variance (ANOVA) with Sidak's multiple-comparison tests (Figure 4A and 4B) or Tukey's multiple-comparison tests (Figures 1D–F, 1H, 1I, 3A–D, and 4C). *P* values are reported in the figures.

Results

Assessment of cells metabolizing ALDEF

ALDEF⁺ cells persist through aging

Muscle biopsies were collected from healthy patients undergoing hip replacement and were dissociated by mechanical and enzymatic treatments. The patients (25 women and 27 men, aged between 28 and 93 years) were divided into three experimental groups on the basis of age—28–48 (*n* = 12), 51–69 (*n* = 25), and over 71 years (*n* = 15)—and the proportion of cells metabolizing ALDEF was analysed in the presence (Figure 1A) or absence (Figure 1B) of the inhibitor DEAB and in association with CD34 (Figure 1C). Variability was noted in the proportions of ALDEF⁺ cells, especially in the groups of patients aged >50 (Figure 1D–F), but the changes were not significant over the years whether ALDEF⁺ cells were associated with CD34 or not (Figure 1E, F). Additionally, no significant difference was noted according to gender (Figure 1G) or when split into its two components, ALDEF⁺/CD34⁺ and ALDEF⁺/CD34⁻. Finally, the ratio of the ALDEF⁺/CD34⁻ cells,

considered as the most myogenic one, was slightly increased in the youngest patients but stable in those older than 50; however, the slope of the curve did not show a significant difference ($r^2 = 0.062$, Figure 1H, I).

ALDEF⁺ cells in a dystrophic context

The stable situation observed earlier was drastically modified in muscle tissue prepared from DMD patients whose muscle structure and function are highly altered owing to adipofibrotic infiltration. Paravertebral muscle biopsies from these young patients operated for orthopaedic spine surgery ($n = 9$; median age 15.4 years) were compared with those harvested from young healthy patients operated for idiopathic scoliosis ($n = 5$; median age 13.75 years). The percentage of ALDEF⁺ cells was dramatically increased in DMD patients, essentially owing to the increase in ALDEF⁺/CD34⁺ population (Figure 2A), which was mainly considered as non-myogenic;

meanwhile, the percentage of ALDEF⁺/CD34⁻ population remained stable

GRMD dogs, a large animal model of DMD, develop the progressive myopathy on both clinical and histological aspects owing to the absence of dystrophin. Here again, the percentage of ALDEF⁺ cells extracted from biceps femoris biopsies dramatically increased ($n = 3$) as compared with those of control dogs ($n = 3$). Surprisingly, we observed a combined increase in the ALDEF⁺/CD34⁺ and ALDEF⁺/CD34⁻ cell populations (Figure 2B), but it should be noted that GRMD dogs were young animals that still present ongoing muscle tissue remodelling at time of surgery. The increase in the ALDEF⁺/CD34⁻ population may reflect the ongoing regenerative activity and/or muscle remodelling at the time of biopsy collection, compared with that in DMD patients who suffer from fixed, established myopathy at their age (15.4 years).

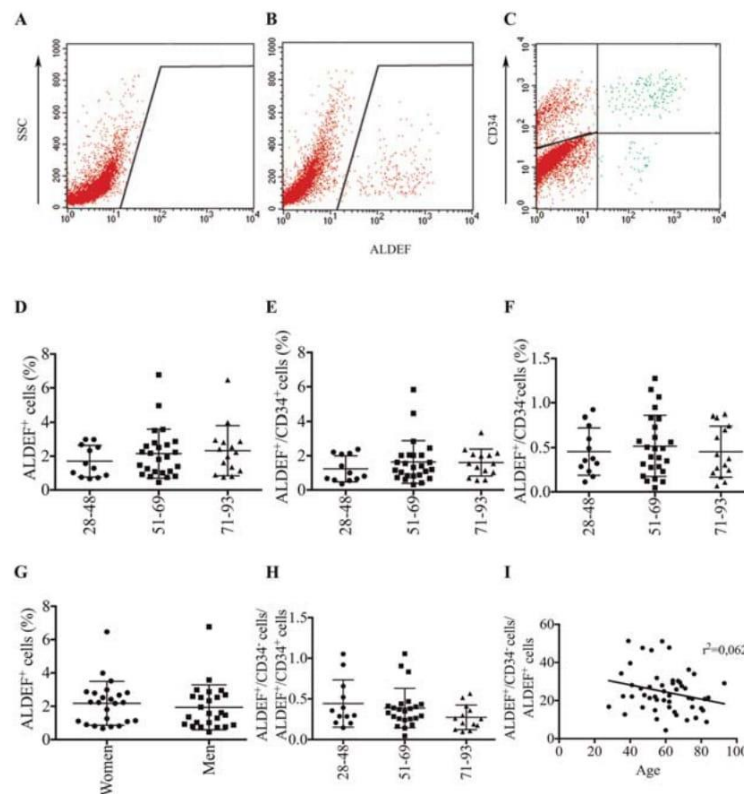


Figure 1 Flow cytometry characterization of ALDEF⁺/CD34⁺ and ALDEF⁺/CD34⁻ populations of cells extracted from patients of different ages. Dissociated cells from human TFL muscle biopsies were incubated with ALDEF substrate in the presence of DEAB inhibitor (A) or in its absence (B) and then with APC-labelled anti-CD34 (C). Representative cytograms are shown. The expression of CD34 can be represented as a function of ALDEF⁺ cells, providing an upper right quarter containing the ALDEF⁺/CD34⁺ cells and a lower right quarter containing the ALDEF⁺/CD34⁻ cells, in which the proportions can be estimated (C). Three cohorts of patients have been designed according to their ages (24–48, 51–69, and 71–93 years), and results have been plotted accordingly. No significant difference was observed between cohorts in the percentages of total ALDEF (D), of ALDEF⁺/CD34⁺ (E), or ALDEF⁺/CD34⁻ (F), nor in the ratio of these populations (H), although a non-significant tendency was observed towards a decrease (I). No significant difference was demonstrated between the cohorts of men and women (G). Data are percentages (mean, SD) of cells positive for the indicated marker. One-way ANOVA with Tukey's multiple comparisons was used for statistics in graphs (D), (E), (H), and (I) and unpaired *t*-test for graph (G).

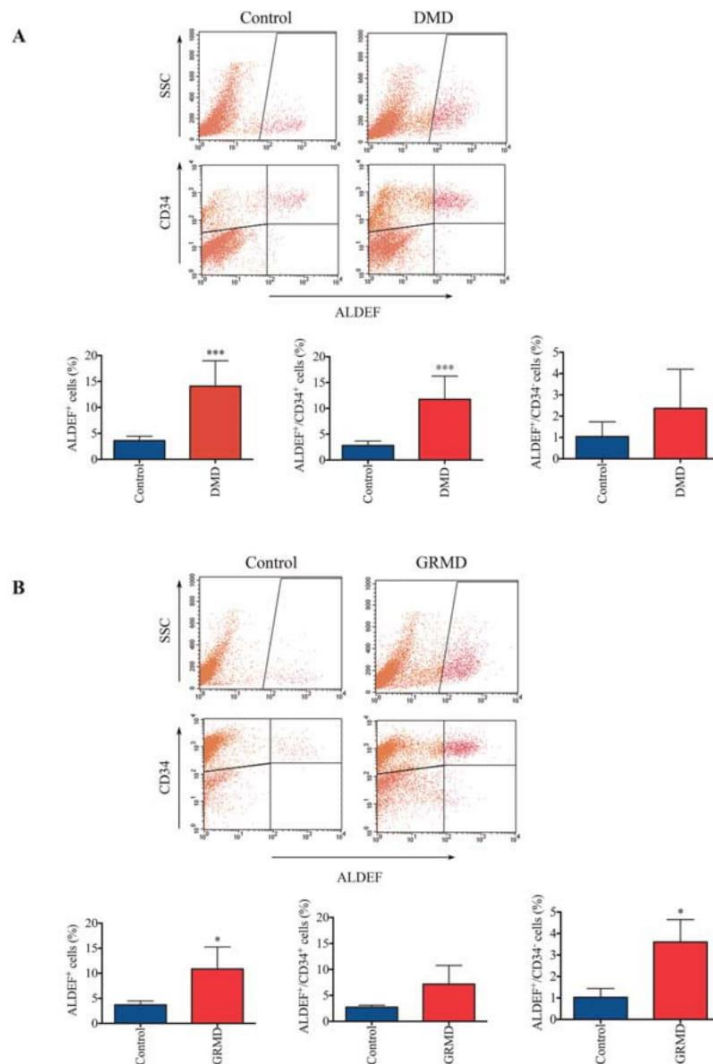


Figure 2 Flow cytometer evaluation of cell populations extracted from healthy and DMD patients, and from control and GRMD dogs. Cells were dissociated from muscle biopsies harvested from healthy ($n = 9$) and DMD patients ($n = 5$) (A) or control ($n = 3$) and GRMD dogs ($n = 3$) (B). Cells were incubated with ALDEF substrate and APC-labelled anti-CD34 allowing to distinguish ALDEF⁺/CD34⁺ and ALDEF⁺/CD34⁻ populations. Representative cytograms are shown; mean and SD are plotted on histograms (right panels). Total ALDEF⁺ cells are increased in DMD patients ($P = 0.0002$) and GRMD dogs ($P = 0.0499$), the ALDEF⁺/CD34⁺ population is significantly increased in DMD patients ($P = 0.0003$) but not in GRMD dogs, while the ALDEF⁺/CD34⁻ population is significantly increased in GRMD dogs ($P = 0.0165$) but not in DMD patients. Unpaired t -test with Welch's correction was applied for control vs. DMD patients and unpaired t -test for control vs. GRMD dogs.

