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# Hematopoietic progenitor populations for cell therapy of autoimmune diseases : characterization and comparison of their mechanism of action in Type I Diabetes and Experimental Autoimmune Encephalomyelitis

Sarantis Korniotis

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Université Paris Descartes

**Ecole Doctorale**

**Génétique Cellules Immunologie Infectiologie Développement**

Institut Necker Enfants Malades INSERM U1151 CNRS 8253

**Hematopoietic progenitor populations  
for cell therapy of autoimmune diseases :  
Characterization and comparison  
of their mechanism of action in  
Type I Diabetes and  
Experimental Autoimmune Encephalomyelitis**

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Thèse de doctorat d'Immunologie

Dirigée par Dr. Flora ZAVALA

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**le 24 Juin 2014**

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*“Life is a continuous ascent to higher and higher removable peaks together with the ecstatic but painful knowledge that there are no extreme peaks”* (Nikos Kazantzakis)

## TABLE OF CONTENTS

<b>Abbreviations</b> .....	8
<b>Introduction</b> .....	12
Chapter I: Hematopoiesis .....	12
I-1 Normal Hematopoiesis .....	12
I-1-1 Hematopoietic Stem Cells (HSCs) .....	12
I-1-2 Multipotent Progenitors (MPPs).....	16
I-1-3 Development of B lymphocytes .....	18
A) Early B cell development .....	18
B) Migration of B cells from the bone marrow to the secondary lymphoid organs.....	20
C) B cell maturation .....	20
D) Other important markers of B cells .....	21
E) Main Subtypes of B cells.....	22
E1) B-1 cells.....	22
E2) Follicular B cells .....	23
E3) Transitional B cells.....	25
E4) Marginal Zone B cells .....	25
E5) Memory B cells .....	26
E6) Plasma Cells .....	27
I-1-4 Regulation of Hematopoiesis.....	27
A) Hematopoietic stem cell niches.....	27
A1) Role of c-kit and CXCL12.....	28
A2) Hypoxia and the stem cell niche .....	29
A3) Osteolineage cells .....	30
A4) The role of N-cadherin in the stem cell niche.....	30
A5) Other cells and factors involved in HSC maintenance .....	31



I-1-5 Influence of Infections – Adaptive Hematopoiesis.....	32
A1) Role of cytokines produced during an infection .....	32
A2) Effector functions of hematopoietic stem and/or progenitor cells .....	34
A3) Regulatory hematopoietic stem and/or progenitor cells.....	34
I-2 Toll Like receptors .....	37
I-2-1 Toll-like Receptors – General.....	37
I-2-2 Types of TLRs and their ligands.....	38
A) TLRs of cell surface .....	38
A1) TLR4.....	38
A2) TLR1/2/6.....	38
A3) TLR5 .....	39
A4) TLR11 .....	39
B) Intracellular TLRs .....	40
B1) TLR3 .....	40
B2) TLR7/8 .....	41
B3) TLR9 .....	41
I-2-3 Signaling pathways of TLRs.....	43
I-2-4 TLRs and diseases .....	45
Chapter II: Autoimmune Diseases.....	46
II-1 Introduction to Autoimmune Diseases.....	46
II-2 Immune Tolerance .....	47
A) Central Tolerance .....	47
B) Anergy .....	49
C) Activators and inhibitors of co-stimulatory signals .....	49
D) B cell tolerance.....	51
F) Peripheral Tolerance of B cells.....	52

G) Ignorance.....	53
Chapter III: Multiple Sclerosis .....	54
III-1 Multiple Sclerosis: Generalities.....	54
III-2 Epidemiology of MS.....	55
III-3 Murine Experimental Models of Multiple Sclerosis.....	55
III-3-1 Induced Animal Models for Experimental Autoimmune Encephalomyelitis (EAE) .....	56
A) Chronic EAE in C57BL/6 .....	56
B) SJL/J R-EAE .....	57
III-3-2 Development of spontaneous EAE .....	59
III-3-3 EAE induction in NOD mice .....	60
III-3-4 Pathology of EAE .....	60
A) Effector CD4 <sup>+</sup> T cells.....	60
B) Th17 Cytokines .....	64
B1) Interleukin 17 (IL-17) .....	64
B2) Granulocyte-macrophage colony stimulating factor (GM-CSF).....	65
B3) Interleukin 23 (IL-23).....	66
B4) Interleukin 21 (IL-21).....	66
C) CD8 <sup>+</sup> T cells in EAE .....	67
D) T-cell trafficking in the CNS.....	69
E) T regulatory cells.....	74
E1) CD4 <sup>+</sup> T regulatory cells.....	74
E2) CD8 <sup>+</sup> T regulatory cells.....	78
F) Role of B cells in EAE.....	80
F1) Effector B cells .....	80
F2) B regulatory cells.....	81

I) B10 cells.....	82
II) Marginal Zone B cells .....	85
III) T2-MZP B cells.....	85
IV) Regulatory B plasmocytes.....	87
V) B-1a B cells .....	87
F3) Stimulatory signals for the induction of B regulatory cells.....	88
I) Toll-like Receptors Signaling.....	88
II) BCR Signaling.....	89
III) CD40 .....	90
IV) Cytokines.....	90
V) Transcription Factors.....	91
F4) IL-10-independent B regulatory mechanisms .....	91
F5) B cells-producing IFN- $\gamma$ and IL-17 .....	95
F6) Human B regulatory cells.....	96
III-3-5 Innate Immune Cells .....	99
A) Macrophages/Microglia .....	99
B) Dendritic Cells (DCs).....	100
C) Natural Killer cells (NK).....	102
D) Natural Killer T cells (NKT)/Invariant NK T cells (iNKT).....	102
III-3-6 Stem cell therapies of EAE .....	104
A) Neural Stem cells .....	104
B) Mesenchymal Stem Cells .....	105
C) Hematopoietic stem/progenitor cells (HSCs/HPCs) .....	107
III-3-7 Current Therapies for Multiple Sclerosis.....	111
Chapter IV: Type 1 Diabetes.....	113
IV1 Generalities .....	113

IV2 Epidemiology of Diabetes.....	113
IV3 Animal Model of Diabetes.....	114
IV4 Pathophysiology of T1D in NOD mice.....	114
A) T effector cells.....	117
B) T regulatory cells.....	118
C) Interleukin 21 (IL-21) in T1D.....	119
D) Dendritic Cells.....	120
E) Macrophages.....	121
F) Natural Killer Cells (NK cells).....	122
G) Invariant Natural Killer T cells (iNKT).....	123
H) Effector B cells.....	123
I) T1D and B regulatory cells.....	124
<b>Results.....</b>	<b>126</b>
<b>Discussion and Perspectives.....</b>	<b>203</b>
<b>References.....</b>	<b>223</b>

## **Abbreviations**

APC: Antibody Secreting Cells

APECED: Autoimmune Polyendocrinopathy, Ectodermal Dysplasia and Candidiasis

ATG: antithymocyte globulin

B6: Black 6

BAFF: B-cell Activating Factor

BBB: Blood Brain Barrier

BCMA: B cell maturation antigen

BCR: B cell receptor

BDNF: Brain-derived neurotrophic factor

BEAM: BCNU-etoposide-cytarabine-melphalan

BM: Bone Marrow

Bregs: B regulatory cells

Cal: Calicheamicin

CD: Cluster of Differentiation

CD40L: CD40-ligand

cDCs: Conventional DCs

CFA: Complete Freund 's adjuvant

CIA: Collagen induced arthritis

CLPs: Common lymphoid progenitors

CMPs: Common myeloid progenitors

CNR2: Cannabinoid receptor 2

CNS: Central Nervous System

CSF: Cerebrospinal fluid

cTECs : epithelial cells of the cortex

CTL: Cytotoxic T lymphocytes

CTLA-4: Cytotoxic T-lymphocyte-associated protein-4

DAMP: danger-associated molecular patterns

DCs: Dendritic Cells

DI1: Delta-like 1

DTH: Delayed type hypersensitivity

EAE: Experimental Autoimmune Encephalomyelitis

ER: Endoplasmic reticulum  
FasL: Fas Ligand  
Flt3-l: Fms-related tyrosine kinase 3 ligand  
FO: Follicular  
G-CSF: Granulocyte-colony stimulating factor  
GC: Germinal Centers  
GITR: glucocorticoid-induced tumor necrosis factor receptor  
GM-CSF: Granulocyte-macrophage colony stimulating factor  
GM: Granulocytes/monocytes  
GMPs: granulocyte/monocyte progenitors  
HIF-1a: Hypoxia-inducible factor  
HLA: Human Leukocyte Antigens  
HPC: Hematopoietic progenitor cells  
HSC: Hematopoietic Stem Cells  
HSPC: Hematopoietic stem/progenitor cells  
Idd: Insulin dependent diabetes  
IDDM2: Insulin-dependent diabetes mellitus 2  
IFN- $\gamma$ : Interferon  $\gamma$   
IFN- $\gamma$ R: Interferon  $\gamma$  receptor  
IFN: Interferon  
IGRP: Islet-specific glucose-6-phosphatase catalytic subunit-related protein  
IH2 : Innate type 2 helper  
IL-21R: Interleukin 21 Receptor  
IL: Interleukin  
IRAK : IL-1R-associated kinase  
Irf4: Interferon regulatory factor 4  
ITIM: Immunoreceptor tyrosine-based inhibition  
KLRG1: Killer cell lectin -like receptor group phenotype G, member 1  
LAG3: Lymphocyte-activation gene 3  
LAP: Latency-associated peptide  
LNs: Lymph nodes  
LPS: Lipopolysaccharide  
LSK: Lineage negative, Sca1 positive and c-kit positive cells  
LT-HSC: Long Term Hematopoietic Stem cells

M-CSF: macrophage-colony stimulating factor  
MAP: Mitogen-activated protein  
MAPK: Mitogen activated protein kinases  
MBP: Myelin Basic Protein  
MDSC: Myeloid Derived Suppressor Cell  
MEPs: Megakaryocyte/erythroid progenitors  
MHC-I: Major Histocompatibility Complex Class-I  
MHC-II: Major Histocompatibility Complex Class-II  
MOG: Myelin Oligodendrocyte Glycoprotein  
MPPs: Multipotent progenitors  
MS: Multiple Sclerosis  
MSC: Mesenchymal Stem Cells  
mTECs: epithelial cells of the medullary  
MyD88: Myeloid differentiation primary response gene  
MZ: Marginal Zone  
NFAT: Nuclear factor of activated T cells  
NK: Natural Killers  
NKG2D: Natural killer group 2, member D  
NKT: Natural Killer T cells  
NO: Nitric Oxid  
NOD: Non-obese diabetic  
NOS: nitric oxide synthase  
NT-3: Neurotrophin-3  
PAMP: Pathogen-associated patterns  
PD-L1: Programmed death ligand 1  
pDCs: plasmacytoid dendritic cells  
PI3K: Phosphoinositol-3-kinase  
PLP: Myelin Proteolipid Protein  
PPR: Pattern-recognition receptors  
PTX: Pertussis toxin  
PVC: Perivascular stromal cells  
RA: Rheumatoid Arthritis  
RAE1: Retinoic acid early transcript 1  
RoR $\gamma$ T: Orphan nuclear receptor  $\gamma$  T

ROS: Reactive oxygen species  
RR: Relapsing/Remitting  
S1P: Sphingosine-1 phosphate  
S1P3: Sphingosine-1 phosphate 3  
Sca-1: Stem Cell Antigen-1  
SCF: stem cell factor SCF  
SCID: Severe combined immunodeficiency  
SLE: Systemic lupus erythematosus  
ST-HSC: Short Term Hematopoietic Stem cells  
Stat: Signal transducer and activator of transcription  
T-bet: Transcription factor T-box transcription factor TBX21  
TCR: T-cell receptor  
TdT: Terminal deoxynucleotidyl transferase  
Teff: T effector cells  
TGF: Transforming growth factor  
Th: T helper  
T1D: Type I Diabetes  
TIM-1: T cell immunoglobulin and mucin domain protein 1 ligation  
TIR: Toll-interleukin-1 receptor  
TLR: Toll Like Receptor  
TNF: Tumor necrosis factor  
TNFR: Tumor Necrosis factor receptor  
TRAIL: tumor-necrosis factor-related apoptosis-inducing ligand  
TRAM: TRIF-related adaptor molecule  
Tregs: T regulatory cells  
TRIF: TIR-domain-containing adapter-inducing interferon- $\beta$   
VEGF: Vasular Endothelial Growth Factor  
VLA: Very late activating antigen  
VNTR: Variable number tandem repeat  
WT: Wild type



## Introduction

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### Chapter I: Hematopoiesis

#### I-1 Normal Hematopoiesis

Hematopoiesis (from Ancient Greek: αἷμα, "blood"; ποιεῖν "to make") is the formation of blood cellular components. These cells are mainly necessary for the maintenance of the integrity of our body serving different kind of functions. Among them, red blood cells primarily carry oxygen and collect carbon dioxide through the use of hemoglobin, and have a lifetime of about four months. There are also cells of the immune system involved in defending the body against both infectious disease and foreign materials.

All blood cells are derived from a specific cellular type, named: **Hematopoietic Stem Cells (HSC)**. In humans, the hematopoiesis starts in the yolk sac of an embryo during the first weeks of development. The hematopoietic stem cells can migrate from the yolk sac to the fetal liver and will arrive in the spleen. Both yolk sac and spleen seem to play the most important role for the formation of red and white blood cells during the 3<sup>rd</sup> and 7<sup>th</sup> month of development. After birth, the differentiation of HSCs occurs only in the bone marrow, which will be the main place of hematopoiesis for the rest of the life<sup>1</sup>. The bone marrow produces about  $1,75 \times 10^{10}$  erythrocytes (red blood cells) and  $7 \times 10^{10}$  white blood cells per day. In mice, HSCs can be first found in the developing yolk sac and blood islands, next in the fetal liver and fetal spleen and bone marrow. Each stage occurs apparently by HSCs entering the fetal circulation.

#### I-1-1 Hematopoietic Stem Cells

The HSCs are characterized mainly by two important and unique properties:

- Their ability to differentiate in all hematopoietic cell types
- Self renewal capacity

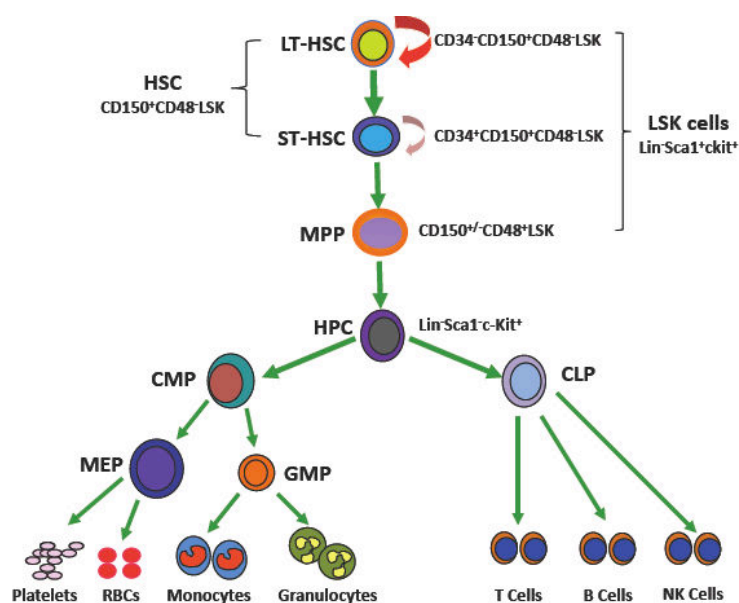
Depending on the influence of several soluble mediators and factors (cytokines) and cell contact signals from stromal cells, these cells, can proliferate while fully maintaining the differentiation potential of the parental cells<sup>2</sup>. At the same time, transplantation of one hematopoietic stem cell in an irradiated recipient can reconstitute completely the whole hematopoietic system including lymphoid, myeloid and erythroid cell lineages.

Actually, the first paper used the term “stem cells” was published in 1932. In that paper, authors suggest that with certain doses of radioactive material, the fundamental damage in the lymphoid tissues is to the stem cell tissues and that damage is to the chromatin of the nuclei of these cells<sup>3</sup>.

In 1963, Till and McCulloch published in *Nature* their paper on cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse bone marrow cells<sup>4</sup>. They demonstrated that in mouse hematopoietic tissue, there are some cells, which can proliferate and give rise to macroscopic colonies when transplanted in irradiated mice. Each colony included hematopoietic cells, many of which were dividing and differentiating in several cell lines.

In 1964, Till et al proposed an important model of stem cell functions in which the decision of a stem cell to self-renew and differentiate is portrayed as a stochastic process. They developed a “birth and death” model for self-renewal and differentiation of stem cells<sup>5</sup>. These two capacities of hematopoietic stem cells have been repetitively shown in irradiated mice whose hematopoietic system had been eliminated. A transplantation of only  $10^4$ – $10^5$  bone marrow cells (= 0.01%-0.1% of the normal quantity) from a healthy donor is enough to restore completely the hematopoietic system. This result emphasizes the enormous capacity of the hematopoietic stem cells for continuous proliferation and differentiation.

The capacity of self-renewal can distinguish HSCs in two main categories: Long term HSC (LT-HSC) which can be self-renewed during the whole life of an individual and are also responsible for giving rise to all cell lineages in an irradiated recipient and, on the other hand, Short term HSC (ST-HSC) which are characterized by a limited capacity of self-renew and they can reconstitute the hematopoietic system for only 2 months (**Figure 1**).



**Figure 1. Typical model of the murine hematopoietic system.** *LT-HSCs: Long term hematopoietic stem cells, ST-HSCs: Short term hematopoietic stem cells, LSK: lineage negative, Sca-1 positive and c-kit positive cells, MPPs: multipotent progenitors, HPCs: hematopoietic progenitor cells, CLPs: common lymphoid progenitors, CMPs: common myeloid progenitors, MEPs: megakaryocyte/erythroid progenitors (MEPs) and GMPs: granulocyte/monocyte progenitors*<sup>6</sup>. Shao L et al, 2013

These last ST-HSCs will then give rise to multipotent progenitor cells (**MPPs**), which lose self-renewal potential but can still fully differentiate into all multilineages in a transitory way (**Figure 1**). These multipotent progenitor cells can give rise to more limited oligopotent progenitors which can be divided mainly into two categories: the Common Lymphoid Progenitor cells (CLPs) and Common Myeloid Progenitor cells (CMPs). All these oligopotent progenitors differentiate into their restricted lineage commitment: CLPs are responsible for the formation of T cell progenitors, B cell progenitors, Natural Killer (NK) progenitors and Dendritic Cell (DC) progenitors. On the other hand, CMPs give rise to granulocyte/macrophage progenitors (GMPs), DC progenitors and megakaryocyte/erythrocyte progenitors (MEPs)<sup>7,6</sup> (**Figure 1**).

For the identification and isolation as well of the HSCs, the first marker, which was widely used, was the CD34 molecule, which belongs to the CD34 family of cell-surface transmembrane proteins together with podocalyxin and endoglycan<sup>8</sup>.

For the first time, a cell subset of CD34<sup>+</sup>CD90<sup>+</sup>(Thy-1) Lin<sup>-</sup> in humans has been shown to be able to give rise to both T and B lymphocytes and display myeloerythroid activities both in vivo and in vitro<sup>9</sup>.

Later on, CD38 and CD45Ra<sup>10,11</sup> are also considered as important markers for the isolation of HSCs which helped the conclusion that the subset of Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup>CD45Ra<sup>-</sup> is enriched for human HSCs and that the candidate for human multipotent progenitors (MPPs) with incomplete self-renewal capacity is enriched in CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>-</sup>CD45Ra<sup>-</sup><sup>12</sup>.

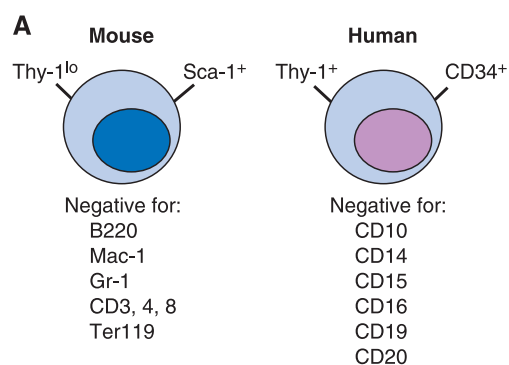
In mouse, lineage marker negative (Lin<sup>-</sup>) bone marrow cells expressing stem-cell antigen 1 (Sca-1<sup>+</sup>) and low levels of Thy.1-1 (Thy.1-1<sup>low</sup>) have been shown to contain HSCs at a very high frequency. This population of Thy.1-1<sup>low</sup>Lin<sup>-</sup>Sca-1<sup>+</sup> is today considered to be the only pluripotent hematopoietic stem cell population<sup>13</sup>. In addition to these characteristics, they express positively the c-kit receptor tyrosine kinase<sup>14</sup> although in some cases where endogenous or exogenous c-kit ligand (SCF) is abundant, the c-kit marker can be down-regulated. These c-kit<sup>+</sup>Thy.1-1<sup>lo</sup>Lin<sup>-/lo</sup>Sca-1<sup>hi</sup> bone marrow (BM) cells are, at the clonal level, all multipotent, but can be subdivided into three populations by the use of surface markers and by their self-renewal capacity<sup>15</sup> :

(a) The totally Lin<sup>-</sup> subset (many but not all of which are CD34<sup>low</sup>) consists of perpetually self-renewing long-term (LT) HSC.

(b) The Mac-1<sup>low</sup> subset is a uniform population of multipotent cells that self-renew in vivo upon transplantation for 6 weeks, which is the end of their productive lifespan (ST-HSC).

(c) The Mac-1<sup>low</sup>CD34<sup>low</sup> cells are clonogenic multipotent precursors whose self-renewal potential is short and, under transplant conditions is difficult to detect; these cells are called multipotent progenitors (MPPs) (**Figure 2**).

LT-HSC give rise to ST-HSC, which in turn give rise to MPP<sup>15</sup>. ST-HSC never give rise to LT-HSC, and MPPs never give rise to either LT-HSC or ST-HSC. Mobilization from BM to blood with treatment of a donor with cytoxan plus G-CSF (Granulocyte-colony stimulating factor) causes all HSC to enter the cell cycle, and many daughter HSCs are released to the blood.



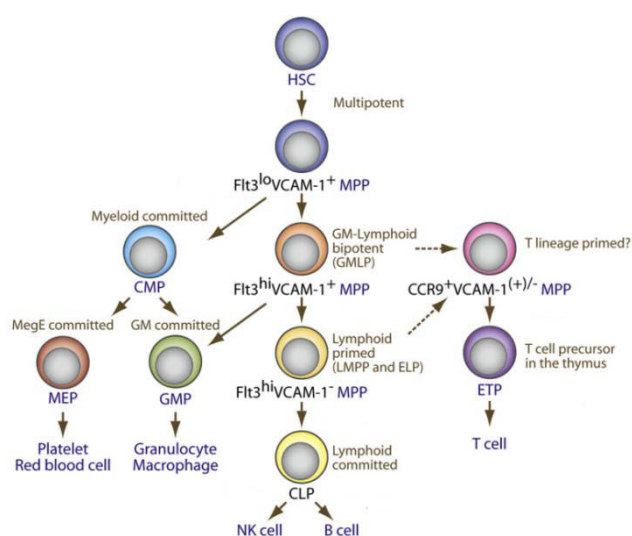
**Figure 2. Differential expression of markers for identification of mice and human hematopoietic stem cells** Mouse HSCs are characterized by the positive expression of *Sca-1* and low expression of *Thy-1* and are negative for the expression of markers *B220*, *Mac-1*, *Gr-1*, *CD3*, *CD4*, *CD8* and *Ter119*. Human HSCs are identified as *Thy-1* and *CD34* positive cells that are negative expression for *CD10*, *CD14*, *CD15*, *CD16*, *CD19* and *CD20*. Weismann et al, *Science*, 2000.

### I-1-2 Multipotent Progenitors (MPPs)

In mice, LT-HSCs give rise to ST-HSCs which then give rise to multipotent progenitors (MPPs) whose further progeny is oligolineage restricted. The commitment to either the lymphoid or the myeloid lineage is popularly viewed as the first step of lineage restriction from HSCs. This sequential developmental lineage is included in the bone marrow cell subset of  $c\text{-kit}^{\text{high}}\text{Lin}^{-}\text{Sca-1}^{+}$  cells (KLS). For the more detailed identification of these stem/progenitor cells, new markers have been used. Both CD150 (SLAMf1) and ERAM1 have been used to positively identify HSCs as neither CD150<sup>-</sup> nor ERAM1<sup>-</sup> cells can support long-term reconstitution<sup>16</sup>.