Anatomical origin does not influence the proportion of ALDEF⁺ cells

We next investigated whether differences in cell populations would be observed among anatomical territories. For this purpose, we compared the populations of ALDEF⁺ cells, associated or not with CD34, from different muscles of NHP *M. fascicularis*: diaphragm (Dia), pharyngeal constrictor (Pha), masseter (Mas), paravertebral multifidus spinae (Para), biceps brachii (Bic), tibialis anterior (Tib), gastrocnemius (Gas), and vastus medialis (Vas) (Figure 3A). The initial proportion of

total ALDEF⁺ cells was higher than that observed in human and reached $8.51 \pm 0.97\%$, and we only observed slight differences in richness between muscle groups (Figure 3B, C), which could be ranked, in decreasing mean proportion as follows: Tib > Pha > Para > Mas > Bic > Vas > Dia > Gas. The population of ALDEF⁺/CD34⁺ cells represented the majority, as in human patients ($6.35 \pm 1.68\%$, i.e. 75% of all ALDEF⁺). The populations of ALDEF⁺/CD34⁻ represented the minority ($1.81 \pm 0.58\%$, i.e. 25% of all ALDEF⁺) and were ranked as follows: Mas > Dia > Tib > Gas > Bic > Vas > Pha > Para. The

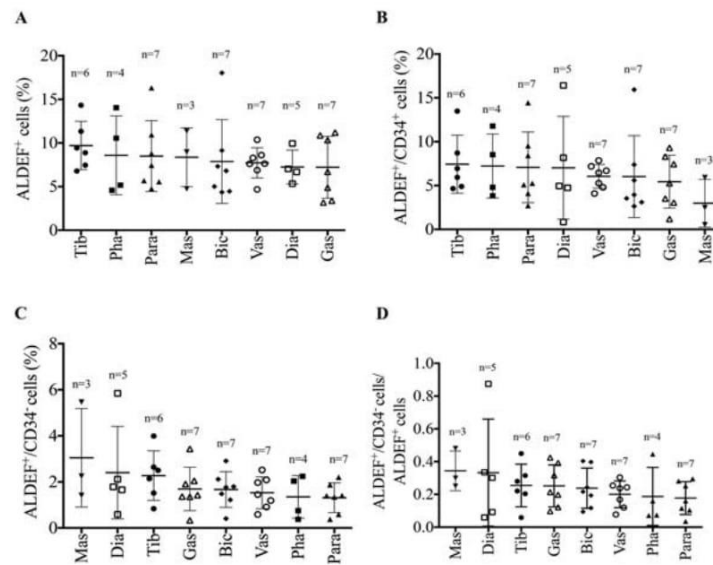


Figure 3 Flow cytometer evaluation of cell populations extracted from distinct anatomic territories. Cells were dissociated from muscle biopsies harvested from several territories of NHP (macaques) of similar ages ($n = 3-7$). Cells were incubated with ALDEF substrate and then with APC-labelled anti-CD34, allowing to distinguish the macaque cell populations. The contents in total ALDEF⁺ (A), in ALDEF⁺/CD34⁺ (B), in ALDEF⁺/CD34⁻ (C) populations, or their ratios (D) were compared and presented variations, and no significant difference between muscle groups could be established. n indicates the number of samples for each muscle group. One-way ANOVA with Tukey's multiple-comparison test.

embryologic origin of these muscles, i.e. somatic or facial, did not influence significantly the proportions of ALDEF⁺ cells; however, the muscle group containing the highest proportion of ALDEF⁺/CD34⁻ cells (Mas) is not a postural or locomotor muscle.

Extracellular markers distinguished sub-populations of ALDEF⁺ cells

ALDH activity represents intracellular, metabolic markers, which can be complemented by the study of associated extracellular markers. We analysed the presence of several extracellular antigens alone and/or in association with ALDEF⁺/CD34⁺ or ALDEF⁺/CD34⁻ clusters to identify in a reliable and comfortable manner the most myogenic over all cell populations. *Figure 4A* shows that the expression of several antigens may discriminate the populations ($n = 7$). Hence, the ALDEF⁺/CD34⁺ cells were frequently expressing CD10 (CALAA), CD49e (integrin alpha5), and CD140b (PDGF beta receptor), while the ALDEF⁺/CD34⁻ cells were preferentially associated with CD9 (MRP-1), CD31 (PECAM), CD36 (PAS IV), CD49a (integrin alpha1), CD49c (integrin alpha3), CD49f (integrin alpha6), CD71 (transferrin receptor), CD106 (VCAM), CD140a (PDGF alpha receptor), CD146 (MCAM), and CD184 (CXCR4). Of note, ALDEF⁺/CD34⁻ cells were frequently associated with some classical myogenic markers (such as CD9, CD106, and CD184), but not all of them because a minority of cells express CD56 (NCAM). Finally, some

markers were exclusive of one population, for example, CD29, CD44, CD47 (not shown), CD61, CD105, CD143, and CD309 were expressed by similar percentages of ALDEF⁺/CD34⁺ and ALDEF⁺/CD34⁻ cells.

The characterization was repeated with cell populations extracted from DMD patient biopsies (*Figure 4B*), focusing on the most discriminating markers identified earlier (CD9, CD10, CD31, CD36, CD49c, CD49e, CD49f, CD140b, CD146, and CD184). Differences were observed between control ($n = 4$) and DMD ($n = 4$) paravertebral biopsies. Total CD9⁺ and CD49e⁺ populations were increased in DMD biopsies. ALDEF⁺/CD34⁻ cell populations were mainly associated with CD9, CD31, CD36, CD49c, CD49e, CD49f, and CD146. The two populations ALDEF⁺/CD34⁻/CD31⁺ and ALDEF⁺/CD34⁻/CD49e⁺ were significantly increased in DMD biopsies as compared with control, and conversely, the ALDEF⁺/CD34⁻/CD184⁺ population was increased in control biopsies as compared with DMD.

Myogenic capacities of ALDEF⁺ cell populations

Because some populations of ALDEF⁺ cells can be defined by the expression of specific extracellular markers, we sorted NHP ALDH-expressing cells on the basis of extracellular markers associated with at least 30% of ALDEF⁺/CD34⁺ or ALDEF⁺/CD34⁻ cells. (i.e. CD9, CD10, CD49e, and CD56). Selected cells were grown, expanded once for amplification, and then committed to differentiation (*Figure 4C*). The

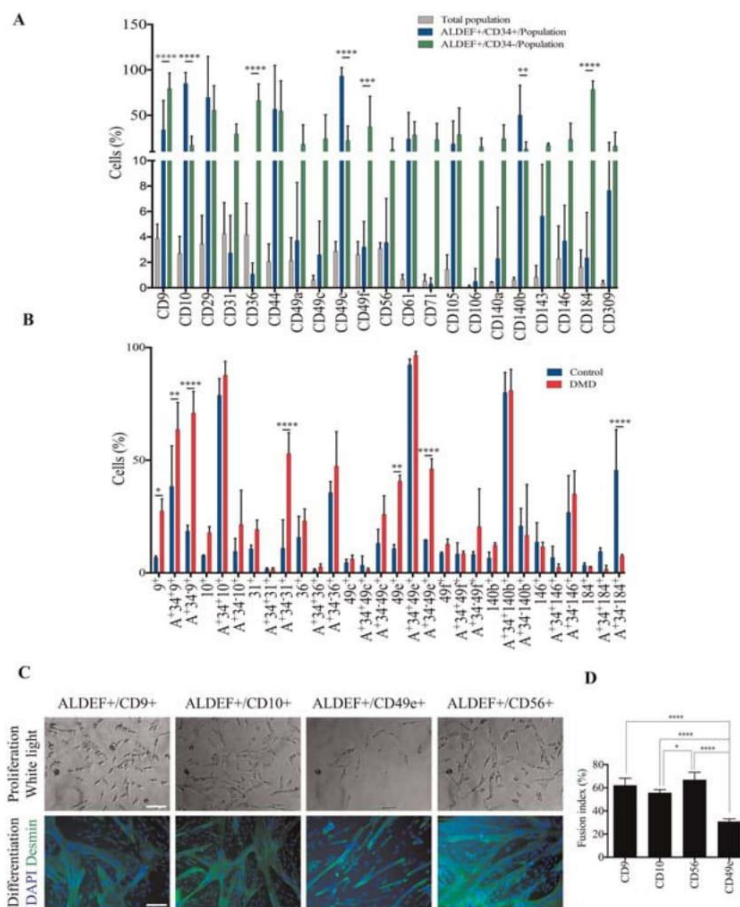


Figure 4 Identification of markers associated with ALDEF⁺ cell populations. Dissociated cells from human control TFL muscle biopsies were incubated with ALDEF substrate and then with APC-labelled anti-CD34 antibody and a second PE-labelled marker. Histograms and data are percentages (mean, SD) of positive cells for the indicated markers. (A) Cell analyses from TFL muscles of healthy controls ($n = 7$). The proportion of a dedicated marker among the whole content of mononucleated cells is presented in grey. The percentages of cells expressing a dedicated marker within the ALDEF⁺/CD34⁺ population and within the ALDEF⁺/CD34⁻ population are presented in blue and in green, respectively. This representation suggests that, for example, CD9 and CD184 are especially co-expressed with ALDEF⁺/CD34⁻ cells from skeletal muscles. (B) Comparison of some selected markers expressed by cells extracted from healthy (blue, $n = 4$) and DMD (red, $n = 4$) paravertebral muscles. For each marker, the three sub-populations are individualized (all cells, ALDEF⁺/CD34⁺, and ALDEF⁺/CD34⁻). Significant differences were observed regarding DMD cell populations expressing CD9, CD31, and CD49e and healthy cell populations expressing CD184. The myogenic differentiation capacities of cells selected on the basis of these markers is compared (C and D). NHP muscle cells ($n = 4$ animals) were incubated with ALDEF substrate and then with APC-labelled anti-CD34 antibody and a second PE-labelled marker—CD9 ($n = 4$), CD10 ($n = 2$), CD56 ($n = 4$), or CD49e ($n = 4$)—and then sorted using a FACSDiva. The selected populations were grown in culture in proliferating medium for two passages (C, left) and then differentiated, fixed, and labelled for desmin expression (green) and DAPI staining (blue) (C, right). Fusion indexes were calculated ($n = 4$ fields per well) (D). Cells selected on CD9 and CD56 were the most myogenic, while cells selected on CD49e were significantly less myogenic ($P < 0.01$) (bottom panel). Data are presented as mean and SD. Two-way ANOVA with Sidak's multiple-comparison test was applied (A and B) and one-way ANOVA with Tukey's multiple-comparison test (C; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$).

proportions of myogenic cells and the fusion indexes were counted after desmin immunostaining (a cytoskeletal marker of myogenic cells, both myoblasts and myotubes). We observed that the cultures performed from CD49e⁺ cells were the less myogenic ones and contained the smallest number of myotubes. In contrast, the cultures associated with CD9⁺ and CD56⁺ were the most myogenic. Cultures performed from CD10⁺ cells presented intermediate values of myogenicity (Figure 4D).

Expansion of ALDEF⁺ populations in primary cultures

We next analysed the long-term ALDEF metabolism in human primary cultures upon expansion to third passage without selection, and we compared it with the classical evolution of the CD56 marker in the same conditions. The proliferation was obtained in a myogenic medium previously described.⁵⁸ As soon as in first passage (7 days in culture), the majority of cells were ALDEF⁺, regardless of the age of donors, and this proportion was close to 100% in

the second (14 days) and third (21 days) passages. These proportions paralleled the expression of CD56 (95% in the second passage) (Figure 5A, B). These results were confirmed by fluorescence microscopy (Figure 5C) showing the fluorescence emitted by myogenic cells in proliferation and by myotubes upon differentiation in the presence of ALDEF *in vitro*. These results indicate that the isoenzymes metabolizing ALDEF are expressed in culture and involved in myogenesis.

The exact nature of isoenzymes metabolizing ALDEF is unknown. ALDEF is a small soluble molecule that remains trapped in living cells, and as it is not a protein, it cannot be cross-linked and fixed using classical protocols, hampering the co-localization of ALDEF and other intracellular markers. To have a broader picture about the presence and roles of ALDH, we attempted their characterization using antibodies and gene expression analysis.

Characterization of aldehyde dehydrogenase isoenzymes

Identification of the isoenzymes expressed by mononucleated cells extracted from biopsies

With the use of flow cytometry, antibodies identified most isoenzymes (Figure 6). From <1% (ALDH4A1) to >20% (ALDH2) of cells contained isoenzymes. Surprisingly, these values differ more or less from the number of cells able to metabolize ALDEF *in vitro*, which has been evaluated to 2–5%. Based arbitrarily on the proportion of cells stained by the Abs, a group of isoenzymes could be constituted in which <5% of the extracted cells were positive, gathering ALDH1A2, 1A3, 3A2, and 4A1. In the second group, >5% of the extracted cells were stained for ALDH1A1, 2, 3A1, 3B1, 3B2, 7A1, 8A1, or 9A1. Not all these isoenzyme-containing cells were metabolizing the ALDEF, but these results call for different methodologies.

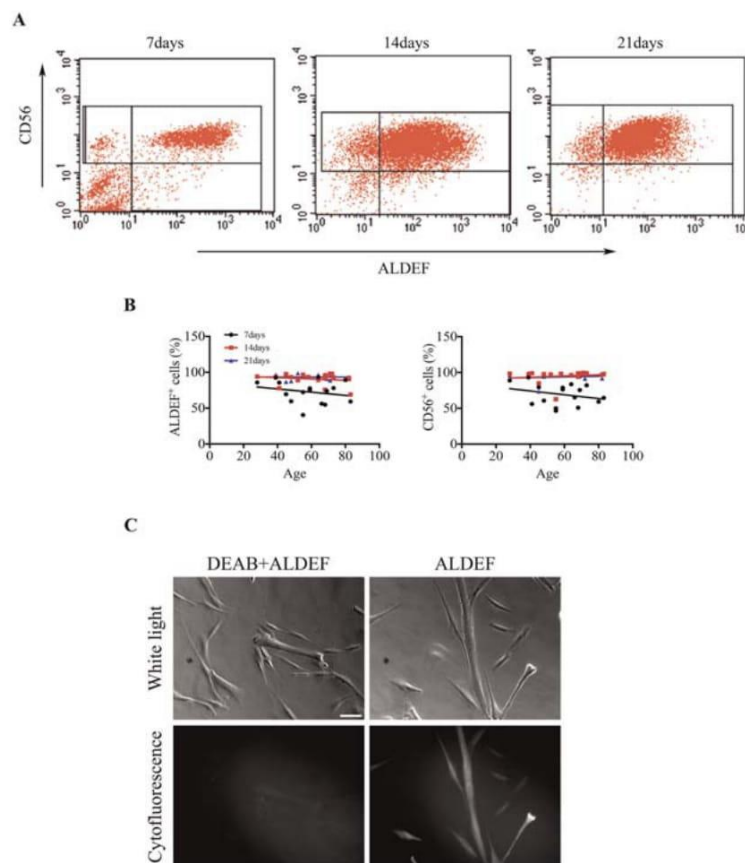


Figure 5 Expression of ALDH activity in culture. Cells were extracted from muscle biopsies of human donors of different ages and grown in culture in proliferating medium. Seven, 14, and 21 days after onset of cultures, ALDH expression was assessed using ALDEF, and the percentage of myogenic cells assessed using PE-labelled anti-CD56 antibody. (A) Increased proportions of cells express ALDEF and CD56 over time, and a few cells remain CD56⁺/ALDEF⁻. This behaviour is observed regardless of the ages of donors (B). The microscopic cytofluorescence observation of cultures following induction of cell differentiation indicates that ALDEF is also oxidized and accumulates in both myoblasts and myotubes (C).

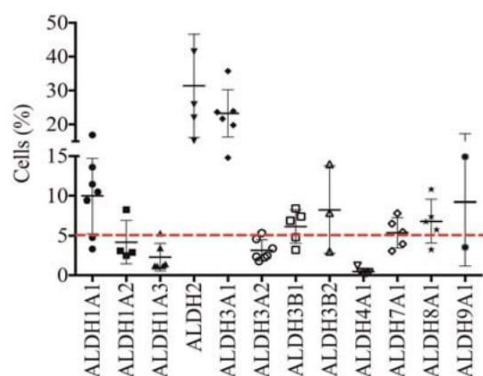


Figure 6 Identification of isoenzymes expressed by human freshly dissociated cells. Human cells were fixed using PFA and permeabilized using saponin, then incubated with antibodies directed against the ALDH isoenzymes indicated, then incubated with a secondary antibody labelled with FITC, and analysed by flow cytometry. Some isoenzymes were present in a high proportion of cells (up to 30% regarding ALDH2), while other isoenzymes were detected in smaller percentages of cells, with values around 5% but raising 10% (ALDH1A1 and ALDH9A1). A baseline was set arbitrarily at 5% to illustrate a maximum percentage of human cells detected using the ALDEF assay. This illustrates the potential discrepancy between the identification of the protein content using Ab, and the enzymatic activity detected by a functional assay. Data are presented as mean and SD ($n = 2-7$).

Abs, which are directed against the ALDH proteins, identify more cells than does the quantification of ALDH enzymatic activity. Indeed, while ALDH2 and ALDH1A2 are thought to be detected by ALDEF,¹⁴ the proteins can undergo several post-translational modifications (oxidation, phosphorylation, nitrosylation, and adduct formation), depending on cellular environment and subcellular localization, which modify their enzymatic activities.⁵⁹

Identification of the isoenzymes expressed by myogenic cells in culture

Human cells were grown in proliferation or in differentiation medium, fixed, and stained for the myogenic marker desmin, and for the isoenzymes showing previously the strongest expression (nine isoenzymes selected). We observed that during proliferation, ALDH isoenzymes labelled both myogenic and non-myogenic cells (Figure 7A). None seemed completely restricted to non-myogenic cells or to myogenic cells. Most presented a cytoplasmic staining, while others showed a nuclear (ALDH3A2) or perinuclear staining (ALDH3B1 and 3B2). During differentiation, ALDH isoenzymes were detected in the myotubes (Figure 7B) with a very weak (ALDH2 and 7A1) or strong intensity (ALDH3A1, 3B2, and 9A1).

Qualitative changes were noted between proliferating and differentiating cultures. Upon differentiation, ALDH1A1 was detected in myotubes but not in non-myogenic cells; ALDH2 staining was dramatically reduced; ALDH3A2 was concentrated in nuclei; ALDH3B1 was detected only in

myotubes. Changes in staining patterns and intensities suggest some roles for several isoenzymes in the course of differentiation *in vitro*.

Histological localization of cell populations in healthy and Duchenne muscular dystrophy muscles

We identified different cell populations harbouring ALDH isoenzymes by immunohistology using snap-frozen muscle biopsies of healthy and DMD patients ($n = 3$). Laminin staining was used to delineate the basal lamina of muscle fibres, and cells were positioned relatively to muscle fibres and basement membranes or microvasculature (Figure 8A and 8B).