Using the staining of CD150 and ERAM1 markers in the LSK cells, three different populations have been recovered with separable expression of Flk2. CD150<sup>+</sup>/ERAM1<sup>+</sup> cells don't express flk2 at their surface whereas CD150<sup>-</sup>/ERAM1<sup>+</sup> and CD150<sup>-</sup>/ERAM1<sup>-</sup> cells express intermediate and high levels of flk2 respectively. According to these complete stainings, we can easily designate these populations as HSCs (CD150<sup>+</sup>/ERAM1<sup>+</sup> KLS), ST-HSCs (CD150<sup>-</sup> /ERAM1<sup>+</sup> KLS) and MPPs (CD150<sup>-</sup>/ERAM1<sup>-</sup> KLS)<sup>17</sup>. Further characterization of MPPs in bone marrow cells identified another marker for their isolation among the Thy-1.1 KLS population, the flt3-l (fms-related tyrosine kinase 3 ligand). Indeed, the flt3-l<sup>+</sup>Thy-1.1<sup>-</sup> KLS population can be divided into three subpopulations based on flt3 and VCAM-1 expression (flt3<sup>lo</sup>VCAM-1<sup>+</sup>, flt3<sup>hi</sup>VCAM-1<sup>+</sup>, flt3<sup>hi</sup> VCAM-1<sup>-</sup>)<sup>18</sup>. The most immature flt3<sup>lo</sup>VCAM-1<sup>+</sup> cells are considered as the 'classical MPPs' and have full multipotent differentiation potential, which differs from the other two subpopulations.

On the other hand, the more mature  $flt3^{hi}VCAM-1^{+}$  MPPs can give rise to GM cells and lymphocytes as efficiently as the classical  $flt3^{low}VCAM-1^{+}$  do. In addition,  $flt3^{low}VCAM-1^{+}$  but not  $flt3^{hi}VCAM-1^{+}$  can give rise to CMPs, suggesting that the  $flt3^{low}VCAM-1^{+}$  population is the main progenitor subset for the myeloid lineage (**Figure 3**)<sup>18</sup>. The most mature  $flt3^{hi}VCAM-1^{-}$  have lower GM and MegE potential with high lymphoid potential.



**Figure 3. Subpopulations of MPPs** In that model, Kondo et al propose three different populations of multipotent progenitor cells (MPPs) based on the expression of Fms-like tyrosine kinase 3 (*Flt3l*) and Vascular Cell Adhesion Molecule 1 (*VCAM-1*). In that model, the myeloid potential has been lost before the stage of Lymphoid Primed Multipotent Progenitor (LP-MPP), responsible for the formation of Common Lymphoid Progenitor cells (CLPs). HSC: Hematopoietic Stem Cell, GMLP: Granulocyte/Macrophage Lymphoid Progenitor, MEP: Megakaryocyte Progenitor, GMP: Granulocyte Macrophage Progenitor and ETP: Early Thymic Progenitor. Kondo et al, *Immunity*, 2008.

The MPP progenitor cells can also be divided according to the expression of Rag.

$VCAM-1/Rag^{+}$  display a high lymphoid potential and very low myeloid potential both in vitro and in vivo. These cells represent the same ability of forming GM colonies as  $VCAM-1^{+}$  MPPs, a result which suggests that the specificity of the lymphoid lineage occurs in  $VCAM-1^{-} Rag^{-}$  cells<sup>18</sup>.

The MPPs that differentiate either in lymphoid or myeloid progenitor cells should migrate to the appropriate place where each differentiation takes place. They start expressing chemokine receptors in order to be attracted to the microenvironments of cells where the corresponding chemokines are produced. That process facilitates the MPPs to move to a specific site either for the myeloid or the lymphoid differentiation. At the same time, they also express cytokine receptors while their interaction with some cytokines is more than necessary for the restricted

differentiation. For myeloid cell development, the cytokine M-CSF (macrophage-colony stimulating factor) should act on its own receptor c-fms and for lymphoid cell development, IL-7 is considered to be the important cytokine<sup>19</sup>.

### **I-1-3 Development of B lymphocytes**

B lymphocytes are some of the most important cell lines of the adaptive immune system. They are characterized mainly by their capacity to produce antibodies and they participate in the protection against a large variety of pathogens. A proof for that is the fact that different abnormalities on either the function or development of B cells can lead to autoimmunity, allergy and immunodeficiency.

#### **A) Early B cell development**

In the normal hematopoietic process, multipotent hematopoietic progenitor cells are responsible to give rise to the common lymphoid progenitor cells which can lead to the differentiation of progenitor cells of B, T and NK cells. B cell development occurs inside the bone marrow where hematopoietic stem cells, originated from aorta-gonad-mesonephros, seed different places and will cause the B cell differentiation<sup>20</sup>. The differentiation from a stem cell to an immature B cell includes four main different steps.

- *Precursor of pro-B cells*: It appears before the rearrangement of the immunoglobulin genes and it is already characterized by the expression of cell surface markers specific for B cells, like CD45R (B220), CD19 and CD38.

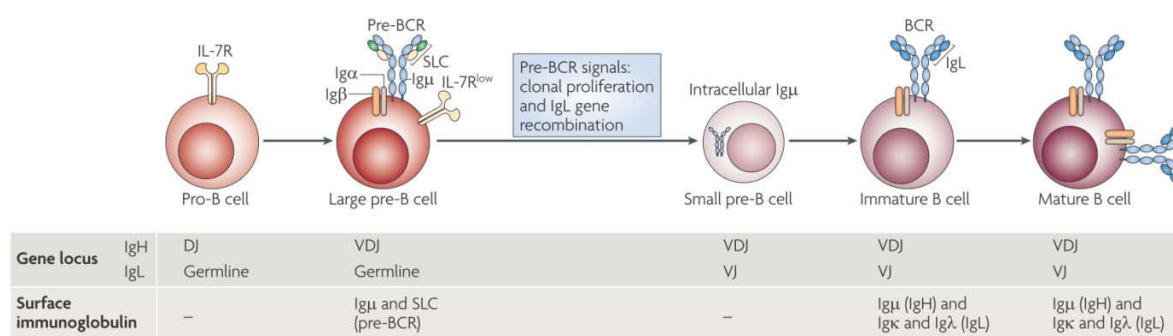
- *Later pro-B cell*: At this stage, we observe the rearrangement of the segments V-DJ of the heavy chain-H and the appearance of the receptors for IL-7, a cytokine that plays an important role in the process of lymphopoiesis.

- *Pre-B cell*: At this stage, the rearrangement of L-chains starts and the isolated  $\mu$ -chains are not yet expressed at the cell surface. Actually, the pre-B cells produce two important proteins:  $\lambda 5$  and Vpre-B. The  $\mu$ -chains associate to the  $\lambda 5$  and Vpre-B and they make together a complex that is associated with the proteins Ig- $\alpha$  and Ig- $\beta$ , they are expressed at the cell surface and are responsible for the transduction of different signals. This transduction seems to be controlled by a receptor of pre-B cells that inhibit further rearrangements of the genes coding for the H-chain and favors the stimulation of genes coding for the L-chain and the cell proliferation. Finally, it is the cell stage where we firstly see the expression of the molecule CD20 at their cell surface.

- *Immature B cells*: It is the stage where the rearrangement of genes coding for the L-chains is responsible for the expression of the molecule IgM at the cell surface. The expression of IgM from the immature B cells engages a phenomenon of feed-back which inhibits any other rearrangements of genes coding for the L-chain. At this stage, immature B cells are the first cells that express the marker CD21 (**Figure 4**).

This differentiation process from a precursor cell to an immature B cell is highly regulated by other non-hematopoietic cells. During the precursor stage, a cell contact between the progenitors and stromal cells seems to be extremely necessary. It is well studied the interaction between some adhesion molecules like VCAM-1 (CD106) at the surface of stromal cells and the ligand VLA-4 (CD49d) at the surface of pre-B cells<sup>21</sup>.

These stromal cells can also provide with other factors, important for the differentiation process like stem cell factor (SCF) or interleukin 7 (IL-7). Apart from the previous different steps concerning the changes in the rearrangement of the genes of immunoglobulines, there are also some other factors which can be equally involved in the following of the differentiation of B cells, like intracellular enzymes or the activation of genes Rag-1 and Rag-2 which are already present at the stage of pro- and pre- B cells. In addition to that, the activity of another enzyme, the TdT (terminal deoxynucleotidyl transferase) is switched off at the stage of pre-B cells<sup>22</sup>. At this last described developmental stage, immature B cells will leave the bone marrow and will egress to the spleen, the lymphoid organ where their differentiation/maturation process into follicular (FO) or marginal zone B (MZ) cells takes place<sup>21</sup>.



**Figure 4** Rearrangement of Immunoglobulin genes during B cell development<sup>23</sup>. Herzog et al, *Nat Rev Immunol*, 2009.



## **B) Migration of B cells from the bone marrow to the secondary lymphoid organs**

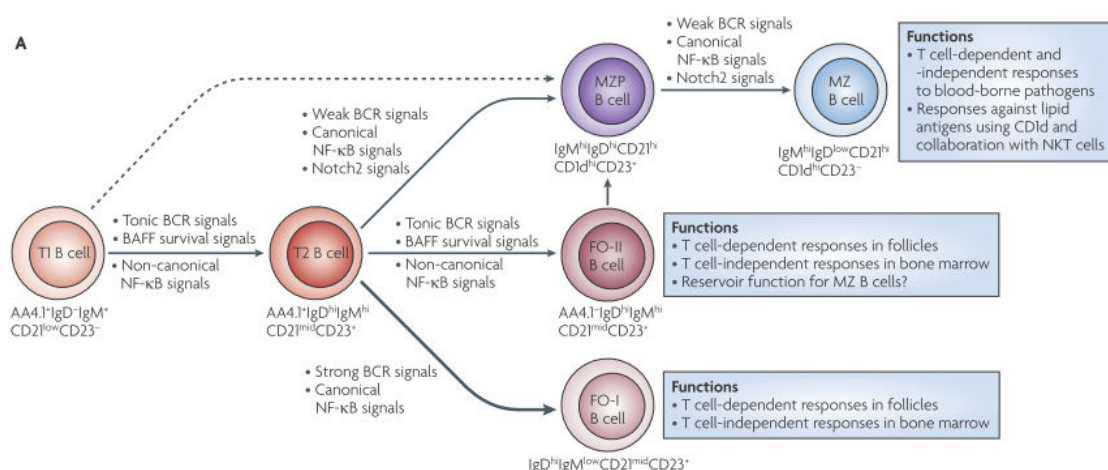
Once they finished the first steps of their differentiation, from the stage of CLPs until immature B cells, they start leaving the bone marrow tissue and they migrate to the secondary lymphoid organs (SLO). Through the bloodstream and the lymph, they go to the spleen, lymph nodes, Peyer Patches and mucosal tissues. They will generate the germinal centers (GC) and then egress back to the circulation. This process is regulated by different factors like adhesion molecules and chemokine receptors. For example, S1P (sphingosine-1 phosphate) receptors that react against their ligands S1P are highly involved in that homing process<sup>24</sup>. Cannabinoid receptor 2 (CNR2) has been shown also to be involved in the retention of immature B cells in the organ of their origin, the bone marrow. After leaving the BM, B cells migrate in the spleen where specialized niches participate in the formation of Marginal Zone (MZ) and the follicular B-cell compartment. Different factors can affect the developmental process of B cells like mutations in the associated kinase Janus kinase<sup>25</sup> and the IL-7 signaling<sup>26</sup>.

## **C) B cell maturation**

In only some days, the immature B cells will differentiate into mature B cells, which express at the cell surface the molecules IgM and IgD. These second steps of B cell differentiation occur outside the bone marrow while immature B cells quit this organ and migrate already in the spleen through the peripheral circulation. To conclude with, the two steps of B cell differentiation: 1) from stem cells to immature B cells and 2) from immature B cells to mature ones, are dependent on the signal pathways by the B cell receptor (BCR) and also the activation of Notch pathway, more specifically of Notch2 and its ligand Dll1 (Delta-like 1) which is present on endothelial splenic cells. In the second lymphoid organs, we can distinguish the lymphoid follicles and the marginal zone. The lymphoid follicles consist of specialized stromal cells and follicular dendritic cells which play an important role in the immune response. On the other hand, in the marginal zone, we find macrophages that keep inside that zone the antigens derived from the circulation. Signals from the engagement of Notch-2 receptor will lead to the differentiation of marginal zone cells and signals from the BCR will favor the differentiation of B cells in the lymphoid follicles.

Immature B cells, after leaving the bone marrow, start expressing the IgD, CD21 and CD22 molecules at their cell surface. These cells at this stage are also characterized by changes in

the density of the expression of other receptors<sup>27</sup>. The immature B cells are also called “transitional” (T1 and T2). The majority of B cells exiting the bone marrow will reside within the lymphoid follicles of the spleen and lymph nodes. Over there, they will meet antigens bound on dendritic cells and they will encounter them to T cells. Because of these interactions, they will differentiate into plasma cells or enter Germinal Center (GC), considered as the main place for the production of high-affinity antibody plasma cells and memory B cell generation<sup>28</sup>. In that place, B cells will make the memory compartments of humoral immunity<sup>29</sup>. An interesting question remains how lymphocytes control their entry either in lymphoid follicles or into and within the GC. Allen CD et al have shown that a combination of molecular interactions by chemotactic gradients and BCR activation is important for that homing process of B lymphocytes<sup>27,30</sup> (**Figure 5**).



**Figure 5. Developmental model of Follicular and Marginal Zone B cells.** Pillai et Cariappa, *Nat Rev Immunol*, 2009

#### D) Other important markers of B cells

For an easy and effective identification of B cells, we use mainly the markers CD19, CD20 and CD22. In addition to these molecules, an important marker for all immature or mature B cells in mice is the B220 (CD45R), just one of the isoforms of the CD45 molecule which is expressed by all hematopoietic cells. Concerning this molecule, we observe differences in its extracellular part but its intracellular part is identical for all isoforms. Moreover, B cells are characterized also by the expression of several surface receptors which are highly involved in the cooperation with the T cells like the MHC-II antigens I-A and I-E for mice and DR, DP and DQ for humans. CD40 is also considered as an important marker of B cells, it belongs to

the family of TNF (*tumor necrosis factor*) receptors and is able to transduce extremely powerful signals on B cells. It can interact with its specific ligand CD40-ligand (CD40L), which is expressed at the cell surface of activated T cells. These interactions are really important since a mutation on the CD40L molecule can lead to immunodeficiency.

An additional marker for the identification of B lymphocytes is the CD72 molecule which interacts with its specific ligand CD5, mainly expressed by T lymphocytes. The expression of CD5 is really interesting because it can be also expressed in a particular subtype of B cells (B-1a) and its expression is necessary for the identification of this cell type (**Table 1**).

Surface marker	Peripheral B cell subset					
	T1	T2	MR/B-2	B-1a	B-1b	MZ
IgM	+++	++	+	+++	+++	+++
IgD	+/-	+	+++	+/-	+/-	+/-
493/AA4.1	++	+	-	-	-	-
B220(6B2)	+	++	+++	+/++	+/++	++
CD21	+/-	+	++	+/-	+/-	+++
CD23	-	+	++	++/- <sup>a</sup>	++/- <sup>a</sup>	-
HSA(J11d/30F1)	+++	++	+	++	++	++
CD43	+/-	-	-	++/- <sup>a</sup>	++/- <sup>a</sup>	
CD5	-	-	-	+	-	-
CD11b/Mac-1	-	-	-	+ <sup>b</sup>	+ <sup>b</sup>	-

<sup>a</sup> part are ++ and part are -.

<sup>b</sup> Only in peritoneal cavity; B-1 cells are CD11b/Mac-1<sup>-</sup> in spleen.

T1, T2 = transitional (maturing) B cells.

MR = mature recirculating B cells.

MZ = marginal zone B cells.

**Table 1. Important cell surface markers for the identification of the different B cell subpopulations<sup>31</sup>. Richard R. Hardy and Kyoko Hayakawa, *Annu Rev Immunol*, 2001**

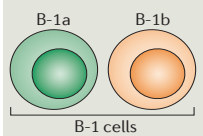
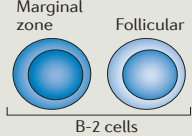
## E) Main Subtypes of B cells

### E1) B-1 cell

One of the main functions of B cells is their ability to produce antigen-specific antibodies after infection, but today it is well known that not all antibody production is dependent on immune activation. Some years ago, an identification of a subset of B cells, which can proliferate and secrete antibodies in response to the bacterial component LPS (Lipopolysaccharide), independently of their specific BCR, provided evidence for the existence of innate-like B cells<sup>32</sup>. It has been also shown that B cells can express several innate receptors like TLR-3-4-7-8-9 but not all B cells can respond to innate immune signals<sup>33</sup>.

Mouse B1 cells have been described for the first time in 1983, as a population of B cells, residing inside the spleen that expresses the CD5 molecule. We already knew that CD5 is a marker of activated T cells (Ly5.1) so at the beginning it was difficult to convince for the existence of a new subset of B cells, expressing the CD5 marker. These CD5<sup>+</sup> cells were shown to secrete IgM antibodies and they were found to be localized mainly in the peritoneal and pleural cavities but are rare in lymph nodes<sup>34</sup>. Some additional studies described the existence of another population of B cells which was very close to these previously identified B-1 cells in terms of the tissue distribution, phenotype and development but that did not express the specific CD5 marker. Either CD5<sup>+</sup> or CD5<sup>-</sup>, these cells are defined as B-1 cells and they develop earlier than follicular B-2 cells during ontogeny<sup>35,36</sup> (**Figure 6**). Based on the expression of the CD5 molecule, we distinguish two subtypes for B-1 cells. The cells that are positive for the expression of CD5 are called B-1a whereas the CD5<sup>-</sup>, B-1b cells.

A distinct B cell population that includes follicular and marginal zone B cells is defined as B-2 cells and it develops later than B-1 cells. Recently, the group of Baumgarth N identified a new population of B cells inside the bone marrow characterized by the phenotype of IgM<sup>+</sup>IgD<sup>low/-</sup>CD19<sup>hi</sup>CD43<sup>+</sup>CD5<sup>+/-</sup>, similar to the splenic B-1 cells and different from antigen-induced conventional B cells which express also CD138, suggesting that the existence of non-terminally differentiated B-1 cells in the spleen and BM are the most significant producers of natural IgM<sup>37</sup>.

<b>Cell surface phenotype</b>	CD5 <sup>+</sup> CD19 <sup>hi</sup> CD1d <sup>mid</sup>	CD5 <sup>-</sup> CD19 <sup>hi</sup> CD1d <sup>mid</sup>	CD5 <sup>-</sup> CD19 <sup>mid</sup> CD1d <sup>hi</sup> CD21 <sup>hi</sup>	CD5 <sup>-</sup> CD19 <sup>mid</sup> CD1d <sup>mid</sup>
	CD23 <sup>-</sup> CD43 <sup>+</sup> IgM <sup>hi</sup> IgD <sup>low</sup>	CD23 <sup>-</sup> CD43 <sup>+</sup> IgM <sup>hi</sup> IgD <sup>low</sup>	CD23 <sup>-</sup> CD43 <sup>-</sup> IgM <sup>hi</sup> IgD <sup>low</sup>	CD23 <sup>+</sup> CD43 <sup>-</sup> IgM <sup>low</sup> IgD <sup>hi</sup>
<b>Frequency in total splenic B cell population</b>	2%	<1%	15%	>70%
				

**Figure 6. Mature splenic B cells** Five subsets of mature B cells are present in the mouse spleen. Bone marrow-derived follicular B cells and marginal zone B cells, which form the B-2 cell population, constitute the majority of splenic B cell sand differ from each other in terms of phenotype and location (they are found in the B cell follicles and marginal zone, respectively). B-1a and B-1b cells are clearly distinct but are minor subsets in terms of their frequency in the spleen. B-1a and B-1b cells can be distinguished phenotypically on the basis of CD5 expression: B-1a cells are CD5<sup>+</sup> and B-1b cells are CD5<sup>-</sup>. Baumgarth N, *Nature Immunol Rev*, 2011

As far as it concerns the development of B-1 cells, there are 2 different models that have been proposed:

- ‘the induced differentiation hypothesis’ which suggests that according to existing specific signals during development, a common progenitor cell can be considered responsible for the formation of both B-1 and B-2 cells,
- ‘lineage hypothesis’ proposing different progenitor cells for B-1 and B-2 cells.

Recently, a new theory has been proposed by N Baumgarth, described as ‘the 2 pathways model’ where the authors identified a precursor cell in the bone marrow and fetal liver which can specifically give rise to both B-1 (B-1b and B-1a) cells. According to their model, possible precursors cells for B-2 cells can also give rise to B cells with characteristics of B-1 cells, under specific conditions. This hypothesis supports the incorporation of the two previous models into the combined ‘two pathways model’<sup>38</sup>. An interesting point concerning the development of B-1 cells is their selection. B-1 cells are known for secreting antibodies that are specific for self-antigens<sup>39</sup> or for a large variety of antigens coming from apoptotic cells<sup>40</sup> and the repertoire of antibodies derived from these cells is formed in the total absence of foreign antigens<sup>41</sup>. Recent studies on the selection of B-1 cells demonstrate that the autoantibodies produced by these cells have undergone a positive selection for their self-reactivity<sup>34</sup> while B-1 cells in mice that lack the expression of the gene Thy.1 (CD90) are not any more able to secrete antibodies specific for Thy.1, specific-antibodies which are normally found in wild type mice. In that process, strong signals through BCR have been shown also to play an important role in the selection of B-1 cells since deletion of positive regulators of BCR signaling or of co-stimulatory molecules, result in decreased numbers of B-1 cells<sup>42</sup>.

## **E2) Follicular B cells (or B-2 cells)**

Follicular B cells are one of the main three subtypes of naïve B cells. Today, follicular B cells are also referred as B-2 cells and once they get mature, they are characterized by their ability to migrate through the blood and lymph to B cell areas (lymph nodes, Peyer’s patches, spleen). The main site where follicular B cells reside is their follicular niche, a place where B cells display an antigen-presenting cell function by priming T-cell-dependent antigens to activated T cells. For many years, investigators were focusing on the signals that can activate follicular B cells in order to differentiate into antibody-producing plasma cells. Many studies have provided evidence that signals delivered via the BCR, CD40 and Toll-like receptors (TLRs) are necessary for the real activation of follicular B cells. The same TLRs are also

expressed by other B cell subtypes like MZ B and B-1 cells but unlike them, the activation of follicular B cells can't be induced only following exposure to TLR ligands, prior stimulation via BCR or CD40 seems to be more than necessary<sup>43</sup>. Apart from their residence in the lymphoid areas, follicular B cells can also migrate in the bone marrow where a specific niche provides them with the essential signals for their activation. In some other cases, it is also possible that activated B cells (GCs) acquire the capacity to migrate to the bone marrow and differentiate into long-lived plasma cells through the interaction of APRIL or/and BAFF produced by stromal cells, with BCMA, a receptor which belongs to the same family of BAFF-R<sup>44</sup>.

### **E3) Transitional B cells**

As it has been mentioned already, immature B lymphocytes in the bone marrow, either mature further at this site or migrate in the lymphoid organs where they continue their maturation process. As long as they stay at an immature stage of differentiation, it has been shown that they can display a transitional phenotype and according to Carsetti and colleagues, they can be subdivided in three distinct subsets (T1, T2 and T3)<sup>45,46</sup> suggesting the involvement of several steps in the maturation process of immature B cells in the spleen. For the identification of these transitional subsets, an important marker is the precursor marker CD93/AA4 and the CD21 which makes them different from MZ B cells. According to the further expression of IgM and CD23 markers, we distinguish T1 cells as IgM<sup>hi</sup>CD23<sup>-</sup>, T2 as IgM<sup>hi</sup>CD23<sup>+</sup> and finally T3 as IgM<sup>lo</sup>CD23<sup>+</sup>.

### **E4) Marginal Zone B cells**

This subtype of naïve B cells is called marginal zone B cells mainly because of their site of localization in the marginal zone of a spleen. This permanent position can be controlled by the activity of some chemokine receptors which have been shown to be implicated in their residence to that zone, such as S1P1 and S1P3, receptors for sphingosine1-phosphate<sup>47-49</sup>. Concerning their functions, MZ B cells can be used to transfer antigen from the marginal zone to the splenic follicles in order to present it to the follicular B cells which will become activated<sup>50</sup>. They can mediate T-cell independent responses to antigens in blood-borne pathogens and during pathogen-driven responses triggered by LPS or other bacterial products that lead to a decrease of integrin adhesiveness and can induce the migration of activated B cells into the spleen where they can differentiate into short-lived plasma cells<sup>51</sup>. In addition to their role in T-cell independent immune responses, they have also been shown to mediate T-

cell dependent responses, being activated directly by T cell antigens. It is interesting the fact that the marginal zone B cells express higher levels of MHC-II, B7-1 and B7-2 so they are considered to get a more enhanced capacity of antigen presentation and activation of T cells<sup>52,53</sup>. Because of their high expression of the molecule CD1d, they can also interact with Natural Killer T cells (NKT) by presenting them glycolipid antigens. NKT cells can also induce the activation of MZ B cells in the spleen via CD40L-CD40 interaction<sup>54</sup>.