In tissues from healthy patients (Figure 8A), some isoenzymes presented an intranuclear staining that covered some parts or the complete surface of the nucleus (ALDH1A1, 1A3, 1B1, 1L1, 3A2, 6A1, 8A1, and 9A1), while other isoenzymes presented mainly a perinuclear staining (ALDH1L1, 2, and 3B1). Some isoenzymes presented a cytoplasmic localization (ALDH1A2, 3A1, 3B2, and 5A1) or a nuclear and cytoplasmic localization (ALDH1L1, 2, 3A2, 8A1, and 9A1). Several nuclei were located inside the muscle fibres, in peripheral or (rarely) central position (ALDH1A1, 1A3, 1B1, 1L1, 3B1, and 8A1). Some isoenzymes labelled cells observed at positions reminiscent of that of satellite cells, that is, beneath the basement membrane and causing an excrescence to the fibre: ALDH1A1, 1A3, 1B1, 1L1, and 3B1. In the absence of the simultaneous staining of extracellular basement membrane and intracellular sarcolemma, it is not yet possible to conclude definitely regarding the status of these cells.

Some isoenzymes were expressed by cells located in the endomysial tissue: ALDH1A1, 1A2, 1B1, 1L1, 3A1, 3A2, and 3B1. Some isoenzymes were typically associated with endothelial or vascular structures (arterioles and venules): ALDH1A1, 1A2, 1A3, 1L1, 2, 3A1, 3A2, 3B2, 6A1, 8A1, and 9A1. Some tissues are especially characterized by the expression of a few isoenzymes: vessel-associated smooth muscle cells express ALDH2, 3A2, 8A1, and 9A1; and nerve bundles strongly express ALDH1L1 and 9A1. These isoenzymes may represent new or supplementary markers of these cell types (Table 3). Immunostainings with antibodies directed against ALDH4A1, 5A1, 6A1, 7A1, and 18A1 were not definitely successful despite attempts using different protocols.

Striking differences were noted upon examination of tissues from DMD patients (Figure 8B and Table 3). As expected, the DMD biopsies presented hallmarks of this myopathy, such as hypertrophic and swollen fibres, centronucleation, and the presence of abundant connective tissue. The isoenzymes presenting a nuclear localization underlined the central position of these nuclei in DMD context. Frequently, we observed an apparent decrease in the number of cells expressing isoenzymes, especially when these cells were juxtaposed to muscle fibres (ALDH1A1, 1A2, 1B1, 1L1, and 3B1) (Table 3). The most spectacular is the huge increase in cells expressing ALDH3A2, 1L1, and 9A1 and located in the endomysial

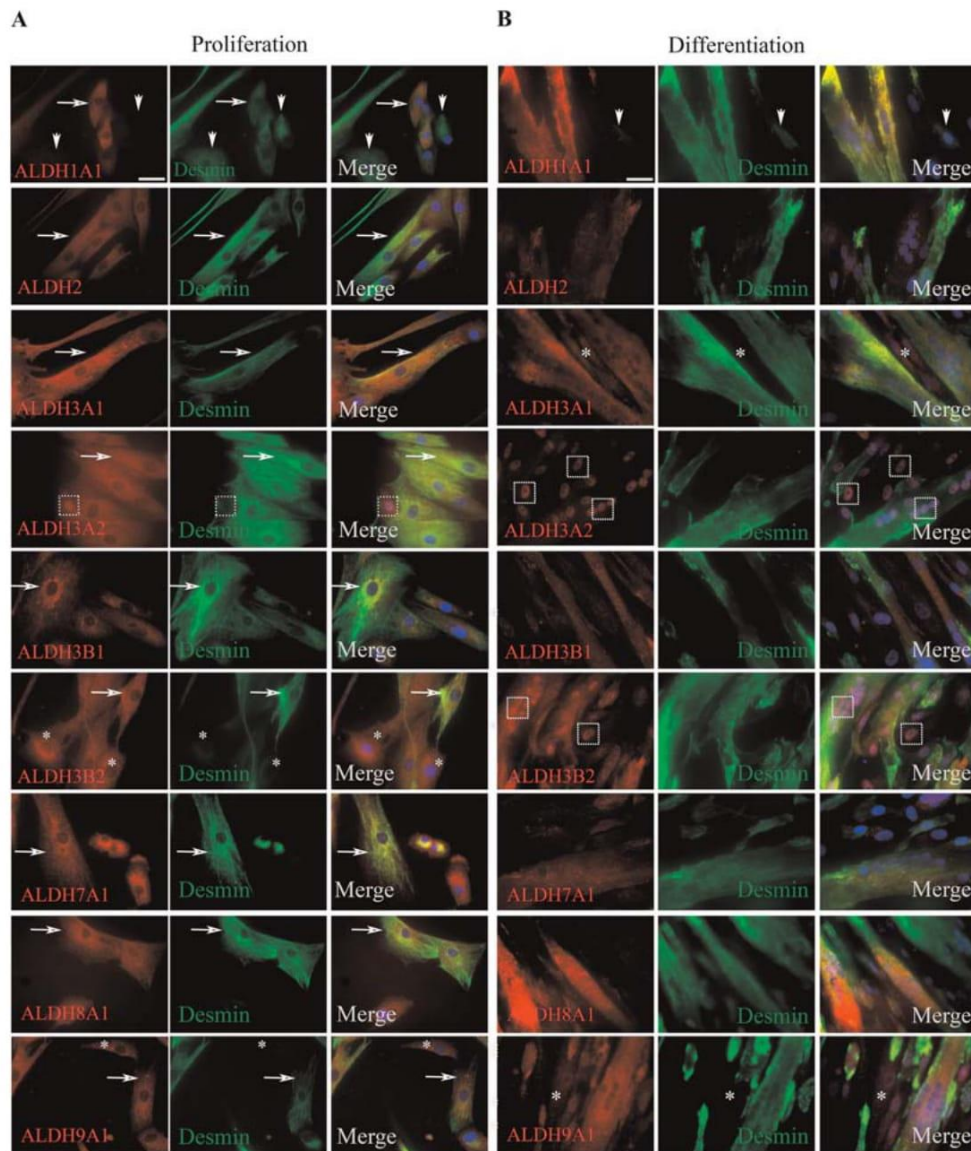


Figure 7 Immunocytochemical phenotyping of primary muscle cells in culture. Human cells were extracted from TFL muscles of healthy donors and grown in culture in proliferation medium (two passages, A) or in proliferation (two passages) and then in differentiation medium (4 days, B). Cells were fixed using PFA and permeabilized using saponin; and then incubated with antibodies directed against ALDH isoenzymes, and desmin; and then incubated with labelled secondary antibodies. Nuclei were stained using DAPI (blue). In proliferation (A), the number of cells expressing isoenzymes (red, first column) generally exceeded the number of cells expressing desmin (green, second column) (ALDH3A2, 3B2, 7A1, 8A1, and 9A1, arrows) except for 1A1 where some desmin⁺ cells were negative for the isoenzyme. ALDH3A2 stained strongly some nuclei. Following differentiation (B), myotubes were generally labelled with a stronger intensity than mononucleated cells (ALDH3A1, 3B1, and 9A1). The intensities of ALDH1A1 and 2 labelling were decreased as compared with cells in proliferation. Several nuclei strongly expressed ALDH3A2, suggesting a translocation from cytoplasm to nucleus. Original magnifications: $\times 40$.

position. These isoenzymes may, therefore, become appealing biomarkers for these cell populations that are overrepresented in the connective tissue of DMD patients.

Expression of isoenzymes at messenger RNA level

The study of isoenzyme expression at the protein level was supplemented by the quantifications of mRNAs by Q-PCR.

These were extracted from crude healthy human muscle, kidney, and liver tissues; from dissociated muscle cells; and from proliferating and differentiating cells in culture (Figure 9).

Expression of isoenzymes ex vivo Human muscle ($n = 4$), liver, and kidney ($n = 2$) were analysed concomitantly for the expression of the genes coding for 18 isoenzymes (Figure 9A).

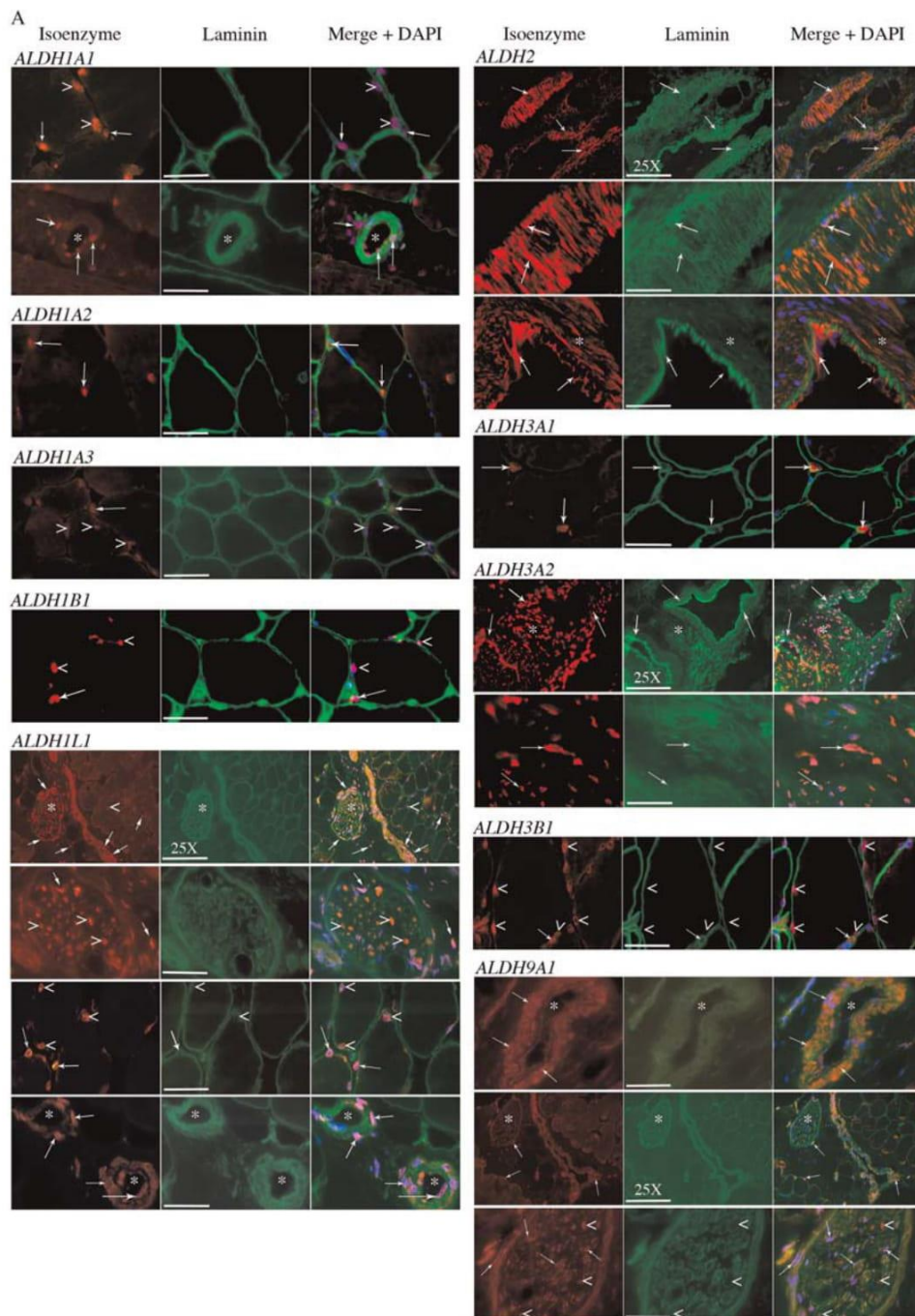


Figure 8 (A and B) Immunohistological localization of isoenzyme-expressing cells *in situ* on cryostat sections. Human skeletal muscle sections were fixed with acetone and labelled for expression of several isoenzymes (red) and laminin delineating the basal lamina of muscle fibres (green). Nuclei were stained in blue with DAPI. Arrowheads point nuclear localization of isoenzymes (intranuclear, perinuclear, or both). Arrows point whole cells regardless of their localization (endothelial, interstitial, neural, myogenic, etc.). Asterisks underline global areas of interest (concentration of cells). The most representative isoenzymes are presented from healthy donors (8A), or from DMD donors (8B) when qualitative differences were perceived between the two groups. Isoenzymes were associated with myogenic-like cells (ALDH1A1, 1B1, 1L1, and 3B1), to endothelial or angiogenic structures (ALDH1A1, 1L1, 2, 3A2, 3B2, and 9A1), to interstitial cells (ALDH1A1, 1A2, 1A3, 1L1, 3A1, 3A2, and 9A1), to neural structures (ALDH1L1 and 9A1), or to connective tissue (ALDH1L1, 3A2, and 9A1). Centronucleation is frequently observed in DMD biopsies (B) (ALDH1A1, 1B1, and 3B1). Original magnification is $\times 100$, and it is $\times 25$ when indicated on the scale bar. Scale bars are $40 \mu\text{m}$ ($\times 100$) and $140 \mu\text{m}$ ($\times 25$).

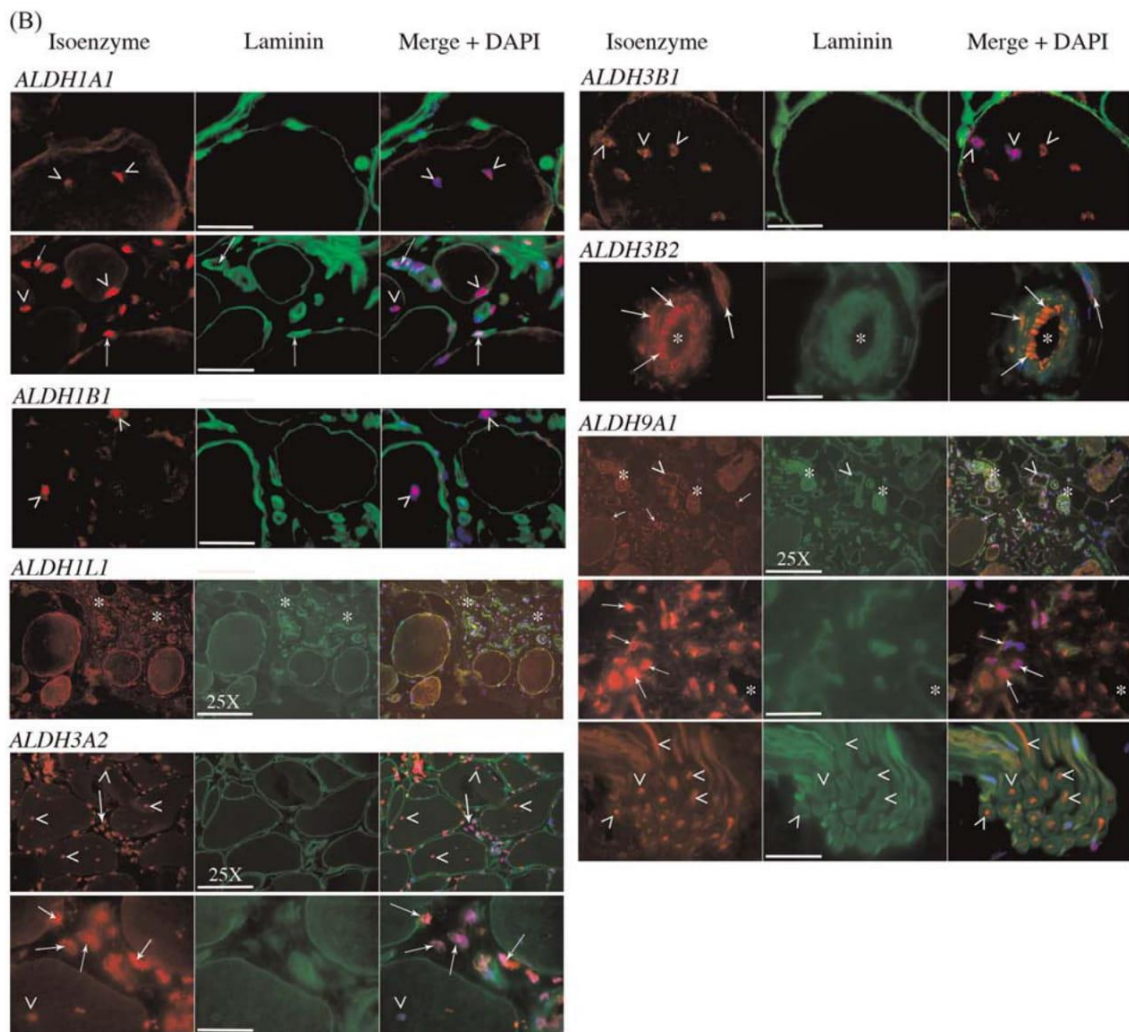


Figure 8 Continued

As expected, the liver contained the highest levels of ALDH isoenzymes, followed by the kidney. Almost all isoenzymes were also expressed in the skeletal muscle tissue, at various levels, but the profile was clearly distinct from that of liver and kidney and may serve to characterize the skeletal muscle tissue. ALDH2 is the most expressed, followed by 1A1, 1B1, 1L1, 3A2, 5A1, 6A1, and 9A1. Some isoenzymes were expressed at very low levels (1A2, 1A3, 1L2, 3A1, 3B1, 3B2, 8A1, and 18A1). The expression of ALDH16A1, which has no catalytic activity,⁶⁰ was extremely low in all human tissues and did not change with conditions (not shown).

Expression of isoenzymes by dissociated muscle cells Just after dissociation, muscle cells ($n = 4$) mainly expressed ALDH1A1, 2, and then 9A1, 1A2, 1A3, 3A2, 6A1, 7A1, 1B1, and 4A1 (Figure 9B). This profile was slightly different

from that observed in crude muscle, and it should be noted that most isoenzymes involved in retinoic acid metabolism were among the most expressed (1A1, 1A2, 1A3, and 1B1). Some structures and cells did not resist to enzymatic and mechanical dissociation, their isoenzyme contents have been eliminated, and the panel focused on markers of myogenic and interstitial cells.

Expression of isoenzymes in cell cultures Most isoenzymes were detected at various levels (Figure 9C) in these specific culture conditions prone to myogenic expansion and differentiation. ALDH1A1, 1A3, 1B1, 2, 3A2, 7A1, and 9A1 were the most expressed. ALDH2, 1A2, 1A3, 3A2, and 6A1 decreased; and ALDH1B1 and ALDH7A1 increased relatively. Some profiles evolved between proliferation and differentiation, but this did not reach statistical significance, except for

Table 3 Location of labelled structures

Location	Intranuclear		Perinuclear		Cytoplasmic		Intra-fibre		Satellite-like		Endomysial		Endothelial		Smooth muscle		Neural		Differences in DMD biopsies
	Control	DMD	Control	DMD	Control	DMD	Control	DMD	Control	DMD	Control	DMD	Control	DMD	Control	DMD	Control	DMD	
ALDH1A1	X some ^a	X some	X some	X some	X	X	X	X	X	X	X	X	X	X	X	X	X	X	^b Satellite-like, rarer than in healthy patients
ALDH1A2			X																↳ Cells, rarely observed
ALDH1A3	X (some)				X				X				X rare ^c						↳ Cells, rarely observed, almost absent
ALDH1B1	X				X			X	X			X	X	X					↳ Satellite-like and endothelial cells
ALDH1L1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	↳ Satellite-like, rarer than in healthy patients
ALDH2			X	X	X most ^e	X						X	X	X	X	X			↳ Not different
ALDH3A1			X	X	X	X				X		X	X	X					↳ Cells not observed, non-specific
ALDH3A2	X				X rare	X				X	X	X	X	X	X	X			↳ interstitial staining
ALDH3B1				X			X	X			X	X							↳ Much more frequent, fibrotic-like cells
ALDH3B2			X		X								X	X					↳ Satellite-like, rarer than in healthy patients
ALDH5A1			X		X	X	X												↳ Not different
ALDH8A1	X		X		X	X	X					X	X	X					↳ Cells, not observed
ALDH9A1	X	X	X		X	X				X	X	X	X	X	X	X	X	X	↳ Rare association to muscle fibres

Isoenzymes ALDH-4A1, 6A1, 7A1, and 18A1 were absent or not detected above background.