### **E5) Memory B cells**

After an infection, we know that our immune system can undergo a clonal process where naïve B cells which are exposed for the first time to an antigen will differentiate into plasma cells producing antibodies specific for the antigen. These differentiated B cells can persist at this stage for many years and they are responsible for clearing away the resolution of further infection by the same antigen. This type of B cells is what we call memory B cells. They can be categorized mainly in two different subtypes according to the expression of surface markers, co-stimulatory molecules, their precursor cells and finally their mutation rate<sup>55</sup>. The most important types of memory B cells are the IgM- and IgD-producing B cells which have been shown to be intrinsically different<sup>56</sup>. The formation of memory B cells occurs normally in the Germinal Center of lymphoid follicles and their precursor cell is a GC cell and its Ig should be mutated and class switched. Conventional B-2 cells are considered to be the main subtype of naïve B cells which can give rise to memory B cells despite the fact that the group of Alugupalli et al managed to demonstrate that B-1b cells can give rise to B cells expressing IgM that confer protection after infection from *Borellia hormsii*<sup>57</sup>.

Several studies today focus on the differences between the IgM- and IgD-memory B cells. It seems that class-switched memory B cells have the ability to differentiate faster and in a more effective way to plasmablasts during the second response whereas IgM-memory B cells can persist longer and display a slower capacity to take part in a secondary response in the presence of serum Ig<sup>58</sup>. The group of Weill has also proposed an interesting concept of ‘layers’ where they support experimentally the idea that memory B cells appear in several layers with different functions<sup>59</sup>.

To conclude with, recent evidences in the field of memory B cells suggest that IgM memory B cells play a crucial role for the persistence of the population whereas IgG1 memory B cells can be considered as the frontline cells of the immune response.

## **E6) Plasma Cells**

Plasma cells develop from B lymphocytes, they can be activated and differentiate into memory B cells or plasma cells which are characterized by their capacity to secrete monoclonal antibodies against a specific antigen. The activation of B cells can be triggered by already activated and differentiated CD4<sup>+</sup> helper T lymphocytes. The production of antibodies is a crucial mechanism for the protection of an individual against foreign exposures. Affinity maturation is also an important process and it is well known that repeated exposure to the same antigen increases the antibody affinity secreted by the plasma cells. They reside in the bone marrow and make up about 1-3% of all bone marrow cells.

### **I-1-4 Regulation of Hematopoiesis**

#### **A) Hematopoietic stem cell Niches**

The microenvironment of each tissue where the hematopoietic stem cells develop has been shown to play a crucial role for the development, maintenance and function of the stem cells. An increasing number of studies have been focusing on the understanding of what we call hematopoietic niches in order to identify all these cell compartments, factors and signals which are involved in the maintenance of hematopoietic stem cells. Schofield had proposed the idea of a stem cell niche, which controls all the key properties of HSCs, in 1978<sup>60</sup>. The main site where hematopoietic stem cells reside and undergo their differentiation process is the bone marrow but we know that they are also able to circulate in multiple tissues. Many groups had studied the tissue of bone marrow in order to investigate the cellular environments that provide HSCs with all these molecules necessary for their survival and function. Within the BM, we can meet several cell types that can be considered as potential candidates for regulating HSCs maintenance, such as mesenchymal stem cells (MSCs), osteoblasts, chondrocytes, glial cells and adipocytes<sup>61</sup>. Two main niches have been shown to exist within the bone marrow, perivascular and endosteal niches<sup>62</sup>.

A study published by Nombela-Arrieta et al described that the majority of HSCs and some progenitor cells, reside in perivascular and endosteal areas of bone-proximal regions, suggesting that a highly vascularized endosteal niche may provide the environment needed for the HSCs maintenance<sup>63</sup>, an observation which is in accordance with a study from Kiel and Morrison who propose that several niches are located closely and are responsible for either

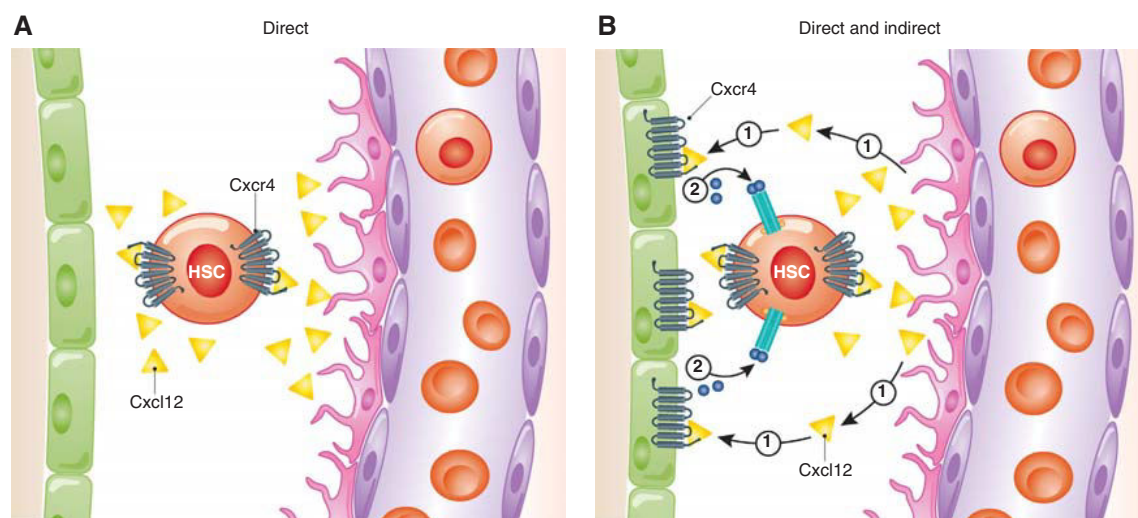


the survival or proliferation/differentiation of HSCs<sup>62</sup>. During the last years, the idea of a spatial organization of HSC niches got a growing number of supporters. A new zone of cells between the perivascular stromal cells (PVCs) has been described. This zone contains many clusters of HSCs, named hemospheres. The importance of these vascular cells had been shown by a deletion of the VEGF2 which resulted in anomalies on the structure of hemospheres and in a reduced number of HSCs in the BM.

### **A1) Role of c-kit and CXCL12**

More recent studies tried to clarify the molecular environment for the HSC maintenance. Since 1988, several groups have paid attention to the role of c-kit and CXCL12 receptors and their specific ligands in HSC function<sup>64-66</sup> and they provided evidence for some reticular cells, close to putative HSCs which express CXCL12 receptors<sup>67</sup>. After years, it was demonstrated that the cell types which are responsible for the production of these factors which seem somehow to control the HSCs maintenance, are endothelial and perivascular cells. Interestingly, it has been also shown that the deletion of the production of these molecules from osteoblast lineages led to a reduction in the number of lymphoid progenitors<sup>68,69</sup>. In addition to the hematopoietic stem cells, it has also been shown that a population of mesenchymal stem/progenitor cells express CXCL12 and SCF<sup>70</sup>. In this concept, we should always keep in mind the possibility that other cell types can also respond to CXCL12 or SCF and it is possible that indirect effects also play important roles in HSC maintenance<sup>71</sup>. Focusing more on the role of the complexes CXCL12/CXCR4 and c-kit/SCF in the maintenance of HSCs, it has been shown that in-vivo administration of antibodies against either c-kit or CXCR4 antagonists<sup>72-74</sup> (see AMD3100) results in the mobilization of the HSCs from the BM to other tissues, like blood or spleen which confirms the hypothesis that one of the functions of these two receptors is to keep HSCs to their BM niches<sup>74</sup>. An indirect effect of the interaction of c-kit/SCF and CXCL12/CXCR4 has been shown and today we believe that the localisation of HSCs close to CXCL12 niches doesn't lead to an exclusive relationship between these cells. Other stromal cells can also interact with the CXCL12 of the niches and it has been shown that these interactions affect the function of HSCs<sup>67,75</sup> (**Figure 7**). Sugiyama et al demonstrated that, HSC location next to CXCL12-expressing cells did not depend on CXCR4 expression by HSCs suggesting that it is possible that CXCL12 acts on non-hematopoietic cells expressing CXCR4 such as stromal cells. Indeed, there is evidence for the expression and function of the CXCR4 receptor by BM stromal cells and additionally, CXCR4 has been shown also to be important for the development of osteoblast

and endothelial cells<sup>76</sup> since the deletion of CXCL12 influenced the function of these cells, including their ability to support HSC maintenance.



**Figure 7. CXCL12 affect HSC function by both direct and indirect mechanisms** *A) the primary function of CXCL12 (yellow triangles) is to support the function of HSCs by acting directly on HSCs. B) CXCL12 can affect HSC function by acting on CXCR4-expressing niche cells, suggesting an indirect mechanism. The action of CXCL12 can reinforce an HSC-supportive environment by either cellular or molecular mechanisms. Ugarte et al, EMBO J, 2013*

## A2) Hypoxia and the stem cell niche

An interesting subject which has been discussed for several years is if the hematopoietic stem cell niches need an hypoxic or not environment. Recent studies support the idea that hypoxic conditions facilitate the quiescence of HSCs since a deletion of hypoxia-inducible factor (HIF)-1 $\alpha$  can trigger the loss of their maintenance and proliferating capacity<sup>77,78</sup>. As it was already mentioned before, one of the regions where HSCs reside is a perivascularized endosteal niche where HSCs should be well oxygenated. However, the preference of HSCs to hypoxic conditions was also supported by their retention inside the bone marrow where there is a strong profile of hypoxia as HSC express continuously HIF-1 $\alpha$  and retain pimonidazole (pimonidazole=molecule which binds to thiol-containing proteins specifically in hypoxic cells and is widely used as a marker of hypoxia)<sup>63</sup>. Actually, it is possible that intrinsic changes in the metabolism of HSC affects their hypoxic profile rather than the hypoxic localization<sup>79</sup>.

### **A3) Osteolineage cells**

Since the discovery that the nature of a potential niche for HSCs is the endosteum, more and more studies focused on the role of the cells which reside in that region, the osteoblast cells. Their involvement in the regulation of the maintenance and trafficking of HSCs has firstly been documented by their ability to secrete factors which have been already mentioned to be highly implicated in the process of HSCs regulation, like granulocyte colony-stimulating factor (G-CSF)<sup>80</sup> and CXCL12<sup>81</sup>. However, the direct role of osteoblasts in that process is not yet well established. It is interesting the fact that the expansion of osteoblast cells through either activation of parathyroid hormones or inactivation of bone morphogenic protein receptor 1 seems to trigger an increase in the number of HSCs<sup>82,83</sup> whereas expansion through a treatment of mice with strontium, displays no effect on HSCs number<sup>84</sup>. Moreover, a recent study by Li JY et al showed that the effect of the parathyroid hormone in HSCs maintenance, is not through the expansion of osteoblasts but through the production of Wnt ligand, Wnt10b, by T cells, which activates the Wnt signaling in both HSPC and stromal cells<sup>85</sup>. The role of osteoblasts has to be clarified in more details and this is why many groups are trying now to investigate if there is a specific more or less differentiated subset which can display properties for the regulation of HSCs even in a transient way<sup>79</sup>.

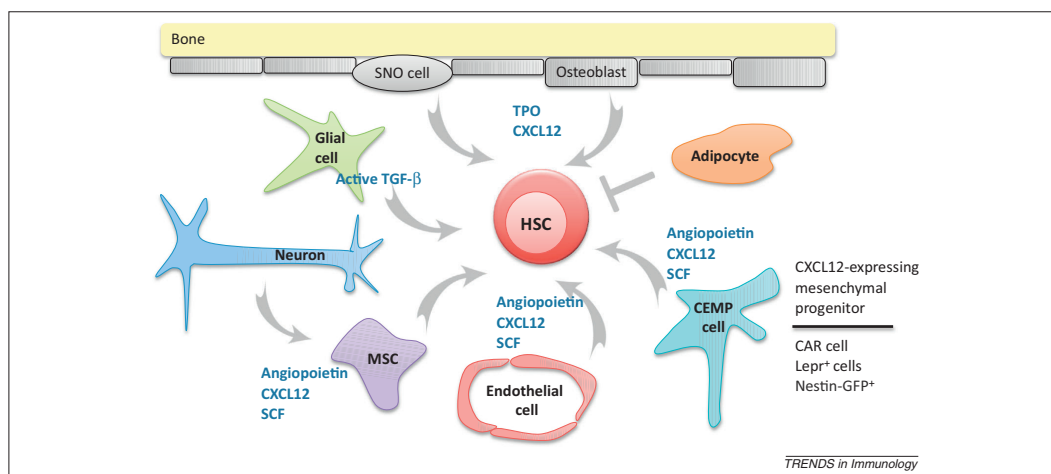
### **A4) The role of N-cadherin in the stem cell niche**

Apart from the involvement of the chemokine receptors in the regulation of HSCs survival and function, several adhesion molecules have been also investigated for their potential effect in HSC maintenance. Among them, the implication of N-cadherin has attracted the interest of many investigators. N-cadherin belongs to the superfamily of cadherins which includes cadherins, adherins, desmogleins, desmocollins and protocadherins<sup>86</sup> and they are dependent on Ca<sup>2+</sup> ions for their function and this is why they got that name. A recent study by Nakamura et al shows that a subset of cells which have been shown to be located close to hematopoietic niches, the osteolineage cells, express high levels of N-cadherin (more in immature cells) and this expression has been involved in the adhesion of HSCs to osteolineage cells<sup>87</sup>. Two other studies present some contrary results, one confirms the important role of this adhesion molecule since when they block the signaling of N-cadherin they find out reduced repopulating activity of the HSCs in the bone marrow<sup>88</sup> and on the other hand, Greenbaum AM et al demonstrated that N-cadherin expression in osteolineages is not necessary for the maintenance of HSCs. They deleted the gene Cdh2 which is encoding for N-

cadherin and this conditional deletion displayed no changes at HSC trafficking, number and repopulating activity<sup>89</sup>.

#### **A5) Other cells and factors involved in HSC maintenance**

It has been shown that endothelial cells can also regulate HSC maintenance through the production of SCF and inactivation of their specific adhesion molecule E-selectin<sup>90,91</sup>. Different studies emphasize also the importance of other cell types in the regulation of HSC maintenance and function inside the bone marrow. A study of Naveiras et al demonstrated that adipocytes can be negative regulators of the hematopoietic stem cell microenvironment since the repopulating activity of HSCs is enhanced in regions of mouse skeleton where adipocytes are few<sup>92</sup>. This observation leads to the hypothesis that competitive adipogenesis may be an important factor in order to ameliorate the efficacy of bone-marrow transplantations. The implication of other cells, like cells of the nervous system has also been shown to affect the egress of HSCs from the bone marrow to the circulation through the regulation of the local secretion of CXCL12<sup>70</sup>. Additionally, it has been shown that the mechanisms controlling the physiological trafficking of HSCs and progenitors cells are also affected by the circadian rhythm. Circulating HSCs and their progenitor cells exhibit robust circadian fluctuations, peaking 5h after the initiation of light and reaching a nadir 5h after darkness. These circadian oscillations are markedly altered when mice have been subjected to continuous light or a shift of 12h. The molecular clock can influence the cyclical release of HSCs and the expression of the chemokine CXCL12 through circadian noradrenaline secretion by the sympathetic nervous system. Several adrenergic signals are derived by nerves in the bone marrow and lead to a decreased nuclear content of Sp1 transcription factor and the rapid downregulation of CXCL12<sup>70,93</sup>. Finally, many studies have focused on the role of the transforming growth factor (TGF)- $\beta$  in the regulation of HSC function. Due to the fact that many different cell types can be considered as good producers of TGF- $\beta$ , a study from Yamazaki et al provided evidence that nonmyelinating Schwann cells are the main source of TGF- $\beta$  production inside the bone marrow and that can affect the number of HSCs since surgical disruption of sympathetic nerves results in loss of Schwann cells which is associated with decreased active TGF- $\beta$  expression and the loss of HSCs<sup>94,79</sup>.



**Figure 8. Distinct stromal cell populations in the bone marrow contribute to hematopoietic stem cell (HSC) maintenance.** *Endothelial cells, mesenchymal stem cells (MSCs) and chemokine CXC ligand (CXCL) 12-expressing mesenchymal progenitors (CEMP cells) are perivascular stromal cells that produce several factors that support HSCs such as CXCL12, angiopoietin and stem cell factor (SCF). CEMP cells have been identified as CXCL12-abundant reticular (CAR) cells, leptin receptor<sup>+</sup> stromal (lepr<sup>+</sup>) cells and Nestin-GFP<sup>+</sup> cells. Osteoblasts and spindle-shaped N-cadherin<sup>+</sup> osteoblasts (SNO cells) produce several factors that support HSCs, including thrombopoietin (TPO) and CXCL12. Sympathetic neurons indirectly regulate HSCs by targeting CXCL12 expression. Glial cells through production of active transforming growth factor (TGF)- $\beta$  and adipocytes regulate HSCs. Bryan AA et al, Trends in Immunol, 2014*

### I-1-5 Influence of Infections – Adaptive Hematopoiesis

HSCs have been considered as dormant cells brought to proliferate and differentiate in cases of cytopenia (eg during chemotherapy, irradiation or bleeding). The production of mature cells in such conditions can be described as homeostatic hematopoiesis. However, in recent years, it has been concluded that the activity of HSCs is constantly modulated by environmental factors. Thus, it was demonstrated that HSCs could respond to bacterial, viral and fungal infections in the absence of cytopenia. It is this mechanism that we call adaptive hematopoiesis.

#### A1) Role of cytokines produced during an infection

It has been shown that certain cytokines can induce a change in the quantitative compartment of stem cells and hematopoietic progenitor cells (HSPC). These cells proliferate in response to TNF and LPS-induced signaling of NF- $\kappa$ B upon infection by E.coli. IFNs play also an important role in the activity of HSCs. Although it was suggested that IFN- $\gamma$  can induce apoptosis<sup>95</sup> of HSCs, it can also increase the ability of progenitors to form colonies upon

infection with *Plasmodium chabaudi*. IFN- $\alpha$  has similar activating effects<sup>96</sup>. Indeed, a study of HSCs isolated from wild-type mice or transgenic mice devoid of IFN-receptor, revealed selective activation of HSCs via an IFN- $\alpha$  signaling. Cytokines produced during infection also appear to affect the hematopoietic niche, since they can induce mobilization of HSPC. For example, G-CSF, including increased serum levels upon systemic bacterial infection, induces a cascade of events leading to the release of proteolytic enzymes which cleave and degrade HSC anchoring molecules. Among these, VCAM-1, VLA-4, CXCL12, CXCR4, and probably integrins and N-cadherin are found. This degradation releases HSPC to leave from their niche, and allows their activation and mobilization in traffic. This strategy is commonly used clinically to recover HSC from a healthy donor. Similarly, TNF- $\alpha$  can trigger the mobilization of lymphoid progenitors. Its effect is mediated by IL-1 $\beta$  which promotes initially granulopoiesis in the bone marrow and extramedullary lymphopoiesis. The HSCs (LT-and ST-HSC) increased spontaneously upon activation of the TLRs, but they can circulate in peripheral blood and lymph nodes as well as at the tissue level<sup>97</sup>. This circulation gives them the possibility of exercising immunosurveillance by interacting with other immune cells and differentiating primarily in mature dendritic cells within the tissues where TLR stimulation occurs. The activity of HSPC is modulated by cytokines that act directly on their microenvironment during infection. However, a close relationship between pathogens and HSCs exists. One of the important studies in this field is that of Nagai et al who has demonstrated the existence of Toll-like receptors (TLRs) at the surface of HSCs<sup>98</sup>. Thus, binding of TLR-2 and TLR-4 agonists at the surface of these cells drives hematopoiesis towards an increased production of myeloid cells on one side, whereas the TLR9 agonist promotes the production of DCs from CLPs<sup>99</sup>. TLRs can also act on cells of the niche by inhibiting osteoclast maturation. This leads to the mobilization and differentiation of HSPC. A potential effect of cytokines by activation of TLRs has also been highlighted in 2005 by Nardini et al. Initially, they showed that a single injection of CpG (TLR9 agonist), increases the concentration of circulating G-CSF for few hours. They also observed that co-administration of G-CSF and CpG had a stronger potential of mobilisation in comparison with administration of CpG or G-CSF alone<sup>100</sup>.

## **A2) Effector functions of hematopoietic stem and/or progenitor cells**

Belyaev et al described the emergence of an IFN- $\gamma$ -dependent atypical progenitor population characterized by the expression of IL7R<sup>+</sup>c-kit<sup>high</sup> in the bone marrow of mice upon infection with *Plasmodium chabaudi*<sup>101</sup>. The in vivo injection of this atypical bone marrow progenitor population increased the survival of mice at the peak of parasitemia and the elimination of the parasite as well. This progenitor population emerges under pro-Th1 immune conditions. Recently, four independent laboratories have identified innate cell populations that would promote the development of immunity and/or CD4<sup>+</sup> Th2 inflammation in the mucous membranes. These cell populations are called "NHC" (for natural helper cells), "MPtype2" (multipotent progenitor type 2) "nuocytes" or "IH2" (for innate type 2 helper) and share the common features of inducing Th2 cytokine response. All these innate cells are activated by IL-25 and/or IL-33, two cytokines produced predominantly by epithelial cells where their increased expression at the mucosal surface is associated with an exposure to an allergen or to helminth parasites. The MPtype2 is an interesting case to highlight the modification of hematopoiesis in its early stages. Indeed, the frequency of multipotent progenitors which are present naturally in mice increases in the mesenteric lymph nodes during infection with helminths. This increase is dependent on the production of endogenous IL-25. Adoptive transfer of MPtype2 cells from wild type mice, induced by IL-25, into IL-25<sup>-/-</sup> recipients (normally susceptible to parasite) conferred protection and was characterized by an increased production of Th2 cytokines as well as a reduction in the number of parasites 20 days after infection<sup>102</sup>. The MPtype2 population induced by IL-25, has been shown to differentiate into multiple cell lines including macrophages, basophils and mast cells after stimulation with SCF and IL-3. This derived progeny of MPtype2 promotes T cell proliferation and Th2-related cytokine production in a MHC-II dependent manner.

## **A3) Regulatory hematopoietic stem and/or progenitor cells**

In recent years, a population of myeloid cells able to suppress immune responses against tumor antigens has been reported by many groups. This cell population is characterized by the expression of the markers CD11b and Gr-1, it is called Myeloid-derived suppressor cell (MDCS) population including immature dendritic cells, granulocytes, macrophages and other myeloid cells. Different studies have provided evidence for possible mechanisms of action of these cells. They have been shown to suppress anti-CD3/anti-CD28 induced T-cell proliferation, downregulate CD3 $\zeta$ -chain through oxidative stress, inhibit the cytotoxicity of

CD8<sup>+</sup> T cells and induce T-cell apoptosis. In 2003, Bronte V et al showed that MDSCs can suppress the proliferation of T cells based on the expression of NO synthase 2 and arginase 1<sup>103</sup>. In the case of experimental autoimmune encephalomyelitis (EAE) disease, an animal model of multiple sclerosis, Zhu B et al have reported that the induction of disease can lead to the increase of CD11b<sup>+</sup> cells in the spleen with suppressive properties in vitro, but the main cell population which can efficiently suppress T cell proliferation and induce the apoptosis of T cells, is found in the subtype of CD11b<sup>+</sup>Ly-6C<sup>high</sup> monocytes. The induction of these cells has been shown to require the production of IFN- $\gamma$  by the activated T cells. Additionally, this cell population expresses also F4/80 and CD93 and is associated with an upregulation of NO-synthase 2 and arginase 1<sup>104</sup>. These observations were reinforced by a study of Moline-Velazquez et al, showing the appearance of these cells in the spinal cord of EAE mice where they have been able to suppress T-cell proliferation in vitro, in a mechanism dependent also on arginase 1<sup>105</sup>. Interestingly, Yi H et al suggested that the presence of MDSCs is responsible for the development of EAE disease since these cells are able to facilitate the differentiation of CD4<sup>+</sup> T cells in Th17 cells, enhancing the production of inflammatory cytokines such as IL-17. This occurs through the production of IL-1 $\beta$  which can bind to its IL-1R on CD4<sup>+</sup> T cells. Depletion of MDSCs could reduce the severity of EAE whereas adoptive transfer of MDSCs restored EAE progression<sup>106</sup>. More recently, an additional study provided evidence for the protective role of MDSCs in EAE, showing that after activation of iNKT cells through  $\alpha$ -Galcer, MDSCs are expanded inside the CNS where they can produce IL-10, NO synthase and arginase-1. Together with activated iNKTs, MDSCs can display suppressive functions and confer protection to EAE mice<sup>107</sup>. The presence and expansion of MDSCs, mainly in the periphery of EAE mice has been recently related with the inhibition of the activated protein C (PC) which is known to display anti-inflammatory effects and confer vascular barrier protection. When administrated a monoclonal Ab against PC (anti-PC) in EAE mice, the clinical incidence was reduced and ex-vivo analysis demonstrated the increase of CD11b<sup>+</sup> MDSCs in the periphery, reducing the number of CD4<sup>+</sup> T cells, suggesting a potential suppressive role of MDSCs against T cell proliferation<sup>108</sup>. A more recent study by Chung JS et al showed also that the expansion and suppressive functions of MDSCs in the context of EAE are co-related also with the expression of the dendritic cell (DC)-associated heparan sulfate proteoglycan-dependent integrin ligand (DC-HIL) receptor on APCs which inhibits T cells activation by binding to syndecan-4(SD-4) on T cells. DC-HIL is required for inducing the expansion of suppressive CD11b<sup>+</sup>Gr-1<sup>+</sup> MDSCs whereas mice deficient for DC-HIL displayed more severe EAE clinical scores, restoring the protection by adoptive transfer



of MDSCs from EAE-affected WT mice<sup>109</sup>.