^asome: Some but not all elements in the category are stained.

^b↳: Indicates a decrease in number (qualitative assessment).

^crare: Small number of elements in the category are stained.

^daxons: Within this neural tissue, axons are stained.

^emost: Most elements in the category are stained.

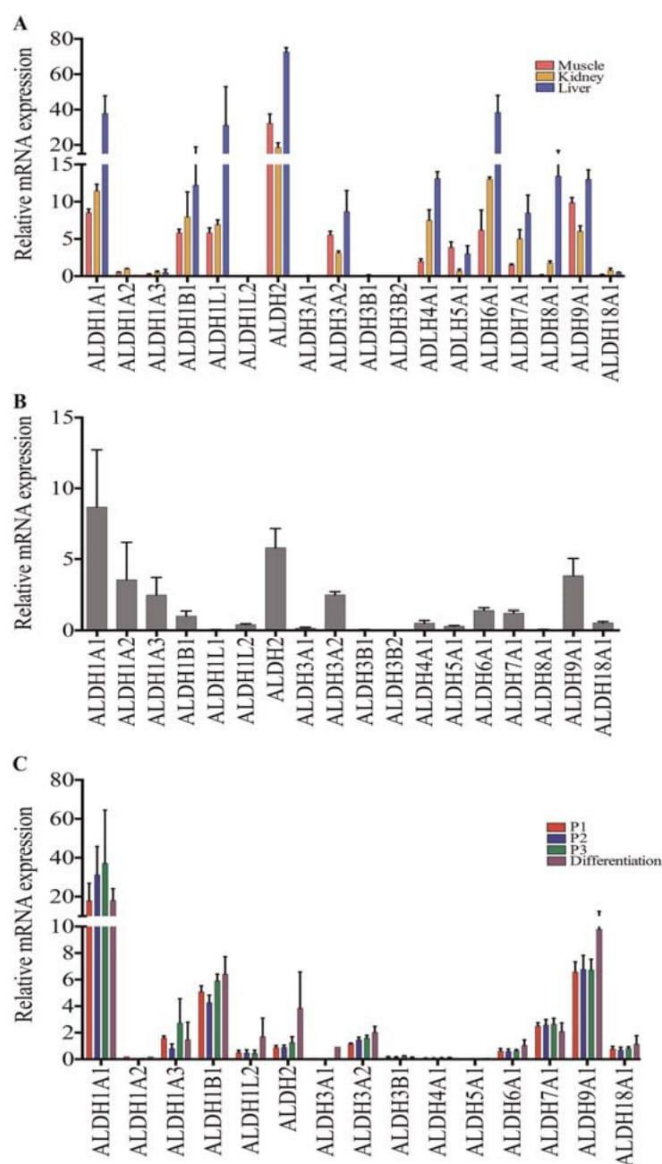


Figure 9 Expression of isoenzyme mRNAs in skeletal muscle tissue and cells. (A) The expression of isoenzymes was assessed and compared in human muscle (red, $n = 2$), liver (blue, $n = 2$), and kidney (yellow, $n = 2$) biopsies by Q-PCR, underlining the presence of ALDH1A1, 1B1, 1L1, 2, 3A2, 5A1, 6A1, and 9A1. (B) The expression was analysed in the total population of freshly dissociated cells from human biopsies highlighting the presence of isoenzymes involved in the metabolism of retinoic acid (ALDH1A1, 1A2, 1A3, and 1B1) and of ALDH2, 3A2, 6A1, 7A1, and 9A1. (C) The expression was analysed and compared in primary cell cultures in proliferation and in differentiation ($n = 4$), underlining the presence of ALDH1A1, 1A3, 1B1, 2, 3A2, 7A1, and 9A1. No significant differences were observed between the proliferation and differentiation stages. Data are presented as mean and SD.

ALDH9A1. ALDH1A1 presented a trend towards decrease upon differentiation, while ALDH2 presented a slight trend towards increase. ALDH3B2 and 8A1 could not be detected, but they were already weakly expressed in the other preparations. ALDH16A1 was very weakly expressed (not shown).

Discussion

This study reports the presence of ALDH-expressing cells in healthy and dystrophic skeletal muscles as evaluated on the side of ALDEF, a convenient marker for functional exploitation of myogenic progenitors, and on the side of isoenzymes

expression in view of delineating fundamental aspects of muscle homeostasis. Both sides documented the important modifications of populations in dystrophic muscles.

ALDEF⁺ populations remain stable with time

In healthy donors, we first observed a slight trend towards decrease with aging, which was not statistically significant. Some variations were noted between patients within the same group of age, but the clinical and physical conditions were unknown at time of hip surgery. While circulating progenitors are generally decreased with aging or in pathological conditions,⁵⁵ our situation resembles the absence of decrease observed by Povsic *et al.*⁶¹ in populations of medullary cells expressing ALDH. The diversity of ALDH isoenzymes allows detoxification of several oxidative remnants, aldehydes and protein-aldehyde adducts that progressively accumulate during aging, which all together would be especially responsible for sarcopenia,^{9,10} as suggested in a variety of other models and tissues.^{7,24,27,28,40,62} The diversity of isoenzymes allows metabolizing several substrates, and several isoenzymes may be expressed by a single cell population, as observed in our flow cytometry, cytofluorescence, and gene expression assays. Moreover, ALDH exerts indirect protective effects through the expression of detoxifying cascades such as glutathione peroxidase and superoxide dismutase.^{8,63} Retinoic acid is diffusible and frequently acts in a paracrine fashion⁶⁴; thus, it may diffuse from a small subset of ALDH⁺ cells and extend a protective area around small territories or niches, at least in healthy conditions. The presence of several ALDH isoenzymes with diverse detoxifying capacities in the muscle tissue suggests their protective role and may explain the persistence of ALDH⁺ cells with aging.

The proportions of ALDEF⁺ cell populations were compared between muscle groups in healthy NHP. Despite inter-individual variabilities, we did not observe significant differences in proportions between muscle groups involved in different physiological muscular functions (positioning, breathing, jawing, and swallowing) or of different embryological origins (trunk, head, and neck). This study in NHP confirmed a trend observed previously that the lowest proportion of ALDEF⁺/CD34⁻ population relates to the paravertebral muscles.⁴⁸ Xu *et al.*⁶⁵ demonstrated the considerable homogeneity in satellite cell contents between muscle groups but underlined some relative differences in richness according to precise physiological requirements.

ALDH⁺ cell populations are imbalanced in dystrophic tissues

The situation in healthy donors contrasts strikingly with the strong increase in the proportion of ALDEF⁺ cells observed

in DMD patients and GRMD dogs and suggests an important contribution to the remodelling undergoing in muscular dystrophy. ALDEF⁺/CD34⁺ cells are significantly increased in DMD patients, but we observed previously that this population was not able to regenerate the skeletal muscles *in vivo* and was associated with a mesenchymal-like behaviour⁴⁸ prone to adipo-fibrotic replacement during regeneration. Indeed, a population of CD34⁺ cells is involved in adipogenesis in human skeletal muscle.^{49,50}

Strong links have been proposed between ALDH expression, retinoids, ALDH-positive cells, metabolic pathways (TGF β , NFKb, BMP, EGF, etc.), tissue environment, proliferation, and fibrosis, although in some cases these links may follow the opposite direction.

First, in models of scarring diseases, the expression of ALDHs and the paracrine synthesis of retinoic acid dramatically trigger or exacerbate tissue fibrosis,^{66,67} suggesting that the increased number of ALDH⁺ cells able to produce retinoic acid may be detrimental to appropriate regeneration or may promote fibrous scarring. Second, in some pathological tissues (e.g. skin, kidney, eyes, and liver), enhanced ALDH activity positively correlates with elevated TGF β signalling pathway, triggering the activation and survival of fibroblasts, which overproduce ECM proteins and ultimately promote a persistent fibrotic and a pro-inflammatory environment.⁶⁶⁻⁷⁰ In the context of DMD, TGF β , Smad, and NFKb are strongly involved in onset and progression of muscle fibrosis.^{71,72} Also, TGF β activates the NFKb pathway in murine dystrophic fibroblastic cells, thus increasing their survival and proliferation.⁷⁰ As NFKb supports the survival and activation of macrophages, in muscle, it may be involved in the crucial phase of degeneration-regeneration that may shift from beneficial in a healthy environment to deleterious when the tissue undergoes inflammation and fibro-adipocytic infiltration. Third, the direct effect of TGF β on ALDH expression varies according to models. While in pancreatic cancer cells the increase in TGF β increases Smad4 but decreases the expression of ALDH1A1,⁷³ the expression of TGF β stimulates the expression of ALDH and the production of ALDH-positive cancer stem cells.^{74,75} Taken together, these reports suggest a link between the TGF β signalling pathway and the expression of ALDHs, leading to proliferation of fibro-adipogenic progenitors and fibrosis, at least in the skeletal muscle.

The difference between control and dystrophic muscles was confirmed upon histological studies. We observed abundant amounts of interstitial cells expressing mainly ALDH3A2, 1L1, and 9A1 isoenzymes in DMD muscles, which could become markers of fibrogenic or fibro-adipogenic cells especially in dystrophic conditions. It is yet unknown whether the population imbalances participate actively in the extension of the disease or reflect the steps of muscle remodelling. In DMD, a detrimental loop including ALDHs and TGF β may be responsible for onset and then expansion of muscle fibrosis in patients.

Our results obtained in DMD patients and GRMD dogs may represent two phases of the disease. On the one hand, the biopsies from DMD patients were obtained at the time of orthosis placement, that is, when muscle regeneration has been exhausted by the cycles of the disease and a fatty fibrotic tissue is replacing the healthy one. Indeed, the non-myogenic ALDH⁺/CD34⁺ population was the most increased. On the other hand, the biopsies from GRMD dogs were obtained from young animals, at early stages of the disease, that is, when muscle is still vigorously regenerating and the myogenic ALDH⁺/CD34⁻ population was the most increased.

It should be noted that the population of ALDEF⁺/CD34⁻ cells, which has been previously associated with myogenicity, is not drastically depleted in human patients or in GRMD dogs. This observation may be important in view of the future use of this population in therapeutic perspective; however, the actual differentiation capacities of ALDEF⁺/CD34⁻ cells extracted from healthy and dystrophic patients remain to be compared. The final role of ALDH⁺ cells and especially of the non-myogenic ALDH⁺/CD34⁺ population in the onset and maintenance of fibrosis in DMD muscles is not elucidated. Nevertheless, the identification of pathways involved may open the avenue to therapeutic tools, such as inhibitors of NFκB or of TGFβ pathways, or antagonists of retinoids.⁷²

Sub-populations are defined among ALDEF-metabolizing cells

ALDEF metabolism alone is useful to isolate muscle cells but not sufficient as a marker of myogenicity. Some initial subsets of ALDEF⁺, such as ALDEF⁺/CD34⁺ cells, do not express myogenic capacities.⁴⁸ Moreover, when reporting that ALDEF⁺/CD34⁻ cells are myogenic, this selects a population on a negative criterion, the absence of a marker (CD34⁻), which is less reliable and comfortable than selection on the basis of pure positive selection. These considerations mandated the search for associated markers directed against extracellular antigens. Such methodology has been developed by others to select specific myogenic cell types,⁷⁶ but it was not based on ALDEF metabolism.

In this study, cells were dissociated using only collagenase; that is, trypsin was avoided to spare extracellular domains of some antigens. Several markers were associated with dissociated muscle cells, some of which are being linked to ALDEF⁺/34⁺ or ALDEF⁺/34⁻ populations. The markers mainly associated with ALDEF⁺/CD34⁺ were CD10, CD49e, and CD140b. The markers mainly associated with ALDEF⁺/34⁻ were CD9, CD31, CD36, CD49a, CD49c, CD49f, CD71, CD106, CD140a, CD146, and CD184. Of these markers, CD9, CD49c, CD49f, CD106, CD146, and CD184 have been previously associated with cells presenting myogenic capacities, whether myogenic or perivascular.^{49,77–84} The association between CD56, which is a marker of human satellite cells and

myoblasts, and ALDEF, was variable at the time of dissociation. The CD56 expression became constant upon culture in myogenic condition.

When we compared the myogenic capacities in cultures of populations selected on CD56, CD9, CD49e, or CD10 markers in association with ALDEF, these populations could be ranked, in decreasing order of myogenicity, as CD56⁺ > CD9⁺ > CD49e⁺ > CD10⁺. Therefore, double-positive selections allow preparing cell populations, in a way that may be further amenable to preclinical and clinical applications, using FACS or magnetic bead selection. In myogenic cultures, most cells exhibited both high ALDEF metabolism and expression of CD56, thus confirming the role of ALDH isoenzymes in myogenesis. This was confirmed later on by the expression of several ALDH genes in culture by Q-PCR.

While it was first designed to allow identification and quantification of ALDH1A1⁺ cells,³ ALDEF can actually be metabolized by several isoenzymes depending upon the tissue and cell population under investigation. For example, the metabolism by ALDH2 and ALDH1A2 has been observed in leukaemia and lung cancer cells overexpressing these enzymes.¹⁴ The presence of ALDH1A1, 1A3, 2, 4A1, 5A1, 6A1, 7A1, and 18A1 has been observed in ALDEF-positive sorted cells.¹³ Of note, the DEAB used to block ALDEF metabolism and provide a negative control does not present the same selectivity towards all isoenzymes,⁸⁵ and the isoenzymes do not present the same enzymatic constants. We frequently observed differences between the percentages of cells expressing a given isoenzyme, and the total percentage of cells metabolizing ALDEF, and this was especially striking regarding ALDH2 and ALDH3A1. On the one hand, this may indicate that in muscle cells, some isoenzymes are present but do not participate in the metabolism of ALDEF in the condition of the assay. On the other hand, the final percentage of ALDEF staining may not reflect post-translational modifications of some isoenzymes⁵⁹ or competition between isoenzymes with differing enzymatic constants for the substrate, or the different levels of blockade by DEAB. There is no doubt that the use of ALDEF reagent allows selecting populations of myogenic cells; however, the exact nature of isoenzymes able to metabolize ALDEF is not clearly known. Therefore, we attempted to complete the landscape of muscle isoenzymes in the skeletal muscles using complementary methodologies.

Several isoenzymes are observed in muscle tissue and muscle cells by histology

Several isoenzymes were identified in different cell populations and structures using histology. Some populations were located close to muscle fibres in position resembling that of myogenic satellite cells (ALDH1A1, 1B1, and 1L1). Some populations were located in positions reminiscent of endothelial capillaries (ALDH2, 3B2, and 9A1), or associated with small

venules or arterioles (ALDH1A1, 2, 3B2, and 8A1). Some populations were located in the endomysium (ALDH1A1, 2, 3B1, and 7A1), and we already noted that some of them were exacerbated and may be involved in the formation of connective tissue in dystrophic muscles (ALDH1L1, 3A2, and 9A1). Finally, some isoenzymes were expressed by neural structures (ALDH1L1 and 9A1). Some isoenzymes therefore show multiple localizations and should be linked to different cell fates or functions: ALDH1A1 (myogenesis, angiogenesis), ALDH1L1 (myogenesis, angiogenesis, and neurogenesis), and ALDH9A1 (angiogenesis, neurogenesis, and formation of connective tissue).

Frequently, isoenzymes labelled nuclei, preferentially inside muscle fibres (ALDH3A1 and 3B1) and also outside the fibres (9A1) or both (3A2). A nuclear localization has been previously observed with ALDH3A1 and 7A1 isoenzymes, where it would directly participate in cell cycle regulation, resistance to DNA damage, and facilitation of repair and reduction of apoptosis.² Nuclear localizations therefore suggest unique roles in muscle cell physiology played by some ALDH isoenzymes.

In muscle cell cultures, several individual isoenzymes are expressed, as assessed by cytofluorescence, suggesting roles in proliferation, regeneration, and differentiation. A similar trend has been observed in several tissues by others (i.e. pancreatic cells, cardiac cells, and haematopoietic cells).^{32,43,46} In conditions favouring myogenicity, ALDH1A1, 1A3, 1B1, 3A2, 7A1, and 9A1 are the most prominent during proliferation and may constitute a signature of myogenesis.

Isoenzyme genes are differentially expressed in distinct muscle contexts

The skeletal muscle has been rarely investigated for expression of ALDH genes. Recently, Terry *et al.*⁸⁶ performed an extensive RNASeq analysis of muscle types in rodents. They reported the expression of all ALDH isoenzymes, at various levels depending on the isoenzyme. ALDH1A1 was the most expressed, followed by ALDH2 and 4A1. Interestingly, for a given isoenzyme, some variations were noted in the level of expression between anatomical and functional muscle groups. To obtain complementary follow-up, we achieved gene expression studies in three different situations: in crude skeletal muscle tissue, in muscle cells obtained after tissue dissociation and elimination of fibres, and in primary cultures. Some discrepancies were noted between the results of protein detection (cytofluorimetry, cytofluorescence, and immunohistology) and mRNA expression (Q-PCR) that may be due to the nature and sensitivities of the reagents and methodologies. For example, ALDH1B1, 2, 4A1, 5A1, 6A1, and 18A1 and to some extent 7A1 are mainly located in the mitochondrial compartment, not in cytosol,¹⁷ and may be more difficult to identify in histology. It was also reported

recently that the process of tissue dissociation itself activates some panels of genes within skeletal muscle cells, which may also create discrepancies between results obtained using different methodologies and cell status.⁸⁷ Nevertheless, overlapping of the results was most frequently encountered, at least on the qualitative point of view.

In crude human skeletal muscle tissues, isoenzymes ALDH1A1, 1A2, 1A3, 1B1, 1L1, 2, 3A1, 3A2, 7A1, and 9A1 were identified by both histological and Q-PCR methodologies, although at lower levels by histology regarding ALDH7A1. Our data confirm some initial northern blot expression studies that reported the presence in crude skeletal muscle tissue of ALDH1A1,⁸⁸ 1L1,⁸⁹ 2,⁸⁸ 3A2,⁹⁰ 4A1,⁹¹ 5A1,⁹² 9A1,⁹³ and 18A1.⁹⁴ At variance, we also observed the expression of ALDH1A3, 1B1, and 6A1, likely because of methodological refinements since the early description or owing to the difference in tissue source.