It has been shown in our laboratory that transplantation of hematopoietic progenitor cells mobilized to the spleen by a cocktail of G-CSF and Flt3-L, at the stage of MPP2, could prevent the T1D in the NOD mice. They acquired the property to amplify the expansion of Foxp3<sup>+</sup> Treg cells in vitro and in vivo, through a cell-to-cell contact mechanism dependent on the activation of the Notch pathway in Tregs via an interaction with its ligand Jagged-2. Mobilisation resulted in hyper-expression of Jagged2 on MPPs interacting with Notch3 on Treg<sup>110</sup> and the secretion of GM-CSF that in NOD mice increases proliferation of Treg stimulated with their TCR<sup>111,112</sup>. These properties confer to these mobilized multipotent progenitors the ability to prevent T1D even at a late stage of diabetes, i.e in NOD mice at the age of 16 weeks, engrafted in small number of cells (1 to 3 x 10<sup>4</sup> per mouse). Fiorina et al have demonstrated that mobilization of HSCs through a CXCR4 receptor antagonist, allows prolonged survival of BALB/c and C57BL/6J mice after allogeneic transplantation of islets<sup>113</sup>. The mobilized HSCs expressed PDL-1 and negative costimulatory molecules. In mice deficient for PDL-1, treatment with the CXCR4 antagonist was not any more able to delay the rejection of allogenic pancreatic islets. In summary, the HSC and progenitor cells are cell populations that are not only intended to provide differentiation into mature cells of the immune system. They are highly dynamic cells that respond to environmental stimuli, interacting with different mature cell types of the immune system, applying immunosurveillance and immunoregulatory functions that started recently to be described.

## I-2 Toll Like receptors

Toll like receptors are pattern-recognition receptors (PRRs) which are characterized by their ability to recognize pathogen-associated patterns (PAMPs) or danger-associated molecular patterns (DAMPs). The first evidence for the existence of these receptors had been provided by Charles Janeway in 1989 who described that some microbial products can be recognized by receptors to link innate and adaptive immune system<sup>114</sup>. The first molecule which had been shown to activate T cells was the pro-inflammatory cytokine IL-1. At this time, many laboratories focused their studies on the clarification of the interaction of this cytokine with its receptor IL-1R. In 1991, Gay N et al demonstrated that a specific domain of IL-1R displays many similarities with the cytosolic domain of a protein, already known in *D.melanogaster*, called Toll<sup>115</sup>. Later on, it was shown that alike the developmental process of flies, many genes involved in immune responses, such as *il-1r*, were regulated by a signaling pathway through the transcription factor of NF- $\kappa$ B in mammals<sup>116</sup>. In 1994, Whitham et al presented also a study in which they described a plant protein, called N protein, where an amino-terminal domain was sharing the same characteristics with the cytoplasmic domain of Toll (in *drosophila*) and IL-1R (in mammals)<sup>117</sup> and was able to provide protection of tobacco against mosaic virus. In 1998, Rock FL et al described five other Toll homologues in mammals and they gave the name TLRs for all of them<sup>118</sup>. Knowing already that the Toll protein was involved in the immune responses in *drosophila*, the idea that TLRs might be involved in the innate immunity in mammals acquired increasing strength.

### I-2-1 Toll-like Receptors – General

TLRs are transmembrane receptors of type 1 that can be divided in two different functional parts. One domain is enriched in leucine repeats and is responsible for the recognition of the PAMPs. Their signaling is dependent on different adaptive molecules such as MyD88, Mal, TRIF, TRAM and SARM<sup>119</sup>. In addition, there is one transmembrane domain and one intracellular TIR (Toll-interleukin-1 receptor) which is necessary for the signal transduction. We can distinguish two groups of TLRs based on their subcellular localisation. TLR1, 2, 4, 5 and 6 can be found at the surface of the cell and TLR3, 7, 8 and 9 reside intracellularly<sup>120</sup>. Today, 10 different types of TLRs have been described in humans and 12 in mice. Only the TLR-1 and -9 are conserved in both species. TLRs are today known to display important protective properties against infections but at the same time, accumulating evidence shows that an inappropriate TLR response can lead to an acute inflammation and autoimmune diseases.

## **I-2-2 Types of TLRs and their ligands**

### **A) TLRs of cell surface**

#### **A1) TLR4**

The most characterized type of surface TLRs is the TLR4, which is widely known to recognize the LPS (Lypoposaccharide). This interaction requires the participation of other binding proteins, CD14 and MD2<sup>121,122</sup>. In resting cells, TLR4 resides inside the Golgi and at the plasma membrane of human monocytes where the trafficking between these regions is really important and can be affected by different factors. At the beginning, the complex of TLR-4-CD19-MD2<sup>98,123</sup>, had been proposed to be necessary for the migration and localization of the receptor at the cell surface, the recognition of the LPS and the activation of the corresponding signaling. Inhibition of this complex did not affect the function of the TLR4, so it seems that there is a continuous circulation between the Golgi and the cell surface until the engagement of the LPS<sup>124</sup> and the interaction with MyD88 and Mal which concludes to the activation of NF-κB signaling. An interesting characteristic of the function of the TLR4 is the internalization into endosomes, which occurs after the recruitment of LPS. This translocation process is dependent on dynamin proteins and GTPases which control either the role of vesicles from the plasma membrane or clathrins associated with the endocytotic process<sup>125-127</sup>. This process seems to serve the activation of TRAM-TRIF pathway and the induction of the IFN signaling in the endosomes<sup>128</sup>. In addition, TLR4 is involved in the recognition of the plant-derived taxol, or endogenous ligands such as heat shock proteins (HSP60 and HSP70), of a domain of fibronectins and oligosaccharides but all these ligands should be present in very high concentrations in order to activate TLR4.

#### **A2) TLR1/2/6**

At the same category we have also TLR2 which is expressed at the plasma membrane, in early and late endosomes but not in the Golgi or ER<sup>129</sup>. It heterodimerizes with other two TLRs, the TLR-1 and 6, and they recognize a big variety of PAMPs of fungi and Gram-positive pathogens. Specifically, TLR2 recognizes lipopeptides that are bound to the bacterial membrane<sup>130</sup> whereas TLR6 recognizes triacylated or diacylated lipopeptides. Concerning the activation and function of this last group of TLRs, it has been shown that surface molecules such as CD14 and CD36 are more than necessary for the downstream signaling pathways<sup>129</sup> as well as CD86 which is involved in TLR2/6 responses but not in TLR2/1 responses. On the other hand, the role of CD14 is stronger since this molecule can be used as a co-receptor for

both TLR2/TLR6 and TLR2/TLR1 complexes. TLR2 activation by viruses in monocytes can trigger the production of IFNs type 1 and its internalization is necessary, in contrast with the production of the cytokine TNF where there is no requirement for internalization, suggesting the existence of two different signaling pathways in distinct regions<sup>131</sup>.

### **A3) TLR5**

TLR5 is a type of Toll-like receptor, which recognizes a conserved protein of bacterial flagella. The specificity of this receptor is important because unlike most other PAMPs, flagellin is a protein, which remains in an unmodified form, a fact that makes difficult to distinguish from the host cellular proteins. Interestingly, flagellin can be found in the intestinal epithelium by pathogenic bacteria like Salmonella<sup>132,133</sup>. CD11c<sup>+</sup>CD11b<sup>+</sup> DCs derived from the small intestine of lamina propria are characterized by a strong expression of TLR5 whose activation promotes the differentiation of Th17, Th1 cells and naïve B cells into plasma cells producing IgA.

### **A4) TLR11**

TLR11 is a receptor present in mice, but not in humans and is known to recognize uropathogenic Escherichia coli. The first ligand for TLR11 has been shown to be a profiling-like protein from Toxoplasma gondii<sup>134</sup>. Infection with the parasite Toxoplasma gondii leads to the induction of a Th1-type response dominated by IFN- $\gamma$  production. Pepper M et al have shown that after an infection by the toxoplasma gondii, TLR11 activation is responsible for the activation of plasmacytoid DCs which produce IL-12, enhance maturation of DCs, upregulate expression of MHC class II and costimulatory molecules associated with their acquired ability to prime naïve CD4<sup>+</sup> T cells<sup>135</sup>. Additionally, TLR11 has been shown to be necessary for the prevention of pathogen-induced immune destruction of self tissue since mice deficient for TLR11 are characterized by non-TLR dependent activation of IFN- $\gamma$  secretion by NK cells resulting in tissue destruction<sup>136</sup>. Recently, it has been reported that TLR11<sup>+</sup> cells can be found also in normal and parasite infected mouse brain suggesting a potential role in CNS infections<sup>137</sup>.

## **B) Intracellular TLRs**

In the second group of TLRs, we can find TLRs that are located not at the plasma membrane but inside the cell and they are present in the ER where they remain only under the control of specific signals from their structure (either from the ectopic domain or the trans-membrane domain). Upon activation from recognition of a ligand, TLR-3, -7 and -9 display a movement from the ER to the endosome. This migration process is ligand-independent and differs from the mechanism of TLR4 where LPS is necessary to trigger the circulation of TLR4 from the Golgi to the plasma membrane<sup>138</sup>. A typical ligand of TLR9, the CpG DNA has been shown to internalize via clathrin-dependent endocytic pathway in endosomes where it is located together with the TLR9 and the adaptor protein MyD88<sup>139</sup> and this localization (where the cleavage of TLR9 by cathepsin occurs) is required for the activation of TLR9 and TLR3 signaling<sup>140-142</sup>. The localization of these TLRs inside the cells seems to be really important since it prevents them from being activated by self nucleic acids which are usually expressed at the cell surface, and on the other hand, they become specialized for the recognition of viral nucleic acids which remain in a more permanent way in the endolysosome, where they can meet TLRs only upon activation as they can migrate rapidly over there from the ER<sup>120</sup>.

### **B1) TLR3**

TLR3 is a kind of intracellular Toll-like receptor, which is mainly expressed on dendritic cells. This receptor can recognize molecular patterns by many viruses such as dsRNA which can activate the dsRNA-dependent protein kinase, PKR74, but the presence of this proteins is not required for the immunostimulatory activity since PKR74-deficient cells remain still able to respond to both dsRNA and its synthetic analogue, polyinosine-polycytosine (poly:IC)<sup>143</sup>. TLR3 is important for an antiviral immune response via the production of type 1 IFN and other inflammatory cytokines, an observation established also by the fact that mice deficient for TLR3 are not resistant against infections of the murine cytomegavirus. In humans, a deficiency of TLR3 is widely associated with susceptibility to the virus of Herpes HSV-1<sup>144,145</sup>.

## **B2) TLR7/8**

The main ligands for the Toll-like receptor 7 are different forms of the imidazoquinoline such as the imiquimod and resiquimod (R848) and analogues of the guanine like loxoribine (with antiviral and antitumoral properties). It is well known that it can also recognize the ssRNA derived for RNA virus from influenza A and HIV (Human immunodeficient virus). The receptor TLR7 is highly expressed on plasmacytoid Dendritic Cells (pDCs) which are characterized by their ability to produce large quantities of IFNs type 1 upon a viral infection. The production of cytokines from this cell type after an immune response against RNA viruses is totally dependent on TLR7, suggesting that TLR7 is used as a mechanism for recognition of viral infections through the ssRNA. The virus is internalized in the endolysosome where it is recognized by the TLR7. This interaction results in the production of the type I IFNs<sup>146,147</sup>. The TLR8 is phylogenetically the nearest TLR to TLR7. Human TLR8 recognize the R848 and ssRNA. Unlike mice lacking TLR7, mice deficient for TLR8 respond normally to these agonists. The TLR8 is expressed in various tissues. It is highly expressed by monocytes and its expression increases after a bacterial infection<sup>144</sup>.

## **B3) TLR9**

TLR9 is a receptor specialized for the recognition of CpG-DNA. Unmethylated CpG motifs can be found in bacterial DNA and we can distinguish mainly two types. The first one is what we call CpG-A, it has a great immunostimulatory activity and it can easily induce the production of IFN- $\alpha$  (by pDCs) but it is less effective for the production of the cytokine IL-12. The fact that TLR9 can trigger the production of IFNs by pDCs suggests that TLR9 can also work as a receptor for viral infection, recognizing viral-derived CpG DNA. CpG-A is considered as the more conventional form and it can induce the production of inflammatory cytokines such as IL-12 and TNF- $\alpha$ . CpG-B is considered to be a better stimulator for the B cells. The recognition of CpG-motifs can trigger alteration in cellular balance and can induce cell signaling via the mitogen activated protein kinases (MAPKs) and NF- $\kappa$ B. In General, CpG-DNA activate the B, NK, DCs and monocytes-macrophages and they are considered to trigger a Th1 type immune response (**Table 2**).

TLRs	Localization	Ligands			
		Exogenous	Endogenous	Synthetic analogs	Fully synthetic molecules
TLR1	Plasma membrane	Lipopeptides (Bacteria and Mycobacteria) Soluble factors ( <i>Neisseria meningitidis</i> )		Triacyl lipopeptides	
TLR2	Plasma membrane	Lipoprotein/lipopeptides (Gram-positive bacteria, Mycoplasma, Mycobacteria, Spirochetes) Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid (Gram-positive bacteria) Phenol-soluble modulin ( <i>Staphylococcus epidermidis</i> ) Heat-killed bacteria ( <i>Listeria monocytogenes</i> ) Porins ( <i>Neisseria</i> ) Atypical lipopolysaccharides ( <i>Leptospira interrogans</i> , <i>Porphyromonas gingivalis</i> ) Soluble factors ( <i>Neisseria meningitidis</i> ) Glycolipids ( <i>Treponema maltophilia</i> ) Outer membrane protein A ( <i>Klebsiella pneumoniae</i> ) Glycoinositolphospholipids ( <i>Trypanosoma cruzi</i> ) Phospholipomannan ( <i>Candida albicans</i> ) Structural viral proteins (Herpes simplex virus, Cytomegalovirus) Hemagglutinin (Measles virus) Lipoarabinomannan (Mycobacteria) Zymosan ( <i>Saccharomyces</i> )	HSP60 HSP70 HSP96 HMGB1 Hyaluronic acid	Diacyl and triacyl lipopeptides	
TLR3	Endolysosome	Single-stranded viral RNA (ssRNA) and double-stranded RNA (dsRNA; Viruses)	mRNA	Poly(I:C) Poly(I:C) <sub>12U</sub>	
TLR4	Plasma membrane	Lipopolysaccharide (Gram-negative bacteria) HSP60 ( <i>Chlamydia pneumoniae</i> ) Envelope proteins (Respiratory syncytial virus and mouse mammary tumor virus) Fusion protein (syncytial virus) Glycoinositolphospholipids ( <i>Trypanosoma cruzi</i> ) Taxol (Plant product)	HSP22 HSP60 HSP70 HSP96 HMGB1 $\beta$ -defensin 2 Extra domain A of fibronectin Hyaluronic acid Heparan sulfate Fibrinogen Surfactant-protein A	Lipid A mimetics (Monophosphoryl lipid A, aminoalkyl glucosamine 4-phosphate)	E6020 E5531 E5564
TLR5	Plasma membrane	Flagellin (Gram-positive or Gram-negative bacteria)		Discontinuous 13-amino acid peptide CBLB502	
TLR6	Plasma membrane	Diacyl lipopeptides (Mycoplasma) Lipoteichoic acid (Gram-positive bacteria) Phenol-soluble modulin ( <i>Staphylococcus epidermidis</i> ) Zymosan ( <i>Saccharomyces</i> ) Heat-labile soluble factor (Group B <i>streptococcus</i> )		Diacyl lipopeptides	
TLR7	Endolysosome	Single-stranded RNA (Viruses)	Endogenous RNA	Oligonucleotides	Imidazoquinolines (Imiquimod, Resiquimod) Guanosine nucleotides (Loxoribine, Isatoribine) Bropirimine
TLR8	Endolysosome	Single-stranded RNA (Viruses)	Endogenous RNA		Imidazoquinolines (Resiquimod)
TLR9	Endolysosome	Unmethylated CpG motifs (Bacteria and viruses) Hemozoin ( <i>Plasmodium</i> )	Endogenous DNA	CpG oligodeoxynucleotides (CPG 7909, CPG 10101, 1018 ISS)	
TLR10	Extracellular	Unknown, may interact with TLR2 and TLR1			
TLR11	Plasma membrane	Profiling-like molecule ( <i>Toxoplasma gondii</i> )			

**Table 2. TLRs: Type, Localisation and ligands.** *Manavalan et al, Frontiers in Physiology, 2011*

### I-2-3 Signaling pathways of TLRs

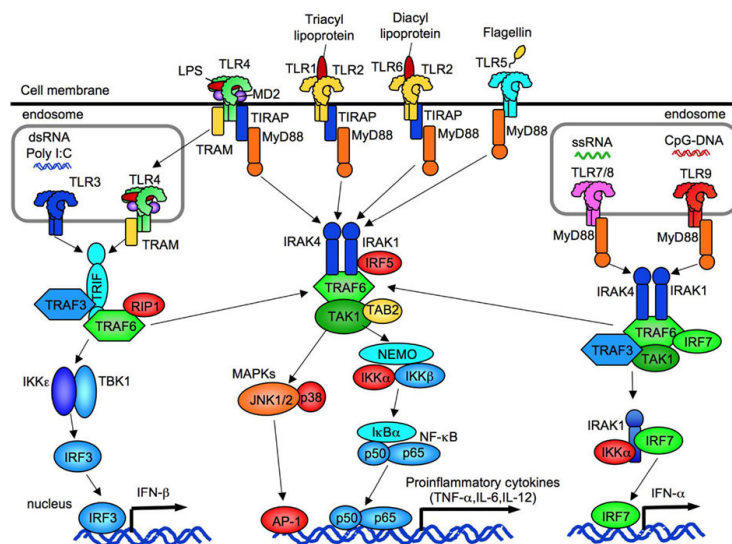
As it has been mentioned already, Toll-like receptors are involved in the host defense against pathogen microorganisms linking the innate and adaptive immune system. Their activation leads to the expression of genes coding for several inflammatory cytokines, co-stimulatory molecules, MHC complex and chemokines through several signaling pathways such as NF- $\kappa$ B and MAP (mitogen-activated protein). These signaling pathways can be either the same upon activation of all TLRs or can differ among some of them, suggesting the existence of functional differences in signaling between TLRs and IL-1R. The differences could be explained after the discovery of the involvement of several adaptor proteins associated with the TIR domain. The signaling pathways which seems to be ‘shared’ by all TLRs and IL-1Rs are dependent on some important factors. I will focus mainly on the three following components.

- The adaptor proteins : MyD88, TIRAP, TRIF, TRAM,
- The protein kinase, IRAK (IL-1R-associated kinase)
- NF- $\kappa$ B signaling

The adaptor protein MyD88 has been shown to play an important role in a large variety of immune responses and has been studied under many different circumstances during the last years. Concerning its structure, it contains two protein interaction domains: the carboxy-terminal TIR which is highly associated with the TIR domain of TLRs, the IL-1R and an amino-terminal death domain that interacts with the IRAK protein to recruit IRAK to the IL-1R complex. Studies with the use of MyD88 deficient mice demonstrated the possible existence of pathways dependent or independent on MyD88 signaling. MyD88 deficient mice, upon exposition to IL-1 or to microbial components which can be recognized by many TLRs, like TLR2, 4, 5, 7 and 9 lose their ability to produce the anti-inflammatory cytokines IL-6 and TNF- $\alpha$ , suggesting their importance for immune responses against a big variety of pathogens. In the same deficient mice, it has been also shown that in some cases of pathogen infections, the NF- $\kappa$ B signaling can be abolished or can still occur, enhancing the idea of a MyD88-independent pathway. In MyD88 deficient mice, after activation with LPS, macrophages upregulate the expression of IFN-inducible genes (glucocorticoid-attenuated response gene and CXC-chemokine ligand 10) and display also a LPS-mediated maturation of dendritic cells upregulating the expression of their co-stimulatory molecules CD40, CD80 and CD86, a phenomenon which is highly associated with their ability to induce proliferation of T cells.



This maturation process has been shown to be a secondary response to the induction of the production of IFN- $\beta$ . At this point, it should be clear that these responses which in some cases seem to be MyD88-independent, they require always the existence of the corresponding TLRs<sup>147-150</sup>. The MyD88-independent responses are usually TRIF-dependent. In addition, TIRAP has been shown to be involved in TLR-2 and TLR-4 activation of MyD88-dependent pathway and TRAM to be more specialized for TLR-4 mediated, MyD88-independent/TRIF dependent pathway. The fact that the activation of the TLR-4 signaling requires the implication of all these adaptor proteins, either in a direct or indirect association, might explain why the LPS (TLR-4 ligand) is such a strong stimulator of immune responses. Concerning the ability of other TLRs (TLR7 and 9) to induce the production of type 1 IFNs, it seems that only MyD88 is required for the signaling pathway. The structure of IRAK contains the central serine/threonine-kinase domain and a N-terminal Death domain and we can distinguish four different types : IRAK1, -2, -3, and -4. The activity of the kinase is dependent on the stimulation of TLR/IL-1R and the signaling through NF- $\kappa$ B. IRAK4 is the form which has been studied mostly in mice and humans and it has been shown that IRAK4 is important for the production of inflammatory cytokines upon exposures to LPS or IL-1 in mice and for the activation of each of five TLRs (2, 3, 4, 5 and 9) in patients<sup>147,151,152</sup>.



**Figure 10. The overview of TLR signaling.** *TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on cell membrane. TLR3, TLR7/8, and TLR9 are expressed in endosome. All TLRs, except for TLR3, activated MyD88-dependent pathway to induce NF- $\kappa$ B and p38/JNK activation. TLR2 and TLR4 signaling require TIRAP and MyD88. TLR3 requires TRIF to activate TBK1/IKK $\epsilon$ . Subsequent to TLR4 internalization, TLR4 signaling activates TRAM/TRIF-dependent pathway. TLR3/4-dependent TRIF-dependent signaling induces IRF3 activation and IFN- $\beta$  production. TLR7/8 and TLR9 induce IFN- $\alpha$  production through IRF. Yang Ling, Frontiers in Physiology, 2012*

#### **I-2-4 TLRs and diseases**

Once it had been clarified the strong involvement of TLRs in many immune responses, different studies started to try to correlate their function in potential therapies of human diseases. First of all, due to the fact that these receptors are specialized in the recognition of pathogens, their clinical implication in vaccines had been studied by different groups demonstrating for example that the vaccine adjuvant monophosphoryl lipid A, a much less toxic form of LPS, is able to promote antibody responses via TLR4 activation. An other interesting finding was also the fact that imiquimod, an already used treatment against genital warts caused by the papilloma virus, is a direct TLR7 ligand. The fear that different endogenous signals can trigger the activation of TLRs leading to the production of cytokines responsible for many inflammatory diseases, led scientists to think about inhibiting the functions of some TLRs as potential therapeutic ways for different diseases. Different examples exist today, such as the use of a synthetic small-molecule inhibitor of TLR4, E6664, for the treatment of patients with sepsis or oligonucleotide-based inhibitors of TLR7 and/or TLR9 for systemic lupus erythema and inhibitory antibody to TLR2 in 2012, against ischemia-reperfusion injury in the heart (pigs) and kidney (mice).

## Chapter II: Autoimmune Diseases

### II-1 Introduction to Autoimmune Diseases

One of the main fields of interest in clinical immunology is the research on a specific category of diseases which are united under the title of ‘autoimmune diseases’. A brief definition could be that these disorders appear when the body’s immune system starts turning against the body itself and trying to attack it as if it was a foreign pathogen. They are characterized by the breaking of the mechanisms of immune tolerance which control the level of activation of the peripheral T and B lymphocytes against autoantigens, expressed somehow by our body’s tissues. According to the American Autoimmune Related Diseases Association (AARDA), today, we have evidence for the existence of more than 50 distinct diseases and syndromes which affect a serious percentage of the population, mainly in Europe and North America, with two thirds of patients being female. Among them, the most usual cases include rheumatoid arthritis, multiple sclerosis, juvenile diabetes, antiphospholipid syndrome, Crohn’s diseases, Graves’ disease, Sjogren’s syndrome, alopecia, myasthenia gravis, psoriasis and lupus erythematosus. The first proof for the existence of this kind of disorders has been shown in 1970s, demonstrating the production of antibodies against red blood cells, correlating that with anemia. Moreover, several viral infections have been associated with the induction of autoimmune disorders.

Autoimmune diseases can be categorized in two different groups. The first one (= **organ specific**) include those that target a particular tissue, attacking autoantigens in this tissue and triggering an immune response limited to this tissue. The second one (= **systemic autoimmune diseases**) is characterized by the attack of several organs, as the autoantigen can be distributed in many different places. A more pathological classification does not exist but we can distinguish them according to the implication of different types of effector T lymphocytes, B lymphocytes and evidence for genetical predisposition.

Despite the fact that our immune system has developed specific mechanisms for limiting the possibility for the existence of autoreactive cells against self-antigens through a process referred as ‘immune tolerance’, in many cases, cells escape this process and can lead to the induction of autoimmune diseases.

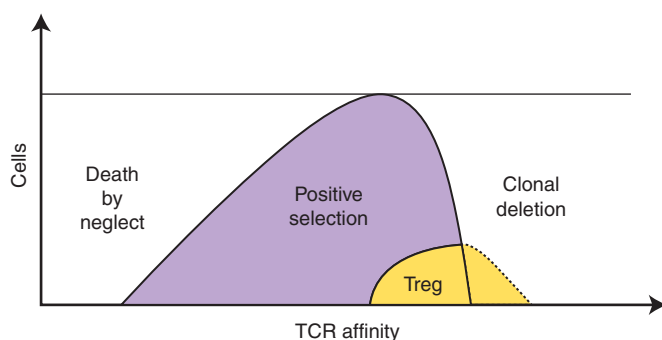
## II-2 Immune Tolerance

It was only in 1980 when for the first time, investigators managed to demonstrate the existence of a mechanism for clonal deletion either for T or B lymphocytes. Analyzing the fate of developing lymphocytes upon stimulation by peptide-MHC complexes in TCR (T-cell receptor) transgenic mice, it had been shown that during the ontogeny of lymphocytes, there is a period, that when interacting with antigens through their receptors, lymphocytes can be led to cell death rather than activation, proliferation and differentiation into effector cells.

T-cell tolerance is really important in order to avoid the induction of autoimmune diseases since T-cells expressing a receptor specific for a self-antigen can be rapidly eliminated. The first type of tolerance is what we call ‘central tolerance’, it occurs in the thymus as soon as a T-cell receptor is formed and expressed at the surface of a T-cell progenitor. That process requires a large variety of autoantigens to be expressed in the thymus in order to maximize the efficacy of the central tolerance. However, not all antigens are expressed, so additional mechanisms of tolerance are needed to restrain the number or function of T cells that are reactive to self-antigens. These mechanisms can act as well on more mature, already circulating, T cells and are referred as ‘peripheral tolerance’.

### A) Central Tolerance

As it was described already in the first part of the introduction, the development of T cells occurs partially inside the bone marrow. In a progenitor stage, they exit bone marrow and migrate in the thymus where they undergo the main process of their differentiation. T-cell receptor rearrangement is a crucial stage for getting either  $\gamma\delta$  or  $\alpha\beta$  progenitors at the CD4 and CD8 double-negative (DN) stage. Through a somatic recombination of TCR genes in double-positive (DP) thymocytes, a large repertoire of different  $\alpha\beta$  TCRs is generated. At this stage, the TCR affinity for self-peptide-major histocompatibility complex (MHC) can affect the thymocyte’s fate. In the thymus, the developing lymphocytes undergo either a positive or negative selection according to their affinity for self-peptide MHC complexes. Those with high affinity TCR will be operated by several mechanisms of tolerance such as clonal deletion, diversion or anergy, a state of unresponsiveness that can be induced mainly in the periphery. Another mechanism, called ‘receptor editing’ can also occur if a secondary gene rearrangement at the TCR takes place, in order to modify the specificity of the TCR for a self-peptide<sup>153–155</sup> (Figure 11).



**Figure 11. A model for the relationship between developmental outcome and TCR affinity for self-peptide-MHC.** It is shown that cells with TCR displaying high affinity for self-antigen die by neglect whereas those with an intermediate affinity are positively selected. High-affinity clones for self-antigens undergo cell death via clonal deletion. In yellow, Tregs which have more self-reactive TCRs, are rescued from deletion via cytokine signaling or by virtue of having a second TCR (dotted line) whereas most conventional T cells have less self-reactive TCRs (in purple)<sup>156</sup>. Yan Xing, 2012, *T-cell Tolerance*

Further in the process of clonal deletion, an important point is to know when and where it occurs. In the thymus, two main different areas exist: the cortex, where both double positive and negative thymocytes reside and also a more inner region, medulla, with only single positive (CD4 or CD8) thymocytes. For the study of this process, two different experimental models have been proposed. The first one includes the use of superantigens (= antigens which can induce non-specific activation of T cells resulting in polyclonal T cell activation and massive production of cytokines) and the second one is through the use of transgenic mice where all T cells express a unique T-cell receptor. Today, different studies suggest that the deletion of polyclonal thymocytes occurs in the thymic cortex whereas deletion of those with a specificity to tissue-restricted antigens occurs in the medulla<sup>157</sup>. Other cell types in the thymus have also been shown to be involved in that process such as epithelial cells of the cortex (cTECs) and the medullary (mTECs) ones. These last ones are known to express the nuclear regulatory protein, autoimmune regulator (AIRE) which is highly implicated in the process of thymic tolerance since mutations in the gene encoding for that protein have been related with the appearance of many autoimmune diseases in both mice and humans. The expression of a large variety of tissue-specific antigens and co-stimulatory molecules by mTECs is a crucial factor at inducing clonal deletion<sup>158,159</sup>. AIRE is able to promote the gene expression of tissue-specific antigens (TSAs) but how this protein can affect the presentation of these antigens by MHC molecules is still unclear. Except for AIRE which is known to be important for the normal formation of the medulla area and maturation of thymocytes, other

signaling pathways are implicated, through a different way, at the induction of tolerance. The members of the TNF superfamily such as NF- $\kappa$ B and CD40 by mTECs have been shown to interact with their ligands expressed by hematopoietic cells, triggering the production of cytokines which can modify the function of mTECs which are formed in the medulla, the necessary microenvironment for inducing self-tolerance<sup>160-163</sup>.

### **B) Anergy**

As it was mentioned already before, in addition to the clonal deletion, there are also other mechanisms which can help at the induction of immune tolerance. One of them is what we call ‘anergy’, a phenomenon where T cells lose their capacity to respond to activating signals. The first and necessary condition for a T cell to become activated is the presence of a TCR signal through an interaction with a MHC-peptide complex (signal 1) and a costimulatory signal by CD28 ligation (signal 2), promoting the production of cytokines such as IL-2. Today, it is well established that only stimulation of TCR is not sufficient either for the production of cytokines or the proliferation of T cells<sup>164</sup>. It has been shown that the complete T-cell activation is lost in the absence of the second signal, inducing T cells to a state of hyporesponsiveness. The IL-2 cytokine is involved in an additional signaling pathway through the interaction with its receptor IL-2R in order to activate the PI3K/AKT-mTOR pathway. Many studies have focused on the role of CD28 receptor expressed by T cells and its ligands B7-1 (CD80) and B7-2 (CD86) expressed at the surface of APCs. During a normal immune response, CD80 and CD86 are increased and bind to CD28 expressed by T-cells. The coincidence of signals 1 and 2 can effectively recruit transcription factors involved in the transcription of the genes for IL-2, NFAT, AP1 and NF- $\kappa$ B whereas the lack of co-stimulation often leads to death of T cells by apoptosis<sup>165</sup>. In addition, it has been shown that the inhibition of the mTOR activation is enough to induce anergy in T cells. Different factors can play a role in that process, such as the activation of AMPK, a sensor of ATP deprivation and hypoxia or the GCN2 pathway and signals from the A2A receptor. The implication of Tregs at inducing anergy has been shown by their ability to express the CD73 and CD50 which are responsible for promoting a hypoxic environment<sup>165,166</sup>.

### **C) Activators and inhibitors of co-stimulatory signals**

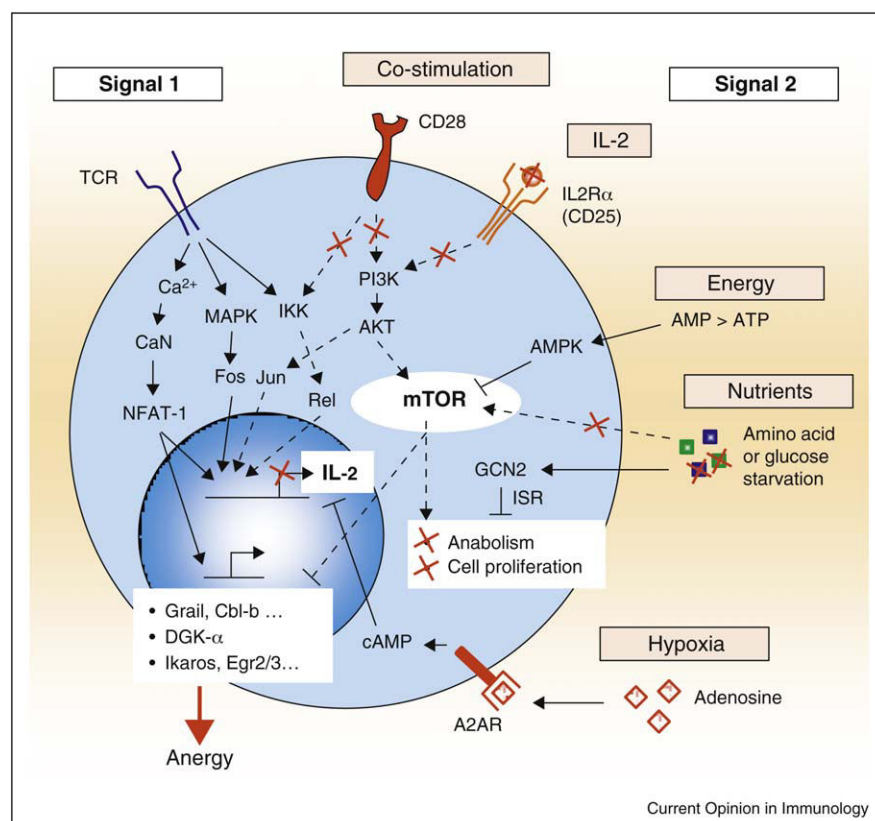
Many examples of immune responses in cases of deficiency for CD28 have suggested that additional surface molecules such as CD40L (CD154) and OX40 may have the same costimulatory functions. In addition to the role of co-stimulation in the initiation of the primary

immune response, it has been shown that other specific receptor-ligand interactions are involved in the amplification of co-stimulatory pathways during an immune response<sup>167,168</sup>.

The co-stimulatory molecules can be grouped into two classes based on their structural homology. The first class includes the molecule CD28 and the inducible co-stimulatory molecule, ICOS. CD28 and ICOS are homodimers which can bind at the surface proteins of the B7 family. CD28 can interact with the B7.1 (CD80) and B7.2 (CD86) molecules due to a highly conserved motif in the CDR3-like loop. ICOS is structurally similar to CD28 but does not bind with B7.1 and B7.2 whereas it is specific for another ligand of the B7 family, the ICOS ligand (ICOSL) (**Figure 12**). Unlike CD28, ICOS is absent or expressed in very low levels on naïve T cells but its expression is greatly increased upon stimulation. Activation of CD28 molecule is the main co-stimulatory signal of naïve T cells whereas ICOS is for the effector T cells. CD28 and ICOS therefore act at different levels of the immune response and are required for the activation of T helper cells (Th), differentiation and expression of effector cytokines.

Two other receptors, CTLA-4 (cytotoxic lymphocyte antigen-4) and PD-1 (programmed death-1), share structural homology with CD28 and can bind also with proteins of the B7 family. The extracellular domain of CTLA-4 possesses 50% homology with that of CD28 and can bind with the same CD80 and CD86 ligands. However CTLA-4 inhibits T-cell activation either by competing with CD28 or by dephosphorylation of the TCR. An interesting study shows that mice lacking the gene *pdc1* (*Pdcd1*<sup>-/-</sup>) encoding for the expression of PD-1, develop spontaneously a lupus-like autoimmune disease, suggesting that inhibition of the ligation of TCR and PD-1, which is necessary for the activation of the PI3K pathway, can lead to anergy. In addition to that, it has been proposed that the activation of the interactions of PD-1 with its ligands PD-L1 and PD-L2 can inhibit T-cell stimulation and expansion of self-reactive cells<sup>169,170</sup>.

The second class of costimulatory receptors belong to the family of TNF receptors (TNFR), including the major costimulatory molecule of B cells, CD40, OX-40, 4-1BB, CD30 and CD27.



**Figure 12. Co-stimulatory molecules and induction of anergy in T cells.** *Chappert and Schwatch, 2010*

#### D) B cell tolerance

Apart from T cells, B cells should also undergo mechanisms of tolerance as they are also highly involved in immune responses through their ability to produce antibodies, cytokines and to act as antigen-presenting cells. The decision between immunity and tolerance can be affected by many factors such as the antigen structure which can determine the T-cell dependence of a B-cell response, target tissue molecules and also their capacity to act as regulatory cells<sup>171–173</sup>. In order to avoid anti-self specificities of the B cell repertoire, two different mechanisms of tolerance have been proposed, with negative selection in different stages of B-cell differentiation. The primary (first mechanism) acts on the distribution of B-cell repertoire during the differentiation and the second one (secondary) can modify the responsiveness of self-reactive B cells in peripheral lymphoid organs<sup>174</sup> affecting both the BCR signaling and the antigen-presenting capacity. In the case of B cells, the central tolerance occurs in the bone marrow, in the organ of their origin.



### **E) Central Tolerance of B cells**

As it has been already discussed in the first part of the introduction, the first event in the generation of the B-cell repertoire is the V(D)J recombination. Successful or not, VDJ heavy-chain gene rearrangements is a kind of first control which determines if the pro-B cells develop into pre-B cells expressing an antigen receptor. Keenan RA et al demonstrated in 2008 that mice deficient for the surrogate light chain (SLC) lose their ability to produce a pre-B cell receptor and have high levels of self-reactive B-cells producing auto-antibodies in the periphery, suggesting an additional checkpoint at the pre-B cell stage where a rearranged Ig heavy chain can be scrutinised before it is paired with the light chain<sup>174,175</sup>. Once the cells get the expression of the B-cell receptor at their surface, they are at an immature stage of differentiation. At this stage, the affinity of B cells to an antigen determines if and how they undergo a tolerating mechanism. Recent studies propose that the ‘receptor editing’ seems to be the principal mechanism of central tolerance and those which still keep self-reactivity, about half of the immature B cells, undergo clonal deletion. Except for the involvement of the BCR affinity, in humans, other signaling pathways have been suggested to affect that process such as MyD88, UNC-93B and IRAK-4<sup>174,176,177</sup>. Finally, it is interesting to mention that in the central tolerance of B cells in the bone marrow, there is no equivalent of the AIRE gene which controls the expression of restricted antigens in the periphery, as B cells interact directly with the antigen. All these mechanisms of central tolerance can be adapted for the conventional B cells, since the B-1 cells are known to produce natural antibodies, display low-affinity self-reactivity and it is not yet very clear what kind of negative selection they undergo, where and when<sup>174</sup>.

### **F) Peripheral Tolerance of B cells**

The differentiation program of B cells continues in the secondary lymphoid organs, firstly as transitional (T1 and T2) cells where they meet self-antigens that are not present in BM. Cells which can display high-avidity interactions will undergo deletion and those with low-avidity anergy or ignorance<sup>174,178,179</sup>. In the case of anergy, BCR signaling loses its coupling with the NF- $\kappa$ B pathway which results in a status of unresponsiveness and failure of B cells to display their properties of antibody production and antigen-presentation. If these ‘anergic’ B cells recognize a cross-reactive antigen, they can immediately access to BAFF or T-cell help which make them survive and undergo negative selection in the Germinal Center (GC) where they become more mature. This transient stage of B cells has been confirmed in both humans and

mice by identifying a population of cells in the B-cell repertoire. This population is called An1 for mice (CD93<sup>+</sup>IgM<sup>low</sup>) and BND for humans (IgM<sup>+</sup>IgD<sup>+</sup>) with functional and phenotypic properties of anergic cells<sup>180,181</sup>. A negative selection of B cells occurs also in the GC against self-antigens presented on follicular dendritic cells, characterizing them by a pro-apoptotic gene expression because of the downregulation of Bcl-2 and upregulation of Bim family molecules, suggesting that the deletion of these B cells occurs after exposure to the antigen and only if they do not get any survival signals from T follicular helper cells. An involvement of a type of macrophages, the tingible-body macrophages (TBM), has been shown by a study of Kranish J et al, proposing that these cells can participate in the removal of potentially apoptotic bodies expressing nuclear antigens from apoptosing GC B-cells, while mice deficient for TBMs are more susceptible to autoimmune diseases, enhancing the importance of self/non-self discrimination in GCs<sup>174,182,183</sup>.

### **G) Ignorance**

It has been shown that a quantity of auto-antigen specific T and B cells can be present in the circulation. A first aid for eliminating the recognition of MHC-peptide complexes by auto-reactive cells is the presence of physical barrier between these auto-reactive cells and the parenchymal cells residing in tissues with expression of auto-antigens. These immunoprivileged sites include as well the brain, the anterior chamber of the eye and testes. However, in some cases, even if there is absence of functional response, the autoreactive cells can have access to the tissues where the antigen is expressed. For example, it has been shown that transgenic mice expressing viral protein in pancreatic  $\beta$  cells do not develop autoimmune disease unless infected by the respective viruses<sup>184</sup>.

## Chapter III: Multiple Sclerosis

### III-1 Multiple Sclerosis: Generalities

Multiple Sclerosis (MS) is a chronic, inflammatory disorder of the Central Nervous System (CNS) which involves autoimmune responses against specific antigens residing mainly in different regions of the central nervous system. MS attacks and destroys the myelinated axons in the CNS triggering serious pathophysiological problems. Actually, there is no specific test for the diagnosis of this disease and usually, neurologists are based on several clinical findings supported from magnetic resonance imaging (MRI) of the brain and tests in ancilar and cerebrospinal fluid (CSF). The results should demonstrate at least two different lesions in the white matter of the CNS and chronic inflammation in the CSF where the symptoms last at least 2-3 days and occur as distinct episodes separated by one month or more. In order to conclude to a MS diagnosis, neurologists should exclude other neurological-related disorders performing examination of oligoclonal bands in the CSF. Within the white matter of the CNS, lesions should be characterized by areas where myelin and oligodendrocytes have been lost and there is infiltration of activated immune cells such as lymphocytes and macrophages.

Concerning the main symptoms for MS, most patients display severe spasticity in the legs, numbness and tingling, fatigue, diplopia in their eyes, optic neuritis by partial or complete loss of vision, bladder disturbances and ataxia.

The cause of this disease is still unknown but many factors, either genetical or environmental seem to affect the appearance of this disorder which leads to the attack of immune cells inside the CNS<sup>185</sup>.

According to the course of the disease, we can distinguish today the following types of MS:

- **Relapsing/Remitting (RR)** MS which is the most common, and is characterized by periods of relapses of symptoms which are followed by periods of remission during which most of the symptoms disappear.
- **Progressive** MS is characterized by the fact that since the appearance of the first symptoms, the disease worsens gradually without periods of relapses and remissions and it is more resistant to the usual drugs for MS.

Apart from these two main forms, there are also some other subforms which share characteristics from both RR and progressive MS. For example, some patients present a kind of secondary progressive MS where the disease worsens but with possible periods of remission, and some others belong to the rare category of Progressive-Relapsing (PR) MS, in

which the disease is progressive from the very beginning with periodical relapses worsening the symptoms.

### **III-2 Epidemiology of MS**

Most people who are diagnosed to suffer from MS are between the age of 20 and 45 years. Geographical parameters such as latitude and climate have been shown to affect the induction of the disease. MS is more common among caucasians, mostly in North Europe than other areas and in the United States of America is estimated that about 300,000 people having MS. Women are more susceptible in the appearance of MS, the percentage is at least two to three times more, providing evidence for the role of some hormones in the incidence or the protection against this neurological autoimmune disease. Scientists suggest the involvement of some virus infections which may favor the evolution of the disease, including Herpes simplex virus, corona virus, Epstein-Barr virus and others. None of these has been confirmed with strong experimental proofs but increasing evidence about the role of some infections in initiating diseases comes out. Genetic factors show that for first-degree relatives of a person with MS, the risk is about 1 in 40 and concerning the case of homozygotic twins who share exactly the same genes, the risks rises to 1 in 4 for developing the disease. This last genetical observation confirms the fact that MS is not a genetic disease such that a genetic mutation or a clear genetic factor is the only and one factor determining the appearance of the disease. Some other factors including the geographical area, infections and/or environmental factors are probably involved in MS. In Europe, the estimated average incidence per year is about 4.3 cases per 100,000. In France, the prevalence of MS is considered lower than other countries with similar latitudes. Today, the risk of MS incidence in France is about 37-58 cases per 100,000 varying by different regions<sup>186</sup>.

### **III-3 Murine Experimental Models of Multiple Sclerosis**

In order to investigate the human disease of MS, there are very well established animal models which are used as experimental tools. It is for sure that the complexity of human Multiple Sclerosis and the heterogeneity in terms of genetical and environmental factors that affect the development of the disease make the use of these animal models an important scientific tool for the investigation of potential therapies. Over more than 30 years, our knowledge on the pathophysiological background of MS has been advanced because of many studies with the use of these animal models.

For MS, there is no a wild-type animal model which develops spontaneously this disease but today, we are able to induce the disease and to mimick very well the most part of human clinical parameters.

Among the used animal models for the research of MS, the most famous one is the Experimental Autoimmune Encephalomyelitis (EAE) which can be induced with the use of various myelin proteins in different mice strains depending on the form of EAE disease that we want to induce. Some of the main symptoms of MS, can be also studied with the use of toxic models of demyelination and remyelination or the use of Theiler's murine encephalomyelitis virus (TMEV) infection of mice.

### **III-3-1 Induced Animal Models for Experimental Autoimmune Encephalomyelitis (EAE)**

#### **A) Chronic EAE in C57BL/6**

It was 80 years ago when the use of vaccinations against rabies virus induced neuroparalytic accident so a question whether this virus is required for the syndrome had been adressed for the first time. Finally, it had been shown that injection of primates with tissue similar to those used for rabies vaccination can be used for inducing encephalitis<sup>187</sup>. Since then, every year, hundreds of scientific papers provided evidence for the amelioration of this model and today, EAE is considered to be one of the most studied animal models for human diseases. An essential factor for the induction of the EAE is the use of a myelin-like protein. The first protein that had been identified was the Myelin Basic Protein (MBP), followed by many others, among them, the most used are the Myelin Proteolipid Protein (PLP) and Myelin Oligodendrocyte Glycoprotein (MOG). Over time, scientists replaced the use of nonhuman primates and large rodents with mice. In mice, we can take advantage of several genetic resources (knockouts and transgenics) in order to study the role of individual immune factors in regulating autoimmune responses against CNS myelin, including cytokines, signaling pathways, transcription factors and T-cell surface receptors, performing a large variety of experiments on the neurobehavioral and neuropathological complexity. The main mouse strain which is used for the induction of EAE, is with the C57BL/6 background where the induction occurs by immunization with MOG peptide representing residues 35-55, emulsified in Freund's adjuvant that is a supplementary complement for *Mycobacterium tuberculosis*. Mice have to be injected also with *pertussis toxin* (*ptx*) on the day of immunization and the day after. *Pertussis toxin* is necessary since it has been shown to provide with additional adjuvant and to facilitate the entry of autoimmune T cells inside the CNS. This protocole is

widely used as it has been shown to work reproducibly and allows to take advantage of the several genetic resources that the C57BL/6 mouse background offers. According to a recent study from Berard JL et al, the dose of MOG peptide can influence the course of the chronic disease with possible induction of EAE with relapses and remission with the use of low doses<sup>188</sup>. On the other hand, we should make clear the fact that there are some limitations concerning the use of this model. It represents only a chronic model of EAE which corresponds to the primary progressive form of MS in humans but it can not display relapses and remissions during the pathological course. The contribution of the induced-EAE model is really important since the majority of treatments that are used today for therapies of MS patients have been identified for the first time in EAE mice. This model helped scientists to clarify more the autoimmune mechanisms, neuroinflammation and cytokine production which affect and are crucial for the initiation and evolution of the disease.