In dissociated muscle cells, ALDH1A1, 1A2, 1A3, 2, 3A2, 3B2, 4A1, 7A1, and 9A1 were identified by both cytofluorimetry and Q-PCR methodologies. ALDH1A2 and 1A3 were increased in dissociated cells compared with crude skeletal muscle, suggesting that these isoenzymes are more abundant in mononucleated cells than in muscle fibres, which are destroyed by the dissociation. Conversely, ALDH1L1, 2, 3A1, and 3B1 are decreased suggesting that these isoenzymes are involved in larger muscle structures; indeed, ALDH1L1 and 2 are observed within vessels, capillaries, and neural superstructures at the histological level, which may not be spared by the dissociation process.

Primary cultures were highly enriched in myogenic progenitors expressing CD56 and contain ALDH1A1, 1A3, 1B1, 2, 3A2, 7A1, and 9A1 as identified by cytofluorescence and Q-PCR. These results suggest that several isoenzymes are involved in myogenesis and/or cell survival *in vitro*. Other cell types have been reported to expand while keeping expression of ALDEF and a progenitor status.^{32,46,95,96} ALDH1A1 was expressed in our primary muscle cultures as previously described, but we also noticed the strong increase in ALDH1B1 and the moderate increases in ALDH7A1 and 9A1 in culture when compared with isolated mononucleated cells, suggesting that these isoenzymes may be positively involved in myogenesis *in vitro*. Meanwhile, ALDH2 was decreased and ALDH1A2 was almost abolished in culture; they may not be specifically involved in myogenesis but rather in homeostasis of other cell types.

Little is known regarding the sequential involvement of isoenzymes during myogenesis. Retinoic acid is involved,^{19–21,23,97–99} and several isoenzymes participating in its production and regulation are expressed in muscle as presented here (ALDH1A1, 1A3, 1B1, and 8A1) and may have additive or compensating effects as illustrated in other systems using knock-out animal models.^{18,100,101}

Specific roles of some isoenzymes have been pointed in dedicated tissues, but the skeletal muscle has been rarely investigated if any (except for northern blot analysis several

years ago). ALDH1A1 is involved in several embryological pathways such as formation of neural, haematopoietic, or cardiac tissues and myogenesis.^{25–29} ALDH1A3 is involved in cardiopulmonary system formation during embryogenesis and in the formation and maintenance of ocular systems. ALDH1B1 is involved in the formation, maintenance, and expansion of pancreatic progenitors³² and subsets of pancreatic cells.⁹⁵ Whether a parallel can be drawn between its unique role in pancreas development and potential roles in the skeletal muscle suggested by our studies is unknown yet. ALDH2 is involved in vascular adaptation, reactivity, and protection of large vessels against ischaemia.³⁸ In our study, cells expressing ALDH2 are mainly located inside vessel walls, or in positions reminiscent of that of endothelial cells. The expression of ALDH2 by endothelial cells would explain the high proportion of ALDH2⁺ cells extracted from muscle biopsies and the relative decrease in their proportion in culture under myogenic conditions. ALDH7A1 is involved in the formation of zebrafish eyes and fins³⁹ through the proliferation of progenitors, together with ALDH2 and 1A2.¹⁰² All these isoenzymes are expressed in various types of cancers through the participation in survival, maintenance, and proliferation of cancer stem cells.

Attempt for a synthetic overview

Gathering the information brought by our tools, we may propose some preferential roles for the different ALDH isoenzymes. We essentially considered their histological localization, the variations in mRNA expression (from crude muscle, dissociated mononucleated cells, and cells grown in a myogenic specific medium), and their expression *in vitro* upon culture in a myogenic medium.

ALDH1A1, 1A3, 1B1, 1L1, and 9A1 would be involved in general homeostasis combining myogenesis and angiogenesis. Of these, ALDH1L1 and 9A1 would be also involved in neural support in the tissue. ALDH1A2, 2, 3A1, 3B2, and 8A1 would be preferentially involved in angiogenesis or endothelial homeostasis. ALDH3A2 would be involved in stromal support. ALDH3B1 and 7A1 would be preferentially involved in myogenic homeostasis. The involvement of ALDH4A1, 5A1, 6A1, 16A1, and 18A1 could not be established, either because of a complete lack of function in muscle or because of inadequacy of tools. ALDH3A2 and ALDH9A1 would be especially involved in the physiopathological process of DMD, because of their increased presence in the fibrotic tissue.

Taken together, our results suggest that, supplementary to ALDH1A1 that was previously described, several isoenzymes present in both myogenic and non-myogenic cells take part in skeletal muscle homeostasis *in vivo* and *in vitro* and would help in defining an identity card of this tissue. Minimally, ALDH1A2, 1A3, 1B1, 1L1, 2, 3A2, 3B1, 7A1, 8A1, and 9A1

deserve future individual investigations, given their high expression level, their association to muscle structures and cells, and their biochemical detoxifying functions.

Limitations of the study and perspectives

This study was hampered by some technical or methodological limitations, represented by the variability of the muscle samples extracted from human patients with intrinsically variable status. Limitations were also met using some antibodies showing variable reactivities between methodologies (flow cytometry, immunohistology, and cytofluorescence). The present study, however, is the first to assess the presence of the ALDH isoenzymes family in the skeletal muscle *in situ* and *in vitro*. Hence, through their presence within several populations in the tissue, their expression in native condition and in culture, their distribution and their persistence with aging, and their imbalance in dystrophic conditions, ALDH isoenzymes are proposed to play roles in skeletal muscle homeostasis. These isoenzymes may therefore constitute new therapeutic targets for pharmacological, molecular, or genetic modulations. Some of them may also define a panel of new specific markers of muscle cell populations. Furthermore, ALDEF⁺ cell populations, in association with dedicated markers, may constitute new promising candidates for regenerative therapy approaches.

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Conflict of interest

The authors declare no competing interests.

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Abstract

Myasthenia gravis (MG) is a rare autoimmune disease mediated by pathogenic antibodies targeting neuromuscular endplate molecules, mainly the acetylcholine receptor, and characterized by immune dysregulation and chronic cell activation. The impaired neuromuscular transmission leads to muscular weakness and invalidating fatigability, and MG crisis can be life-threatening. The treatments are associated with serious side effects, mandating the research of innovative therapeutic solutions.

Mesenchymal Stem Cells (MSC) are multipotent progenitor cells possessing broad immunoregulatory capacities, and acting via cell-cell contacts, production of extracellular vesicles and secretion of soluble mediators. To boost their immunosuppressive capacities, MSC can be primed by high doses of pro-inflammatory cytokines such as interferon- γ (IFN- γ), which however may impact MSC fitness and immunogenicity. Our team developed an alternative approach, in which MSC are cocultured with peripheral blood mononuclear cells (PBMC), thus becoming conditioned MSC (cMSC). The transfer of research grade cMSC improved the clinical outcomes in a humanized MG mouse model (NSG-MG).

In a clinical perspective, research grade cells were first replaced by clinical grade MSC, and we initiated the identification of typical signatures that will allow their use. Ideally, the use of PBMC should be replaced by a cocktail of their activating molecules.

Here, we characterized thoroughly gene expression, phenotypic profile, and secretome changes induced by PBMC conditioning, when compared to non-stimulated (rMSC) and IFN- γ (γ MSC) cells. We studied the functional capacities of these cells *in vitro* and *in vivo*. Additionally, we identified molecules produced during cell cultures that may be responsible of conditioning or immunomodulation.

RNAseq study showed that compared to rMSC gene expression profile, conditioning by PBMC deregulated the expression of 244 genes. Compared to rMSC and γ MSC, the signature of cMSC included up-regulation of *CCL2*, *CCL11*, *DPP4*, *ICAM-1*, *IL6*, *PDCD1LG2*, *TNFRSF11B*, *TNIP1*, *TNIP3*, *ZC3H12A*, and down-regulation of *CILP* and *LGALS1*. γ MSC deregulated 2089 genes, bearing a classical signature consisting in up-regulation of *IDO1*, *TGF- β 1*, *CXCL9*, *CXCL10*, *CXCL11*, *HLA* and *HLA*-associated genes. Main pathways related to cMSC were extracellular matrix remodeling, signaling by interleukins and immunosuppression, while IFN- γ response, JAK-STAT signaling and inflammatory response were associated with γ MSC. Gene signatures for each treatment were confirmed by qPCR.

cMSC and γ MSC also presented phenotypic differences when compared to rMSC. Signatures of cMSC included increased CD26, CD54, CD105, CD273, and CD318 expression, without HLA modulation. At variance, IFN- γ activation increased expression of CD54, CD274, CD317, HLA-ABC and HLA-DR molecules. General phenotypic profile studied by mass cytometry revealed 10 different metaclusters; rMSC and cMSC had close profiles, sharing most of the metaclusters except for one, that was characterized by a strong immunomodulatory imprint. γ MSC showed a completely different phenotypic profile.

Regarding functional capacities, *in vitro*, conditioned medium (CM) produced by cMSC had higher immunosuppressive capacities on T cell proliferation and induced higher percentage of Treg when compared to rMSC and γ MSC CM. The analysis of cMSC secretome revealed 44 molecules that were significantly upregulated by cellular conditioning. Meanwhile, proteomic analysis of soluble factors secreted by PBMC alone, MSC alone and both cells in coculture allowed the identification of 22 molecules that are potentially implicated in cellular conditioning.

In our NSG-MG mouse model, cMSC reduced significantly the clinical score of mice when compared to untreated ones, 2 weeks after injection till end-point of the experiment.

These results provide clues for defining the mechanisms of action of conditioning by PBMC, and suggest the use of cMSC or their CM to operate immunomodulation in the context of MG, or other auto-immune diseases.