As it has been mentioned already, the EAE model, mainly induced in C57BL/6 mice represents some important limitations. First of all, apart from the fact that this model provides evidence only for the chronic form of MS and not for the RR type, the main cell type which has been shown to display a pathological role is the CD4<sup>+</sup> T cells. Unlike human MS, in the case of EAE, the main area of the central nervous system where the inflammation and demyelination occurs is the spinal cord. MS is mainly a brain disease with demyelination of cerebral and cerebellar cortex. Finally, in human MS, we know that B cells can also be implicated in the pathological background via their production of autoantibodies, their professional function as antigen-presenting cells as well as their ability to produce pro-inflammatory cytokines such as IL-6. In C57BL/6 EAE model induced with a myelin peptide, the study of the role of B cells is not easy to be identified but instead, an induced model with the use of total myelin protein is suggested for studying the particular role of B cells<sup>189-193</sup>. That model helped also to investigate in more details the role of CD8<sup>+</sup> T cells in CNS autoimmunity and it has been confirmed by many studies that these cells can respond to myelin-antigens and to migrate to the site of inflammation triggering inflammatory responses. More recently, Ford and Evavold provided evidence for the exact epitope of MOG protein which can induce specific CD8<sup>+</sup> T cell responses in C57BL/6 mice<sup>194,195</sup>.

## **B) SJL/J R-EAE**

For a more particular study of the RR form of MS in mice, another mouse strain has been demonstrated to be the ideal one. It is called SJL/J and the course of EAE disease displays several remissions and relapses helping scientists to focus on the pathological characteristics

of this form of disease. This type of EAE is induced with the use of the immunodominant epitope of PLP (PLP<sub>139-151</sub>) and represents a better way to study the more usual type of human MS. More recently, Zamvil et al managed to induce EAE in SJL/J and PL/J mice demonstrating that an acetylated N-terminal-derived peptide from MBP can trigger the induction of EAE in PL/J mice. At the same period, Miller et al demonstrated that a relapsing EAE disease can be induced in SJL/J mice by the use of syngeneic homogenate from the spinal cord<sup>195-197</sup>. Despite the fact that during the first years after the discovery of EAE scientists were believing that the main myelin component that is able to induce EAE is MBP, and that the ability of PLP to induce EAE in SJL/J mice is because of a kind of contamination with MBP, today more and more evidence supports the idea that many independent myelin antigens share the capacity to induce EAE. For getting a RR-EAE, the most used way is by immunizing with PLP<sub>139-151</sub> in SJL/J mice. The first clinical signs appear between the 12<sup>th</sup> and 18<sup>th</sup> day after immunization and interestingly, relapses can be observed after recovery from the initial attack. The use of a myelin antigen in order to induce EAE is considered as an active way of induction.

In addition to that, EAE induction can be done also by a passive transfer of PLP<sub>139-151</sub>-T-cells to healthy, pertussis toxin (ptx)-injected non-EAE mice which can give rise to both chronic and RR-EAE, suggesting a mixture of disease course. The process of passive transfer is a little bit more complicated since we should immunize donor mice with PLP peptide and at the 10<sup>th</sup> day of disease, peripheral lymphoid cells are recovered to be restimulated in vitro with the peptide and then to be transferred to naive, ptx-injected healthy recipients. The same passive process can be followed also for the induction of a chronic type of EAE with the use of the MOG peptide.

The important role of pathogenic CD4<sup>+</sup> T cells at inducing EAE disease has been shown with the use of many experiments. In the case of the adoptive passive transfer, the contribution of autoreactive CD4<sup>+</sup> T cells has been demonstrated by their direct ability to induce EAE in healthy mice. In order to clarify the essential role of antigen-specific CD4<sup>+</sup> T cells for that process, the development of T cell receptor (TCR) transgenic mouse models has greatly facilitated the study of antigen-specific immune responses.

### III-3-2 Development of spontaneous EAE

In contrast to MS, which seems to occur in a spontaneous manner, the classic EAE model presented above has to be induced with substances that boost the immune system. To offer the possibility of studying disease aspects without confounding factors present in conventional EAE such as adjuvants, spontaneous EAE models were designed. RAG1-deficient H-2u mice, which only express T cells transgenic for MBP<sub>1-11</sub>, but no other lymphocytes, spontaneously develop chronic EAE. However, other lymphocytes are present (because the RAG1 gene is left intact), which is a more physiologic situation, but only 14% of the mice develop EAE<sup>198</sup>. In contrast, 50% incidence is achieved when using TCR<sup>MOG</sup> x IgH<sup>MOG</sup> double-transgenic mice (with a TCR recognizing MOG<sub>35-55</sub> and B lymphocytes producing antibodies that comprise the heavy chain of a demyelinating MOG-specific antibody). A spontaneously occurring atypical RR-EAE, rather resembling the “Western” variant of MS, is achieved with TCR<sup>1640</sup> x IgH<sup>MOG</sup> double-transgenic female mice on the SJL background, in which the TCR is specific for rodent MOG<sub>92-106</sub>. Herein, the acute phase was characterized by acute ataxia, followed by classic EAE symptoms during relapses<sup>199</sup>. A similar shift from atypical loss of balance and coordination, resulting in the animals rolling in an axial rotatory manner to typical EAE is observed when immunizing C3H/HeJ mice with PLP<sub>190-209</sub>. However, the disease course is monophasic in this model<sup>200</sup>. Atypical symptoms and lesion topology can also be achieved by adoptive transfer of MOG-specific Th17 cells into lymphopenic hosts<sup>201</sup>. Disease induced by adoptive transfer of MOG<sub>97-114</sub>-specific T cells into C3HeB/Fej (H-2k) mice additionally features proprioception defects, spasticity and hyperreflexivity<sup>202</sup>. Herein, lesions were found to be located not only in the spinal cord but also in the cerebellum and brain. Both two last studies pointed out that the Th17:Th1 ratio had to be greater than 1 to cause attack of the brain and thus induce atypical EAE<sup>203</sup>.

It is interesting to mention that recently, the group of VJ Kuchroo in the USA, taking advantage of TCR transgenic technology managed to generate two transgenic TCR lines (5B6 and 4E3) on SJL/J background that are specific for PLP<sub>139-151</sub> and both develop spontaneously EAE whereas Wekerle et al generated a MOG<sub>92-106</sub>-specific transgenic TCR line (TCR1640) which develops a spontaneous RR-EAE in SJL/J mice<sup>195,204-206</sup>.



### III-3-3 EAE induction in NOD mice

Several studies have demonstrated that the EAE disease can be induced also in the Non-Obese Diabetic (NOD) background of mice which are known to develop diabetes spontaneously, upon immunization with the MOG<sub>35-55</sub> peptide. These EAE mice display a relapsing-remitting course of disease which is followed by a chronic stage. Taking into account the fact that it is usually the case in human MS that patients with diagnosed RR-MS can display later a secondary progressive pathology, this animal model is very close to human course and can allow very effective clinical studies. The group of Kuchroo generated a CD4<sup>+</sup> TcR transgenic model for MOG<sub>35-55</sub> driven EAE on the NOD background and surprisingly, these mice are characterized by both CD4<sup>+</sup> and CD8<sup>+</sup> MOG<sub>35-55</sub> reactive T cells so it has been suggested to be a nice model for studying immune responses induced by both T cells. This animal model is called 1C6, and it has been shown that CD8<sup>+</sup> MOG<sub>35-55</sub> reactive T cells can induce EAE disease when transferred in lymphocyte-deficient NOD.Scid mice, even the fact that the incidence seems to be lower than this induced by CD4<sup>+</sup> T cells<sup>195</sup>.

### III-3-4 Pathology of EAE

EAE disease is considered as CD4<sup>+</sup> T-cell mediated disease since it has been described to be the main cell type able to induce EAE by adoptive transfer in naive-healthy ptx-pretreated mice. Different studies have provided evidence for the specific CD4<sup>+</sup> T cell types which play a crucial role in the initiation of disease and for the role of other immune cells with either effector or regulatory properties.

#### A) Effector CD4<sup>+</sup> T cells

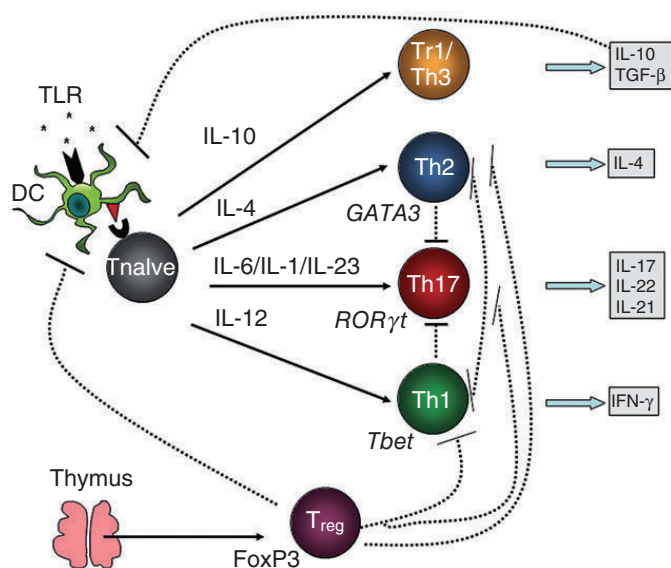
Naive T cells reside mainly in the secondary lymphoid organs. Once they recognized their cognate self-antigen in the context of MHC class II molecules expressed by the antigen-presenting cells (mainly by DCs) in the presence of the necessary co-stimulatory signals and cytokines secreted by other immune cells, CD4<sup>+</sup> T cells become activated and differentiate in a specific effector phenotype. Two main types of differentiated T helper cells have been firstly shown to participate as effector cells. Each one has a particular phenotype and cytokine signature. The first division has been made by Mosmann et al in 1986 providing evidence for the existence of IL-4-producing Th2 and IFN- $\gamma$ -producing Th1 cells<sup>207</sup>. Later on many studies focused on the phenotype of Th2 cells showing that these cells are also characterized by the production of multiple cytokines such as IL-5, IL-13 and IL-10 and are able to provide help to B cells in order to secrete IgG1 antibodies.

Th1 cells can be generated from naïve CD4<sup>+</sup> T cells but a crucial cytokine has been shown to favor the differentiation of naïve CD4<sup>+</sup> T cells into Th1 cells. IL-12 is a cytokine, produced by activated antigen-presenting cells (APCs), and its presence is considered to be an important molecular factor for the differentiation of naïve T cells in Th1 cells. In addition to IL-12, small amounts of IFN- $\gamma$  and activation of TCR signaling can induce the expression of the transcription factor T-bet which is upregulated in a Stat1 (signal transducer and activator of transcription 1) dependent manner. The expression of the transcription factor T-box transcription factor TBX21 (T-bet) is an important factor for the Th1 signature since its expression can induce the expression of IL-12 receptor (IL-12R) on T cells keeping them in a stable and effective status to respond to IL-12. As it has been mentioned already, the main cytokine produced by the Th1 cells, is IFN- $\gamma$ . That means that Th1 cells should be able to produce large amounts of IFN- $\gamma$  and in order to ensure this production, the transcription factor Stat4 has been shown to transactivate T-bet whose upregulation controls the production of IFN- $\gamma$ . IL-12 is also implicated in that process by regulating the expression of Stat4. All these factors and pathways are today known to be necessary in order to determine the commitment of T cells to the Th1 lineage<sup>208–210</sup>.

In the context of EAE and MS, as also in other autoimmune diseases like psoriasis and rheumatoid arthritis, it has been shown by a large number of studies that the adoptive transfer of Th1-reactive cells can induce the disease, so for many years, this cell type and its key cytokine IFN- $\gamma$  were considered to be the main pathological factors for some organ-specific autoimmune diseases. Additionally, IFN- $\gamma$  has been identified to be present in lesions of MS patients. The resistance of T-bet deficient mice (Tbx21<sup>-/-</sup>) to MOG<sub>35-55</sub>-induced EAE disease and the fact that administration of neutralizing antibodies against the p40 unit of IL-12 protected mice against EAE, had made scientists to get a strong conclusion that Th1 cells are the main effector T-cells responsible for the pathogenesis of MS and EAE<sup>211,212</sup>. In 1990, for the first time scientists started getting doubts about the unique crucial role of Th1 at inducing EAE. The establishment of knockout mice for ifng and ifngr as well as il12p35, provided evidence that these mice were not resistant to EAE but even more susceptible than the WT. A new era started at the immunological field of EAE. In 2000, it has been demonstrated that the already known p40 subunit of IL-12 is not only paired with p35 to form the active unit of IL-12 but also with another subunit which is called p19 that is responsible for the formation of a new cytokine, called IL-23. After this discovery, more attention had been given to the role of this cytokine, showing that despite the fact that IL-23 is unable to induce the differentiation

and/or commitment of Th1 cells, it triggers the production of the cytokine IL-17 by CD4<sup>+</sup> T cells. A stronger evidence for the role of IL-23 in EAE has been provided by Cua DJ et al in 2003, when they showed that mice deficient for IL23p19 (not able to produce IL-23 but they can still produce IL-12) were totally resistant to any model of induced autoimmunity, including EAE<sup>213</sup>. It was becoming increasingly clear that the role of IL-23/IL-17 had to be investigated in more details. An additional problem was that until that time, the main types of helper T cells were not known to be able to produce the IL-17 cytokine so it was not easy to explain how it is possible that this cytokine can play an important role in the pathogenesis of EAE. So, the idea of an additional, new one, T helper cell lineage (Th17) with IL-17 cytokine to be the signature cytokine for the differentiation from naïve cells and IL-23 to be the key cytokine, which determines the commitment of this new lineage, has been born. Th17 cells are found in the inflamed CNS of EAE and IL-17 is upregulated in MS lesions and in other sites of inflammation of autoimmune diseases<sup>214-217</sup>. Th17 differentiation, survival and expansion are dependent on a large variety of cytokines and transcription factors that work together to establish the commitment of Th17 cell lineage. Today, it is known that TGF- $\beta$  (transcription growth factor  $\beta$ ) can synergize with the cytokine IL-6 to generate Th17 cells. IL-6 has been shown to trigger the expression of IL-21, which, through a positive autocrine feedback loop induces more IL-21 production upregulating the expression of the IL-23 receptor alpha chain. In addition to these pathways, it has been demonstrated that Th17 cells can be induced also by IL-1 $\beta$  and IL-23 independently of TGF- $\beta$  and, they seem to be more pathogenic. Recently, it has been shown by two studies published back to back that IL-23 is necessary for the pathogenic nature of Th17 cells which can also display this effector role through the production of the following cytokines: IL-17F, IL-17A, IL-21, IL-22, IL-9, IFN- $\gamma$  and mainly GM-CSF. Concerning the involvement of transcription factors in the differentiation program of Th17 cells, RoR $\gamma$ t (orphan nuclear receptor  $\gamma$ ) has been shown to drive the expression of genes coding for IL-17A and IL-17F among others, but also STAT3, interferon regulatory factor 4 (IRF4), aryl hydrocarbon receptor (AHR), Notch and BAFF. It has been shown that STAT3 can be activated by IL-6 and IL-23 which upregulates the expression of RoR $\gamma$ t whereas it is known that IRF4 is related to RoR $\gamma$ t since IRF4 deficient mice display impaired RoR $\gamma$ t expression and are unable to produce IL-21 and to induce the expression of IL-23R<sup>214-216,218-224</sup> (**Figure 13**).

Today, in addition to Th1, Th2 and Th17, two other helper cell subsets have been identified. Th9 cells are characterized by the production of the cytokine IL-9 and are induced by TGF- $\beta$  plus IL-4 and produce high levels of IL-10 but very few of IL-17 or IFN- $\gamma$ . The effector functions of Th9 are related to models of allergic diseases and it has been shown that these cells are implicated in the progression of asthma through mast cell responses<sup>225,226</sup>. The role of Th9 cells has been studied also in the EAE model. The group of Rostami has shown that IL-9 is important for the T-cell activation and differentiation in autoimmune inflammation of the central nervous system and that IL-9<sup>-/-</sup> mice are more resistant to EAE in comparison with WT mice<sup>227</sup>. More recently, Elyaman et al provided additional evidence for the factors, which are responsible for the induction of IL-9, showing that Notch and Smad3 cooperate to induce IL-9 and participate in regulating the immune responses<sup>228</sup>. The last type of T helper cells, Th22 is characterized mainly by the production of IL-22, IL-26 and IL-13, of which the IL-22 is the most important functional cytokine. The phenotype of Th22 is promoted by the presence of IL-6 and TNF- $\alpha$  along with the help of plasmacytoid dendritic cells<sup>229</sup>.



**Figure 13.** The differentiation and regulation of CD4<sup>+</sup> T cell subsets<sup>230</sup>. *Fletcher JM et al, 2010*

## B) Th17 Cytokines

### B1) Interleukin 17 (IL-17)

IL-17 is considered to be the most important functional cytokine of Th17 cells, the main pathogenic cell subset in the context of EAE and MS. IL-17A (IL-17) was soon identified as one of the cytokines produced by T cells in response to IL-23<sup>231</sup>. The increased frequency of IL-17 secreting cells in myelin immunized mice led to the conjecture that they constitute the critical effector population in autoimmune demyelinating disease. This was supported by the demonstration that IL-23 polarized, IL-17 secreting myelin-reactive CD4<sup>+</sup> T cells are highly encephalitogenic following transfer into naive syngeneic recipients<sup>232,233</sup>.

Additional experiments have indicated that the encephalitogenicity of IL-23 polarized cells is not solely attributable to the production of IL-17 itself or of other cytokines traditionally ascribed to the Th17 panel. The course of EAE is unperturbed, or only ameliorated, in IL-17A knock-out mice and in wild-type mice treated with neutralizing antibodies specific for IL-17A<sup>234,235</sup>. Conversely, ectopic expression of IL-17A did not worsen EAE<sup>236</sup>. Genetic targeting of other Th17 associated cytokines, such as IL-17F or IL-22, also failed to confer resistance to the disease<sup>237</sup>. The dispensability of IL-17A is not explained by redundant activities of IL-17F, since mice treated with an IL-17 receptor-Fc fusion protein, that blocks both cytokines, only had a modest impact<sup>235</sup>. Furthermore, IL-17F knock-out mice succumb to EAE, despite treatment with an IL-17A neutralizing antibody reduces disease severity<sup>236</sup>.

Based on the above discussion, it could be argued that it is more appropriate to view IL-17 as a surrogate marker of IL-23 modulation, rather than as a master effector cytokine, in the context of CNS autoimmunity<sup>238</sup>. This does not mean that IL-17 is completely irrelevant to the pathogenesis of inflammatory demyelinating disorders. Several lines of evidence indicate that, in certain instances, IL-17 facilitates leukocyte trafficking across the blood–brain barrier (BBB) and infiltration within the CNS. In some EAE models, CNS infiltrates induced by myelin-reactive Th1 cells are confined to the subpial white matter, whereas those induced by Th17 cells reactive to the same epitope extend into the parenchyma in finger-like projections<sup>232</sup>. Kebir and colleagues reported that human Th17 cells migrate across BBB endothelial cell monolayers more efficiently than Th1 cells and that IL-17 and IL-22 directly stimulate CNS endothelial cells to express chemokines and downregulate tight junction proteins<sup>239</sup>. IL-17 also stimulates astrocytes to produce ELR<sup>+</sup> CXC chemokines that could, in turn, attract neutrophils to the BBB and activate them to release vasoactive substances<sup>240</sup>.

Leukocyte infiltration of the brainstem, as opposed to the spinal cord, appears to be particularly dependent on IL-17 and impeded by IFN- $\gamma$ <sup>241</sup>. The biological basis for this regional difference in IL-17 dependence is unknown.

## **B2) Granulocyte-macrophage colony stimulating factor (GM-CSF)**

Th17 is considered today to be the crucial T-cell lineage for the pathogenesis of many organ-specific autoimmune diseases. Similar to the other subsets of helper T-cells, Th17 is mainly characterized by the ability to produce some signature cytokines such as IL-17, IL-22 etc. Granulocyte monocyte colony stimulating factor (GM-CSF) is an inflammatory cytokine whose production has been shown to be IL-23 driven. Traditionally, it is known to induce the proliferation and differentiation of macrophages, monocytes and granulocytes. GM-CSF can display its effects through different signaling pathways such as the Jak-stat, the MAP-kinase and the PI3K (phosphoinositol-3-kinase) pathway, following its binding to a heterodimeric receptor which is composed of the common signal-transducing subunit  $\beta$  with the receptors for IL-3 and IL-5, and a GM-CSF specific  $\alpha$ -chain as well. Actually, in 2011, two studies published back to back in Nature Immunology by the group of Rostami A and Becher B demonstrated the pathogenic role of GM-CSF at inducing EAE, suggesting that the transcription factor RoR $\gamma$ t is also responsible for upregulating the expression of GM-CSF and that T cells which lack the expression of cytokines IFN- $\gamma$  and IL-17 but are still able to produce GM-CSF keep their capacity to induce EAE whereas T cells that can not produce GM-CSF lose their ability to induce CNS inflammation, despite the fact that they still produce IL-17 and IFN- $\gamma$ <sup>242,243</sup>. The group of Bernard had provided the first evidence for a potential pathogenic role of GM-CSF in the context of EAE in 2001 showing that administrated injections of recombinant GM-CSF, following active immunization with myelin antigens can induce EAE in mice deficient for GM-CSF, which are normally resistant to that disease. Concerning an insight in the mechanism of action of this cytokine, it has been shown that GM-CSF can trigger the mobilization of a subset of monocytes with CD11b<sup>+</sup>Ly6C<sup>hi</sup> phenotype into the circulation and later inside the CNS where they can act as CD11c<sup>+</sup>MHC-II<sup>+</sup> professional dendritic cells, promoting the activation of Th17 cells by the production of IL-6 and IL-23<sup>244-247</sup>.

### **B3) Interleukin 23 (IL-23)**

As it has been described already, IL-23 cytokine is a key cytokine for the differentiation and commitment of the pathogenic Th17 cells. Recently, several studies support the idea that despite the fact that IL-23 is necessary for inducing the expression of IL-17, IL-23 can promote EAE independently of IL-17. IL-23 is required for the induction of GM-CSF, which has been shown to be the crucial pathogenic cytokine in the context of EAE. However, IL-23R signaling seems to be necessary for the accumulation of the pathogenic effector T cells inside the CNS but it is still unknown if this receptor is required for the upregulation of chemokine receptors and adhesion molecules needed for the homing of Th17 cells into the CNS or controls their retention once they have migrated to the site of inflammation. More recently, an additional role of IL-23 has been shown in synergy with the cytokine IL-1 $\beta$ . Sutton CE et al demonstrated that innate  $\gamma\delta$  cells, which express IL-23R and the transcription factor ROR $\gamma$ t, produce the inflammatory cytokines IL-17, IL-21 and IL-22 in response to IL-1 $\beta$  and IL-23 even without engagement of T-cell receptor. These activated  $\gamma\delta$  cells are found in the brain of EAE mice and they may suggest an alternative way by which IL-1 and IL-23 mediate CNS inflammation<sup>241,248–251</sup>.

### **B4) Interleukin 21 (IL-21)**

IL-21 is an inflammatory cytokine known to be produced by activated CD4<sup>+</sup> T cells (mainly Th17 cells), follicular T helper cells and Natural Killer T cells (NKT). It belongs to the IL-2 family of cytokines, and its receptor, IL-21R, shares common chains with receptors for other cytokines such as IL-2, IL-4, IL-7, IL-9 and IL-15 but it has also its own specific receptor  $\alpha$  chain. The interaction of IL-21 with its receptor IL-21R activates Jak1 and Jak3 pathways which are responsible for the signal transduction via Stat1 and Stat3. Different roles have been proposed for IL-21 either at inducing effector properties or providing signals for the regulatory phenotype of a type of B cells<sup>252</sup>. IL-21 can activate NK cells by inducing the expression of killer inhibitory receptor and the production of IFN- $\gamma$ <sup>253,254</sup>. As it has been mentioned already, IL-21 is a cytokine produced in large quantities by Th17 cells and it has been shown to act through an autocrine mechanism in order to keep stable the rate of Th17 cells generation and commitment through a synergy with TGF- $\beta$ <sup>219,221</sup>. Among effector B cells, IL-21 is known to be involved in the regulation of immunoglobulin production and differentiation of B cells into plasma cells regulating the expression of two factors important for that process, Blimp-1 and Bcl6<sup>249,250</sup>. In the context of EAE, Vollmer et al have shown

that IL-21 administration before MOG<sub>35-55</sub>-induction of EAE enhanced the inflammation into the CNS through boosting NK cell function and inducing IFN- $\gamma$  secretion whereas its administration had no similar effect after EAE had progressed<sup>257</sup>. More recently, Raveney et al demonstrated that the pathogenic T cells which are infiltrated into the CNS in order to induce inflammation are characterized by the expression of the nuclear receptor NR4A2 that is necessary for activating IL-21 signaling<sup>258</sup>. Although IL-21 seems to be a crucial pathogenic cytokine for the development of EAE disease, mice deficient for its receptor IL-21R have been shown to display more severe clinical scores. Liu R et al have reported that the impact of IL-21R deficiency was associated with a defect of CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells and a downregulated expression of Foxp3 suggesting a temporal influence of IL-21 on the activity of immunoregulatory cells. These observations are in accordance with a study by Piao WH et al who demonstrated that in the RR-EAE animal model (SJL mice) the blockade of IL-21 either after or before the induction of the disease, enhanced the inflammation into the CNS associated with a defect in the Tregs which lost their capacity to prevent EAE<sup>259,260</sup>.

### C) CD8<sup>+</sup> T cells in EAE

CD8<sup>+</sup> T cells are considered as an additional type of effector T cells which has been shown to be highly implicated in the pathogenic pathways altered in EAE. More attention had been focused on the role of CD4<sup>+</sup> T cells because of the fact that susceptibility to MS is associated with MHC-II genes and additionally that a subset of TCR V sequence genes that had been detected within MS patients expressed TCR sequence motifs similar to peripheral CD4<sup>+</sup> T cells. The observation that depletion of CD4<sup>+</sup> T cells in MS patients doesn't lead to complete recovery, suggested the hypothesis of the implication of other cells at mediating EAE disease<sup>261</sup>. Abdul-Majid KB et al demonstrated<sup>262</sup> that induction of EAE with MOG<sub>1-125</sub> in CD8-deficient DBA/1 mice resulted in a reduction in demyelination and CNS inflammation as compared with wild-type animals<sup>262</sup>. CD8<sup>+</sup> T cells have been shown to be enriched in the CNS where the inflammation and demyelination occurs and CD8<sup>+</sup> T cell clones specific for myelin antigens have been isolated from the periphery of MS patients. Concerning their mechanism of action, it is believed that CD8<sup>+</sup> T cells can display their effector properties through different ways. It is known that they have the possibility to kill target cells and contribute to the death of several cells of the CNS such as oligodendrocytes, astrocytes, microglia and neurons. In 2001, for the first time, the group of J Goverman demonstrated that MBP-specific CD8<sup>+</sup> T cells isolated from WT mice can mediate CNS autoimmunity through the production of IFN- $\gamma$  and display different clinical signs in comparison with CD4<sup>+</sup> T-cell

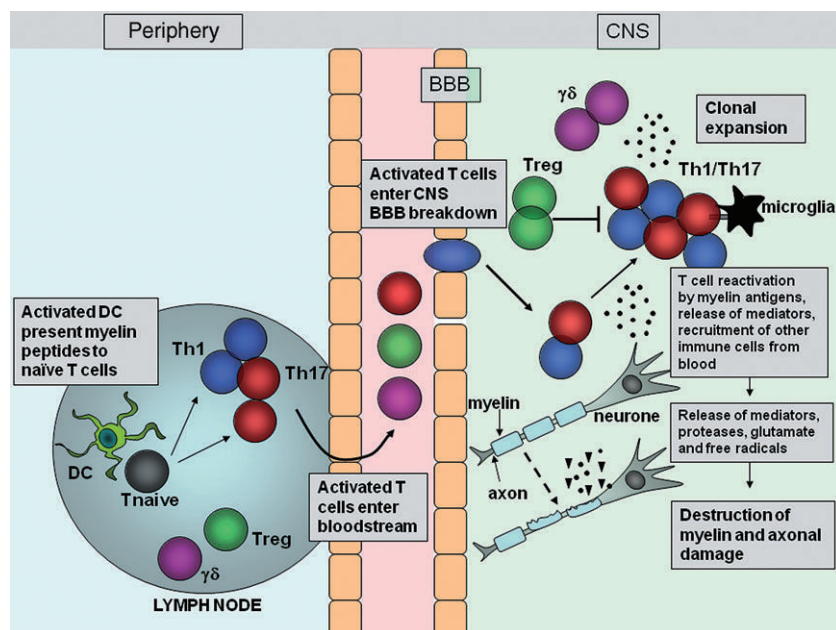


mediated CNS autoimmunity<sup>261,263</sup>. A possible cytotoxic effect by CD8<sup>+</sup> T cells has been shown by the presence of neutrophils and necrotic cell damage in a context of CD8-induced EAE. A recent study from Saxena et al demonstrated the contribution of CD8<sup>+</sup> T cells in the loss of oligodendrocytes. Transfer of preactivated hemagglutinin-specific CD8<sup>+</sup> T cells to a mouse model where Ag influenza hemagglutinin is expressed only in oligodendrocytes led to microglia activation, loss of oligodendrocytes and demyelination, suggesting that CD8<sup>+</sup> T cells can induce in vivo lysis of oligodendrocytes and can be considered mediators of CNS autoimmunity<sup>264</sup>. Interestingly, Leuenberger T et al showed that CD8<sup>+</sup> T cells are not able to play either a proinflammatory or regulatory role in the absence of CD4<sup>+</sup> T cells despite the fact that CD8<sup>+</sup> T cells are accumulated inside the CNS<sup>265</sup>. In order to display their effector functions, naive CD8<sup>+</sup> T cells can differentiate in different subtypes of effector CD8<sup>+</sup> T cells. This activation process, similar to what already had been mentioned for CD4<sup>+</sup> T cells, requires the recognition of peptide:MHC class I complexes on APCs, presence of cytokines and co-stimulatory molecules provided by CD28-B7. For a lineage commitment to CD8<sup>+</sup> Tc1 cells, characterized by the production of IFN- $\gamma$ , T-bet should be expressed whereas IL-12 production by APCs is also essential for inducing the differentiation of naive CD8<sup>+</sup> T cells in Tc1 cells. Gata4 is induced by IL-4 to trigger the differentiation of Tc2 cells. Similar to CD4<sup>+</sup> Th17 cells, a subset of CD8<sup>+</sup> Tc17 cells can be generated through the expression of the transcription factor Ror $\gamma$ t which is induced by TGF- $\beta$ , IL-23, IL-6 and IL-21 and induces the activation of STAT3 signaling pathway<sup>261</sup>. An important factor for the generation either of Th17 or Tc17 cells is the IRF4. In a recent study, Huber et al demonstrated that mice deficient for IRF4 are resistant to EAE disease and the adoptive transfer of WT CD8<sup>+</sup> T cells could give rise to Tc17 cells residing in the periphery but were unable to infiltrate the CNS. Adoptive transfer of a small number of CD4<sup>+</sup> T cells was also unable to induce the disease but when they were transferred together with CD8<sup>+</sup> T cells, EAE could be induced and Th17 cells were accumulated in the CNS. These observations suggested that the secretion of IL-17 by Tc17 cells was required for the infiltration, accumulation and induction of CNS-autoimmunity by CD4<sup>+</sup> Th17 cells<sup>266</sup>. CD8<sup>+</sup> T cells can act as effector cells through cytotoxic or non-cytotoxic mechanisms. It is known that the largest part of their cytotoxicity is mediated by the expression of granzyme, perforin and FasL (CD95). In addition, they can also produce pro-inflammatory cytokines such as IFN- $\gamma$  and TNF- $\alpha$  enhancing inflammation<sup>267-269</sup>. Interestingly, today, it has been shown that a specific epitope of the MOG-peptide, the MOG<sub>42-50</sub>, can selectively induce a CD8<sup>+</sup> T cell response, binding to H-2D<sup>b</sup> molecule<sup>194</sup>. The field of CD8<sup>+</sup> T cells in the context of CNS autoimmunity needs to be further investigated

since new evidence suggests that in the neurons, there is a large quantity of MHC-I peptides<sup>270</sup>.

#### **D) T-cell trafficking in the CNS**

The CNS is considered as an immune-privileged site against the attack of immune cells since it can efficiently control the entry of immune cells across its barriers to the perivascular and subarachoid spaces and can be well protected in physiological conditions. It has been shown by many studies that antigenic material including foreign tumors, tissue grafts, bacteria, viruses and viral vectors can't pass through the Blood Brain Barrier (BBB) to induce inflammatory responses. In the context of MS and its animal model of EAE, an essential step for the induction of the disease is the breakdown of the Blood Brain Barrier (BBB) and the infiltration of activated or not immune cells inside the CNS where they display their inflammatory properties attacking myelin proteins and triggering demyelination. EAE, as T-cell mediate disease requires the homing of T cells from the lymphoid organs where they reside, to the CNS. Leukocyte extravasation from the bloodstream is a multistep process that depends on several factors including fluid dynamics within the vasculature and molecular interactions between circulating leukocytes and the vascular endothelium (**Figure 14**). An important step is the presence of chemokines on the vascular endothelial cell surface. After being activated upon recognition of an antigen from DCs, activated helper T-cells start expressing chemokine receptors, receptors for adhesion molecules and other factors that enhance their tendency to move inside the CNS. However, within the CNS, infiltrating leukocytes that cross the endothelium face additional boundaries to parenchymal entry, including the abluminal presence of localizing cues that prevent egress from perivascular spaces<sup>271</sup>.

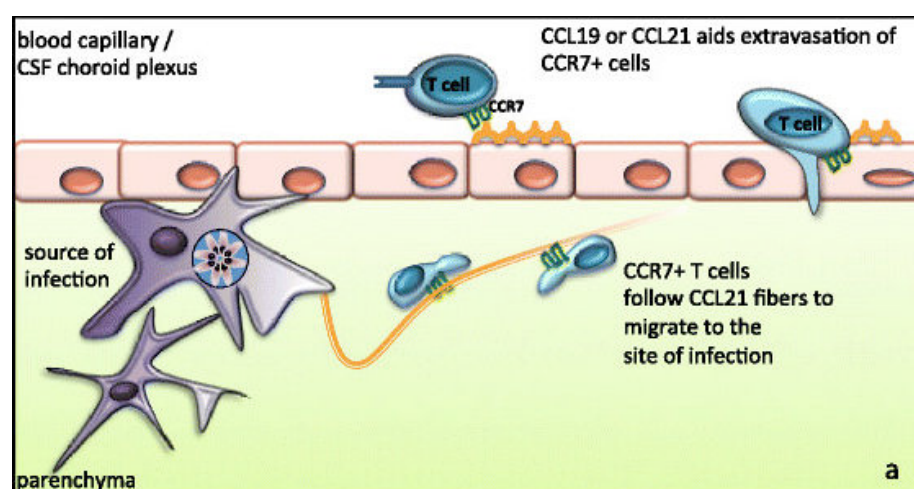


**Figure 14. Migration and effector function of T cells in the central nervous system (CNS) during experimental autoimmune encephalomyelitis (EAE).** After immunization with myelin antigens, complete Freund's adjuvant (CFA) and pertussis toxin, dendritic cells (DC) are activated in the lymph nodes by Toll-like receptor (TLR) agonists within the mycobacterium tuberculosis component of CFA, and present myelin antigen to naive T cells. The activated myelin-specific T cells enter the bloodstream and traffic to and enter the CNS. Breakdown of the blood–brain barrier (BBB) occurs, allowing recruitment of other inflammatory cells into the CNS. T cells entering the CNS encounter their cognate myelin antigens and become reactivated by local APC. T cells expand and release inflammatory mediators which help recruit other immune cells to the site of inflammation. Activation of local microglial cells and infiltrating cells results in production of proteases, glutamate, reactive oxygen species and other cytotoxic agents which promote myelin breakdown. Damage to the myelin sheath surrounding axons is followed by axonal damage and neurological impairment<sup>230</sup>. Fletcher JM et al, 2010

In the case of inflammation, endothelial cells display an upregulation in terms of the expression of several adhesion molecules, chemokines, selectins and integrins which interact with effector T cells expressing corresponding receptors, determining the type of T cells that has to move to a particular site of inflammation to display its functions. Integrin mediated adhesion of circulating lymphocytes to the vascular endothelium is followed by crawling and extravasation across the vascular wall. This process has been shown to be controlled by different proteins such as Platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) whereas its blocking causes leukocytes to accumulate at the apical side of the endothelium or on the abluminal side between the endothelium and the basement membrane<sup>272</sup>. CD99 expression is also necessary for leukocytes to leave from the luminal endothelial cell area through the paracellular pathway<sup>273,274</sup>. Other molecules, such as the junctional adhesion

molecules (JAMs), ESAM, and VE-cadherin are expressed exclusively at endothelial cell junctions and may contribute solely to paracellular transmigration. Within the CNS, the entry of leucocytes across the endothelium is mediated via the trans-cellular pathway, leaving interendothelial cell junctions intact because of the specialization of the endothelial tight junctions at the BBB. T-cells can interact with the vascular wall either by rolling or immediate capture and the T-cell transmigration occurs exclusively at the levels of the post-capillary venules<sup>275</sup>. During inflammation, vascular endothelium in most tissues upregulate E- and P-selectin and chemokine ligands of chemokine receptors expressed by cells of both innate and adaptive immune system. The expression of inflammatory chemokines in human or murine BBB including CCL2, CCL4 and CCL5 are highly implicated in the interaction with activated mononuclear cells expressing their chemokine receptors CCR1, CCR2 and CCR5 suggesting a role in the induction of EAE<sup>276-278</sup>. Vajkoczy et al showed that T cells interact with the spinal cord white matter microvasculature and that alpha4-integrin is required for the G-protein-dependent adhesion of T cells to microvascular VCAM-1<sup>279</sup>. More recently, CCR6 has been considered as one of the most important chemokine receptors for migration of T cells through the choroid plexus since CCR6<sup>-/-</sup> mice are resistant to EAE, with no accumulation of activated Th17 cells inside the CNS whereas the transfer of myelin-specific T-cells of wild-type mice into CCR6<sup>-/-</sup> mice reconstituted disease. CCR6 is a chemokine receptor for the chemokine CCL20 which has been shown to be highly expressed by epithelial cells of the choroid plexus<sup>250,280</sup>. CCR6 has been shown to be expressed also by B cells and Treg cells so the detailed conditions under which CCR6 affects the EAE course should be investigated further. Another chemokine that has been shown to play a critical role for the migration of T-cells in the CNS in the case of neuroinflammation is the CCR7. CCR7 interacts with its two ligands CCL19 and CCL21. CCL19 and CCL21 are known as homeostatic, or lymphoid, chemokines due to their role in guiding B-cells, naïve T-cells and mature dendritic cells (DCs) into lymphoid tissue under physiological conditions<sup>281,282</sup>. While several chemokines were expressed in parenchymal tissues and inflammatory cells during EAE, only CCL19 mRNA transcripts were expressed on the endothelial cells of post-capillary venules in normal as well as EAE brains and spinal cords. Additionally, CCL21 transcripts were induced at post-capillary venules during EAE whereas the infiltrated T-cells inside the CNS have been shown to be positive for the expression of CCR7. Interestingly, CCL19 mRNA levels in CNS tissue were found to increase with EAE progression while CCL21 levels were very low<sup>283</sup>.

The expression of these lymphoid chemokines in the CNS during EAE led to the idea that CCL19 and to a lesser extent CCL21, may function to signal circulating lymphocytes that would normally home to the lymphoid tissues. During neuroinflammation, the expression of CCL19 and CCL21 would help to retain CCR7<sup>+</sup> T-cells within the CNS and favor interaction with mature DCs suggesting that the interactions CCL19-CCR7 may contribute to the entry, local retention of memory T-cells and maintaining cycles of neuroinflammation (**Figure 15**). Kivisakk et al examined the expression of CCR7, CCL19 and CCL21 in brain autopsies and CSF samples from MS patients<sup>284</sup> finding large numbers of CCR7<sup>+</sup> cells in the inflammatory cuffs of MS lesions, expressing also MHC-II and CD86, indicating antigen presenting capacities and that these cells may be maturing DCs since CD3<sup>+</sup> T-cells in acute and chronic MS lesions did not express CCR7 in contrast with the CSF of MS patients where the majority (about 90%) of T-cells expressed CCR7. In humans, CCL19 and CCL21 expression levels have been determined in tissue samples of patients with neuroinflammation including MS whereas evidence has been provided also for a potential role of CCR7 to recruit other types of immune cells to the CNS including B-cells which also express the chemokine receptor CCR7<sup>271,285</sup>.



**Figure 15. Model proposing the role of CCL19/CCR7 during neuroinflammation.** *CCL19 and CCL21 expression at the blood–brain barrier (BBB) may aid extravasation of CCR7<sup>+</sup> leukocytes.* Noor S, *J Neuroinflammation*, 2012

Additionally, the role of the chemokine CXCL12 has been shown to be highly involved in the homing process of T lymphocytes inside the CNS. CXCL12 or stromal cell-derived factor (SDF-1) is a chemoattractant for monocytes and lymphocytes and endothelial cells of the CNS express it. The receptor of CXCL12, CXCR4, has been detected to be expressed in numerous types of cells including astrocytes, microglia, oligodendrocytes and endothelial

cells of the BBB<sup>286–288</sup>. Interestingly, some studies have shown that in the endothelium of the BBB, there is a source of the chemokine ligand CXCL12 as well as its receptor, suggesting a role for CXCL12 in recruiting CXCR4<sup>+</sup> circulating lymphocytes. Krumbholz et al provided evidence for the expression of CXCL12 protein along the blood vessels of the BBB as well as in astrocytes in both inactive and active MS lesions. CXCL12 expression was mainly localized to the vasculature of the spinal cord along the abluminal surface of the endothelial cells of the BBB in mice 10 days post-immunization. At the peak of the disease, CXCL12 polarity is lost and its localization shifts toward a more luminal expression pattern, which was associated with dense perivascular infiltrates of CXCR4 positive mononuclear cells. Interestingly, the use of a CXCR4 antagonist, AMD3100, during the induction of EAE led to significant worsening of disease associated with more widespread parenchymal infiltration, increased demyelination and larger lesion areas suggesting that abluminal expression of CXCL12 at the CNS vasculature is important in restricting the parenchymal access of CXCR4-expressing immune cells<sup>289</sup>. Recently, it has been shown that the expression of CXCL12 is related with the role of the pro-inflammatory cytokine IL-1 $\beta$ . Injection of mice with IL-1 $\beta$  induces pathologic relocation of CXCL12 at the BBB while injection of TNF- $\alpha$  does not.

Except for these chemokines, other factors have been shown also to participate in the homing process such as the adhesion molecule p-selectin and the integrins  $\alpha$ 4 $\beta$ 1 and LFA-1 since antibodies for  $\alpha$ -integrins can block the migration of T-cells through the BBB<sup>290</sup>. Finally, many studies have focused on the role of the sphingosine-1 phosphate, a lysophospholipid, since it has been shown to play a crucial role for the T-cell trafficking inside the CNS. Based on that, an oral treatment for RR-MS patients is already in use, the FTY720 (fingolimod) which seems to act through an active phosphorylated metabolite, FTY72-P that resembles S1P and alters lymphocyte trafficking through the blocking of S1P1 receptor<sup>291</sup>. Interestingly, one study from J Govermans' lab showed that using an agonist specifically for S1P1 receptor (AUY954) can ameliorate the EAE incidence indicating an important role of plasmacytoid DCs which can migrate to the CNS and are necessary for the protective effect of AUY954<sup>292</sup>. More recently, Garris CS et al showed that the phosphorylation of S1p1 is necessary to avoid Th17 polarization and exacerbation of CNS-inflammation<sup>291</sup>.

## E) T regulatory cells

### E1) CD4<sup>+</sup> T regulatory cells

The existence of populations, which can negatively regulate autoimmune responses, is one of the most important discoveries in the field of cellular therapies against autoimmune diseases, participating in the immune tolerance. Today, we can distinguish two main types of T regulatory populations, natural and inducible Tregs. The first one is characterized by the expression of the molecule CD25 at the surface and the transcription factor forkhead box P3 (FoxP3) in the nucleus. The expression of Foxp3 by the Tregs has been shown repetitively by several studies to be essential for them to display their regulatory functions. In the thymus, Foxp3 expression seems to start at the late double-positive stage whereas CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs are detected in mice from day 3 after birth, an observation in accordance with the finding that thymectomy on day 3 after birth produces organ-specific autoimmune disease which can be prevented by inoculation of CD4<sup>+</sup>CD25<sup>+</sup> natural Tregs<sup>293,294</sup>. The generation of natural Tregs is a result of positive selection of CD4<sup>+</sup> T cells with high affinity for self-antigens in the thymus whereas the inducible Tregs are derived in the periphery upon response to foreign antigens. Additionally, mutations of the *foxp3* gene can lead to the deficiency of nTregs and development of inflammatory/autoimmune disorders in mice and humans. Interestingly, ectopic expression of Foxp3 in naïve T cells is sufficient to convert them into functionally nTreg-like cells upregulating the expression of Tregs-associated molecules such as CD25, cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR)<sup>295</sup>. It has been proposed that expression of Helios, an ikaros family transcription factor, may specifically identify nTregs, allowing specific tracking of Tregs from different origins in health and diseases<sup>291</sup>. More recently, several studies support that Helios<sup>-</sup> can be readily identified within naïve (CD45RA<sup>+</sup> CD31<sup>+</sup> CCR7<sup>+</sup> CD62L<sup>+</sup>) Foxp3<sup>+</sup> Tregs, suggesting a finding inconsistent with the notion that lack of Helios expression identifies mainly Ag-experienced iTregs that should express also memory markers<sup>297</sup>.

The inducible Treg cells contain different T cell populations such as T regulatory-1 (Tr1), Th3 and subsets of CD8<sup>+</sup> T cells with suppressive properties and they are mainly characterized by displaying their function through the production of anti-inflammatory cytokines such as IL-10 and TGF- $\beta$ . It is difficult some times to distinguish the different regulatory populations because firstly, the regulatory function of nTregs is also cytokine-dependent and additionally, the expression of CD25 is upregulated in effector T cells, so it has been proposed that the use of the CD127 can be considered as an additional molecule

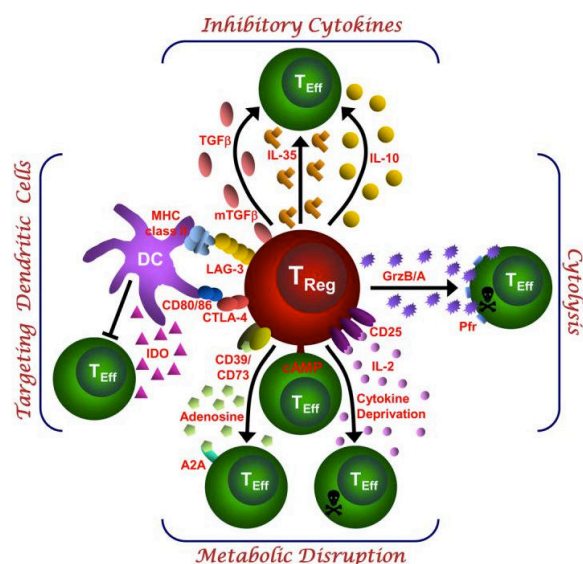
helpful for a more clear identification of nTregs<sup>298</sup>. Many studies have showed that in MS patients there is impairment in Treg numbers and function<sup>299-301</sup>. Recently, Fletcher JM et al provided evidence for the existence of a new phenotype of CD39<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs which can suppress the production of inflammatory cytokines IFN- $\gamma$  and IL-17 and that this subset seems to be impaired in patients suffering from RR-MS<sup>302</sup>. In the context of EAE mice, either the use of mice deficient for Tregs or an adoptive transfer of this population demonstrated their capacity to protect against the development of EAE. Khom AP et al showed more specifically the therapeutic effect of CD4<sup>+</sup>CD25<sup>+</sup> T cells after an adoptive transfer in MOG-induced EAE mice and at the same time, Zhang X et al performed a study demonstrating that this protective property is IL-10 dependent. The protective role of CD4<sup>+</sup>CD25<sup>+</sup> Tregs through the production of either IL-10 for the case of nTregs or TFG- $\beta$  for inducible Tregs has been shown with the use of Tregs from mice deficient for the production of these anti-inflammatory cytokines, which are unable to keep their capacity to suppress the autoimmune responses.

An additional experimental protocole, which enhanced the role of T regulatory cells, was by the use of the anti-CD25 monoclonal antibody (PC61) which preferentially depleted Tregs, showing that the presence of Tregs influences the threshold for disease development<sup>304</sup>. T regulatory cells have been shown to be present in both periphery and CNS where they can display their protective properties. McGeachy et al showed that in case of CNS-inflammation, the main producer of IL-10 inside the CNS is the CD4<sup>+</sup>CD25<sup>+</sup> T cells, displaying potent suppressive capacity also in vitro<sup>305</sup>. Interestingly, during a recovery phase of EAE, there is a large number of Tregs accumulating inside the CNS, and of those, one in three can produce IL-10 and express the molecule CD103 which may define a specific subpopulation or just a marker of activation<sup>306</sup>. Different factors have been shown to influence the function and the numbers of T regulatory cells such as the PDCD5 molecule which increases acetylation of Foxp3 in synergy with Tip60 or administration of SM934 which increases Tregs and can induce suppression of Th17 and Th1 immune responses<sup>307</sup>. Additionally, vitamin D3 can induce IDO<sup>+</sup> DCs able to produce IL-10 and TNF- $\alpha$ , decrease MHC-II and CD80 and trigger the expansion of Tregs whereas the role of pituitary adenylate cyclase-activating polypeptide has been shown to control the balance in the number of Tregs as well<sup>309</sup>. Tregs have been shown to display their suppressive effects either by the secretion of anti-inflammatory cytokines or through cell-to-cell contact-dependent mechanisms. Tregs are characterized by the expression of several molecules such as CTLA-4 (CD152) and lymphocyte-activation



gene 3 (LAG3) that together with the expression of costimulatory molecules expressed by APCs contribute to the contact-dependent suppressive mechanism<sup>310</sup>. There is strong evidence that CTLA-4 expression by natural Tregs has a key role in Treg-mediated suppression since CTLA-4 blockade in normal mice leads to the development of organ-specific autoimmune diseases similar to those induced by Treg depletion. Additionally, blockade of CTLA-4 by anti-CTLA-4 monoclonal antibody abrogates in vitro CD4<sup>+</sup>CD25<sup>+</sup> Treg-cell mediated suppression<sup>295</sup>. Signals to Tregs through interaction with the CD80 and CD86 molecules on APCs have been shown to activate Tregs to exert suppression. CTLA-4 expressed on Tregs can trigger the induction of the enzyme indoleamine 2,3-dioxygenase (IDO) in DCs by interacting with their CD80 and CD86 since IDO catalyzed the conversion of tryptophan into kynurenine and other metabolites, which have potent immunosuppressive effects by inducing de novo generation of Tregs<sup>311</sup>. In addition, due to the fact that CTLA-4 upregulates the expression of lymphocyte function-associated antigen-1 (LFA-1), one mode of contribution of CTLA-4 to Treg-mediated suppression might be that it augments the physical interaction between Tregs and APCs, thus enhancing the activation of Tregs or their suppressive interaction with other T cells or APCs<sup>312</sup>. Lymphocyte activation gene 3 (LAG3, also known as CD223) is a CD4-associated adhesion molecule that binds to major histocompatibility (MHC) class II molecules, is expressed at the surface of Tregs upon activation and it has been shown that anti-LAG3 antibodies abrogate Treg suppression and the suppressive capacity of Tregs from LAG3<sup>-/-</sup> mice is impaired in vitro as well<sup>310</sup>.

Interestingly, Collison LW et al have provided evidence for the existence of a population of T regulatory cells, which can mediate its suppressive function not via the cytokines IL-10 and TGF- $\beta$  but through the secretion of IL-35. They called this population iT<sub>R</sub>35 cells. iT<sub>R</sub>35 cells did not require the expression of Foxp3 for their development and have been shown to be strongly suppressive in vitro whereas in vivo they need a cytokine milieu of IL-10 and IL-35 for their generation<sup>313</sup>. More recently, a study by Liu Y et al reported the identification of new population of Tregs, developing in the CNS in response to autoimmune inflammation and is characterized by the phenotype of CD4<sup>+</sup>FoxA1<sup>+</sup>CD47<sup>+</sup>CD69<sup>+</sup>PD-L1<sup>hi</sup> Foxp3<sup>-</sup>. The adoptive transfer of these cells conferred protection to EAE mice in a FoxA1- and PD-L1-dependent manner. The development of this population is induced by interferon- $\beta$  and requires T-cell intrinsic IFN- $\alpha/\beta$  receptor signaling. Interestingly, it was demonstrated that in a case of relapsing-remitting multiple sclerosis, clinical response to treatment with IFN- $\beta$  was associated with an increased frequency of suppressive FoxA1<sup>+</sup> Treg cells in the blood<sup>314</sup>.



**Figure 16. Basic mechanisms used by Treg cells** This schematic depicts the various regulatory T ( $T_{reg}$ )-cell mechanisms arranged into four groups centred around four basic modes of action. ‘Inhibitory cytokines’ include interleukin-10 (IL-10), interleukin-35 (IL-35) and transforming growth factor- $\beta$  (TGF- $\beta$ ). ‘Cytolysis’ includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. ‘Metabolic disruption’ includes high affinity IL-2 receptor  $\alpha$  (CD25)-dependent cytokine-deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine-purinergic adenosine receptor (A2A)-mediated immunosuppression. ‘Targeting dendritic cells’ includes mechanisms that modulate DC maturation and/or function such as lymphocyte activation gene-3 (LAG3; also known as CD223)–MHC-class-II-mediated suppression of DC maturation, and cytotoxic T lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule, by DCs<sup>315</sup>. Vignali DA et al Nat Rev Immunol, 2008

The hope that a simple transplantation of T regulatory populations would be sufficient to reverse EAE disease encountered problems in terms of the quantity, their ability to migrate to the site of inflammation and the duration of their existence so, many scientists have focused on how it is possible to make these cells expand inside the body in order to take advantage of their regulatory functions. The expansion of T regulatory cells is dependent on cytokines such as TNF- $\alpha$  and the activating effect of TNF- $\alpha$  on Tregs was firstly demonstrated by the group headed by J Oppenheim, showing that the presence of TNF- $\alpha$  induced in-vitro the expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, enhancing their production of TGF- $\beta$ <sup>316</sup>. Interestingly, the role of TNF- $\alpha$  at inducing either pro-inflammatory or anti-inflammatory responses has been shown to differ depending on the inflammatory conditions. In the case of rheumatoid arthritis, it has been reported that in both human and mice, anti-TNF treatment can reduce the incidence of disease whereas in case of Type I Diabetes and lupus-like disease TNF- $\alpha$

displays anti-inflammatory properties<sup>317</sup>. In the case of EAE, Zhang Qi et al showed that the neutralization of TNF- $\alpha$  increased iTreg differentiation and could protect against EAE through a signaling pathway involving the induction of TNF receptor II and the activation of Akt<sup>318</sup>. More recently, Batoulis et al demonstrated that anti-TNF- $\alpha$  treatment reduced pathogenic T cell immune responses and delayed the onset of EAE but had no effect on disease severity once EAE had been established<sup>319</sup>. In addition to TNF- $\alpha$ , IL-2 can induce Treg expansion when administrated before immunization with myelin MOG<sub>35-55</sub> peptide for the induction of EAE<sup>320</sup> and IL-9 is highly involved to regulate the signaling of Jagged2-Notch1 which has been shown to expand Tregs<sup>228</sup>.

A recent study by Ray A et al showed that B cells can promote the expansion of T regs in an IL-10 independent manner through the expression of glucocorticoid-induced TNFR ligand (GITRL) despite the fact that Hoehling et al suggested that after B-cell depletion there are no changes in Treg-cell proliferation, their accumulation in CNS and their capacity to display suppressive properties. On the other hand, a study performed by Mann MK showed that in B-cell deficient mice there is impairment in the numbers of Tregs in the CNS and production of IL-10 through the lack of B7 signaling by the B cells<sup>321,322,323</sup>. Interestingly, it has been shown that during recovery from RR-EAE, the population of Tregs that is accumulated in the CNS is characterized by the expression of CD62L<sup>high</sup>, CTLA-4, ICOS and TGF- $\beta$  and are very efficient suppressors of immune responses<sup>324</sup>. Other factors which have been also shown to be involved in the expansion of Tregs is the chemokine receptor CCR6 and CXCR3 whose expression by CD4<sup>+</sup> Tregs is necessary for both the migration and suppressive effect of these cells inside the CNS<sup>325</sup> via PD-1 as well<sup>326</sup>. Recently, Peterman F et al showed that the cytokine IL-23 which is known for its role in the maturation of Th17 cells, can also prevent the development of Treg cells through the activation of  $\gamma\delta$  T cells which make the effector  $\alpha\beta$  T cells able to inhibit the suppressive effect of Tregs and prevent the conversion of conventional Tregs into Foxp3<sup>+</sup> Treg cells<sup>327</sup>.

## **E2) CD8<sup>+</sup> T regulatory cells**

Except for CD4<sup>+</sup> Tregs, evidence has been provided also for the existence of CD8<sup>+</sup> T cells able to display regulatory properties producing anti-inflammatory cytokines such as IL-10 and TGF- $\beta$ . It was demonstrated that mice deficient in CD8<sup>+</sup> T cells present a more severe type of EAE disease<sup>328</sup>. Distinct populations of CD8<sup>+</sup> Tregs have been identified, among them the subsets of CD8<sup>+</sup>CD28<sup>-</sup> and CD8<sup>+</sup>CD122<sup>+</sup>. Najafian N et al showed that CD8<sup>+</sup> T cells lacking

the expression of CD28 display their regulatory effects against EAE through the inhibition of APCs activation. CD8<sup>+</sup> T cells can also contribute to the resistance of CD28<sup>-/-</sup> mice to CNS autoimmunity since the depletion of CD8<sup>+</sup> T cells make these knock out mice susceptible to EAE whereas the adoptive transfer of CD8<sup>+</sup>CD28<sup>-</sup> cells can induce resistance of CD28<sup>-/-</sup> mice to EAE<sup>269,329</sup>. Additionally, the role of CD8<sup>+</sup>CD122<sup>+</sup> regulatory cells has been investigated in the model of EAE. It has been shown that this subtype of CD8<sup>+</sup> T cells can produce IL-10, which control the proliferation of CD8<sup>+</sup> T cells and their production of IFN- $\gamma$ . Lee Y et al reinforced these observations demonstrating that the adoptive transfer of CD8<sup>+</sup>CD122<sup>+</sup> T regulatory cells can confer protection to EAE whereas the mice become less resistant to EAE after depletion of CD122<sup>+</sup> cells<sup>330-333</sup>. Recently, Chen ML et al. showed that a newly described subtype of CD8<sup>+</sup> Treg cells which expresses at the surface the latency-associated peptide (LAP) can exhibit suppressive functions both in vitro and in vivo through the production of cytokines TFG- $\beta$  and IFN- $\gamma$ <sup>334</sup>. The role of INF- $\gamma$  by CD8<sup>+</sup> Tregs has been shown also by another study where authors demonstrated that TCR-peptide-reactive CD8aa<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> Tregs are able to induce apoptosis of the pathogenic T cells via the production of IFN- $\gamma$  and perforin but not through Fas/FasL interactions<sup>335</sup>. Several subsets of CD8<sup>+</sup> Tregs have been described to express CD25, Foxp3, CD122 and/or HLA-G that lack expression of CD28. Recently, a new population of CD8<sup>+</sup> T cells expressing HLA-G without Foxp3, has been shown to display suppressive functions that are mediated by HLA-G, immunoglobulin-like transcript-2 (ILT-2) and IL-10 whereas CD8<sup>+</sup>Foxp3<sup>+</sup> cells are not able to perform similar effects<sup>336-338</sup>. Additionally, CD8<sup>+</sup>CD28<sup>-</sup> Tregs which occur naturally, have been shown to suppress inflammation through the production of IL-10 in the murine model of inflammatory bowel disease whereas the corresponding human cell population can induce the upregulation of ILT-3 and ILT-4 on other immune cells such as monocytes and DCs<sup>339,340</sup>. In the case of EAE, mice deficient for the expression of the non-classical MHC class Ib molecule Qa-1 are more susceptible to EAE disease, suggesting a potential protective effect of Qa-1-CD8<sup>+</sup> T cells which has been shown to be related also with expansion of Vb8.2<sup>+</sup>CD4 cells<sup>341,342</sup>. It is obvious that much remains to be learned about the potential complex interactions that can occur between cytotoxic/pro-inflammatory (Tc1/Tc17) CD8<sup>+</sup> (and CD4<sup>+</sup>) T cells and the various types of T cell subsets, both at the peripheral lymphoid tissue level and within CNS lesions.

## **F) Role of B cells in EAE**

### **F1) Effector B cells**

As it has been clear in the previous part, EAE is considered as CD4<sup>+</sup> T-cell mediated autoimmune disease, emphasizing the crucial pathogenic role of CD4<sup>+</sup> T cells at inducing the disease. On the other hand, B cells are known to participate in several immune responses by their capacity to produce antibodies, inflammatory (or not) cytokines and to act as antigen-presenting cells. The first evidence that B cells can participate in the pathogenesis of EAE came in rats when Willenborg et al showed that immunoglobulin-deficient rats were resistant to EAE induction upon immunization either with guinea pig spinal cord homogenate or purified MBP<sup>343</sup> but the role of Ig seems to be necessary only for active induction since passive transfer of activated lymphocytes from MBP immunized anti-IgM treated mice can result in EAE course<sup>344</sup>. Further evidence for the existence of anti-myelin proteins in a case of EAE disease has been provided by a study of Schluegener HJ et al showing that using MOG monoclonal antibodies, the EAE incidence was accelerated in Lewis rats<sup>345</sup>. Later on, many studies using different animal models, showed the role of the complement in the antibody-induced demyelination. Different studies using either knock out mice for FcR $\gamma$  or reducing the deposition of complement C1, C3 and C9 within the CNS by the administration of a recombinant form of human complement receptor 1 showed the important and restricted role of complement in the pathogenesis since FcR $\gamma$  is not necessary<sup>346-348</sup>. Focusing on the role of more specific FcR receptors with either lower or higher affinity, which are known to activate immune cells, the role of the complement needs to be investigated in more details since evidence show that complement may be required for the antibody-induced demyelination but its role on the progression of EAE may be regulatory or protective<sup>349</sup>. In order to clarify the role of B cells in the pathogenesis of EAE, the establishment of transgenic mice deficient for the B cells because of the disruption in the IgM heavy chain ( $\mu$ MT) was really helpful. Three different studies using this mouse model, showed that the  $\mu$ MT mice were susceptible to EAE following immunization with MBP, MOG and in human rMOG-immunized  $\mu$ MT as well where the production of MOG-specific antibodies was playing a crucial pathogenic role<sup>350,351</sup>. The model of human rMOG-induced EAE had been established as a useful model for studying the role of B cells. More recently, an anti-CD20 depletion strategy (anti-CD20 depletes all the B cells from the stage of pre-B cells but not the plasma cells) in human rMOG-induced EAE induced diminished T cell responses, suggesting an antigen-presenting role for B cells. In transgenic TCR and BCR mice, it has been demonstrated that a direct T-

cell:B-cell interaction with self-antigen, specific B cells acting as APCs, can trigger the activation of autoreactive T cells and the production of autoreactive antibodies, despite the fact that B cells seem to be unable to induce EAE disease alone<sup>199,352</sup>.

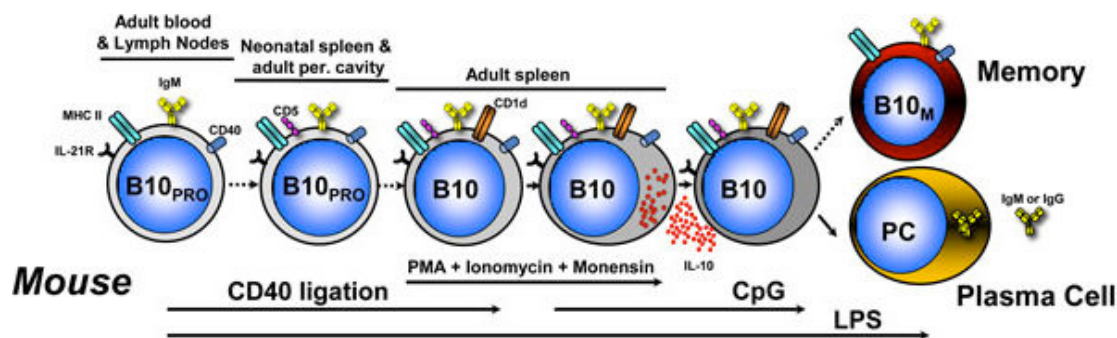
## **F2) B regulatory cells**

In addition to their effector functions and their participation in the pathogenesis of immune-related diseases, B cells have been shown to display also other properties either through antigen-presentation or via the production of cytokines. Since 1970, investigators started providing evidence for possible (negative) regulatory functions by B cells, which can be used for therapeutic purposes for the treatment of autoimmune diseases. Although, an observation made in 1950 suggested an association between B cell deficiency and autoimmunity, showing a possible regulatory function in B cell subsets<sup>353</sup>, the first immunosuppressive role of B cells had been described in 1968 by Morris and Moller. They reported the induction of a suppressive signaling of immunoglobulins either through an indirect modulation of DCs from activating the Fc $\gamma$  receptors or through the use of ITIM motifs that can be fixed to the inhibitory receptor Fc $\gamma$ RIIB<sup>354,355</sup>. In 1974, Katz SI et al reinforced these studies showing that the adoptive transfer of splenocytes that have been depleted for B cells, so there was no production of antibodies, lost its capacity to inhibit delayed type hypersensitivity (DTH), but at the same time, Neta R et al suggested that the protection against DTH seems to occur before the appearance of circulating antibodies<sup>356,357</sup>. In 1988, Youinou P et al provided evidence for the expansion of a population of B cells, positive for the expression of CD5 with longer half-life to be present in many cases of patients suffering from autoimmune diseases<sup>358</sup>. A protective role of naïve B cells has been also shown in the context of allograft rejection in a minor-histocompatibility antigen dependent manner. In 1996, Wolf SD et al taking advantage of the B-cell deficient mice ( $\mu$ MT) in C57BL/6J strain, showed that these mice, unlike wild-type mice, were unable to recover from EAE disease, suggesting that B cells may play an important suppressive role for the protection against this disorder<sup>346</sup>. In 2002, Fillatreau et al, reported that not all B cells, but only these cells which are IL-10 competent are responsible for this protective effect since chimeric mice with IL-10 deficiency restricted to B cells could develop a non-remitting EAE with increase in the production of IFN- $\gamma$  by T cells, enhancing the pathogenic profile of Th1 cells<sup>359</sup>. IL-10 was already known to be an anti-inflammatory cytokine with a large variety of functions and of course a strong potential in suppressing autoimmune-inflammatory responses. The regulatory role of IL-10 by B cells has been

reported in different autoimmune models such as colitis, where at the peak of the disease, the frequency of IL-10-producing B cells increases and is considered to be responsible for the downregulation of signaling pathways of IL-1 and STAT3 (signal transducer and activator of transcription 3) which are needed for the Th-2-driven inflammatory responses. In the case of arthritis, Mauri C et al showed that the activation of splenocytes with anti-CD40, isolated from arthritic mice, can induce the differentiation of IL-10-competent B cells which can confer protection to arthritic mice upon adoptive transfer<sup>360,361</sup>. During the last years, different subsets of B cells have been shown to display regulatory functions through the production of IL-10<sup>362,363</sup>. Among them, the more widely studied are the following ones:

### **I) B10 cells**

This subset of regulatory B cells with suppressive functions has been described for the first time by the group of Tedder TF in 2008, when they showed the existence of a unique subset representing only 1%-2% of spleen B220<sup>+</sup> cells in wild-type mice, characterized by the expression of CD1d<sup>high</sup>CD5<sup>+</sup>, expressing largely IgD<sup>low</sup>IgM<sup>hi</sup> and less than 10% IgG or IgA but not CD23 and CD93<sup>364,365</sup>. B10 cells are strong producers of the anti-inflammatory cytokine IL-10 and are responsible for normalizing inflammation in mouse models, either deficient for CD19 or wild-type in which B cells have been depleted with the use of anti-CD20 mAb<sup>366</sup>. The identification of these B10 cells can't be done phenotypically since the markers CD1d and CD5 are expressed also by other B cells subtypes such as marginal zone (MZ B cells), follicular (FO), transitional, memory and B-1a B cells. Their IL-10 competence, as analyzed by an intracytoplasmic staining for IL-10, upon ex-vivo stimulation with LPS (TLR4 ligand) or CpG (TLR9 ligand) and additional exposure to phorbol-esters (phorbol-12-estate-myristate-13-acetate (PMA)) and ionomycin for 5h, is an essential tool for the identification of B10 cells. B10 cells are derived from a functional progenitor population (B10<sub>pro</sub>) which shares exactly the same phenotype with mature B10 cells, is unable to produce IL-10 spontaneously but develop into B10 cells only if activated through CD40L or alternatively exposed to LPS or CpG (**Figure 17**).



**Figure 17. Linear differentiation model of B10 cell development *in vivo* in mice.** In mice, B10<sub>pro</sub> cells are found in the CD5<sup>-</sup>CD1d<sup>-</sup> adult blood and lymph node B cell subsets and within the CD1d<sup>-</sup>CD5<sup>+</sup> neonatal spleen and adult peritoneal cavity B cells subsets. CD40 stimulation induces B10<sub>pro</sub> cells to become competent for IL-10 expression, while LPS induces B10<sub>pro</sub> cells to become IL-10-competent for IL-10 expression and induces B10<sub>pro</sub> cells to produce and secrete IL-10. CD1d<sup>hi</sup>CD5<sup>+</sup> IL-10-competent B10 cells in the adult spleen are induced to express IL-10 following stimulation with phorbol esters (phorbol-12-myristate-13-acetate (PMA)) and ionomycin or LPS plus PMA and ionomycin for 5h. Following a transient period of IL-10 expression, a small subset of B10 cells can differentiate into antibody-secreting plasma cells (PC). B10 cells also possibly differentiate into memory B10 cells (B10<sub>M</sub>)<sup>364</sup>. Kalampokis I et al, *Arthritis Res Ther*, 2013

The development of B10 cells is dependent on many different factors. The BCR diversity and the signaling through CD19 play a key role in the normal development and/or expansion of peripheral B10 cells. A combination of signals from both innate and adaptive immune system can control the production of IL-10 by Bregs, including stimulation through BCR, overexpression of the CD19 molecule at their surface and signaling through CD40 ligation. On the other hand, the expression of CD22 affects negatively the production of IL-10 since it reduces the signaling dependent on CD19 and BCR. Recently, it has been shown that the activation of the molecule T cell immunoglobulin and mucin domain protein 1 ligation (TIM-1) which can be induced also by the stimulation of the BCR, represents a common functional marker for all regulatory B cells triggering their production of IL-10<sup>367</sup>. Additionally, the expression of the endoplasmic reticulum calcium sensors STIM-1 and STIM-2 (Stromal interaction molecule -1, -2) have been reported to be essential for the activation of BCR, proliferation and production of IL-10 by Bregs whereas their depletion restricted to B cells leads to impairment of B regulatory properties<sup>368,369</sup>. Interestingly, the factors, which are known to affect the development of B10 cells, are not always the same with those affecting their suppressive functions. Despite the fact that B10 cells develop normally in mice deficient for the adaptor protein MyD88, their function is increased upon stimulation through the Toll-like receptors 4 and 9 (TLR-4 and TLR-9) and MyD88 which induce the production of IL-



$10^{370}$ . Recently, it has been shown that apoptotic cells expressing ligands for TLRs can be recognized by B cells to induce their differentiation into IL-10-competent Bregs<sup>371</sup>. A more complete mechanism for the function of B10 cells has been proposed by the group of Tedder, demonstrating that the IL-21 cytokine, in combination with anti-CD40 and BAFF, are responsible for the *in vitro* expansion of IL-10<sup>+</sup> Bregs, an important observation for clinical applications. Despite the fact that T cells are thought to help Bregs to become fully active during their maturation, T-cell interactions are not viewed as being vital in the development of Bregs. This is supported by the fact that T cell-deficient mice contained Breg and the frequency of Breg was 5 times higher than in wild-type mice. In addition, it has been observed that CD1d, MHC class I, and MHC class II were not required for regulatory B cell development<sup>252</sup>. The increased number of Bregs in T cell-deficient mice suggests that T cells may inhibit the maturation of Bregs. However, it remains unclear if T cells truly inhibit the development of Bregs or the inflammatory state in immunodeficient mice induces the increased numbers of Bregs. In the context of EAE, B cells have been shown to display regulatory properties during the phase of induction of the disease since the depletion of B cells 7 days prior to immunization exacerbated the clinical score in contrast with their depletion at the day 14 post-immunization that ameliorated the EAE incidence, reducing the number of pathogenic MOG-specific T cells inside the CNS and the production of inflammatory cytokines. Matsushita et al reported that the same non-protective effects appear in case of a selective depletion of B10 cells by means of CD22 mAb. The protective effect of these CD1d<sup>high</sup>CD5<sup>+</sup> B10 cells is dependent on IL-10 since the adoptive transfer of B10 cells from mice deficient for IL-10 did not confer protection to EAE mice. The regulatory effect of B10 cells in EAE requires the production of IL-21 and a cognate interaction with CD4<sup>+</sup> T cells. B10 cells cannot display their suppressive properties once the disease has been established. B10 cells seem to act at the induction of the disease whereas Tregs perform their regulatory functions at the later phase of the disease.

### **Other types of IL-10<sup>+</sup> B regulatory cells**

In addition to B10 cells, which are characterized by the restricted phenotypic expression of CD1d<sup>high</sup> CD5<sup>+</sup>, the use of a combination of markers is considered to be a better strategy for the identification of B regulatory cells.

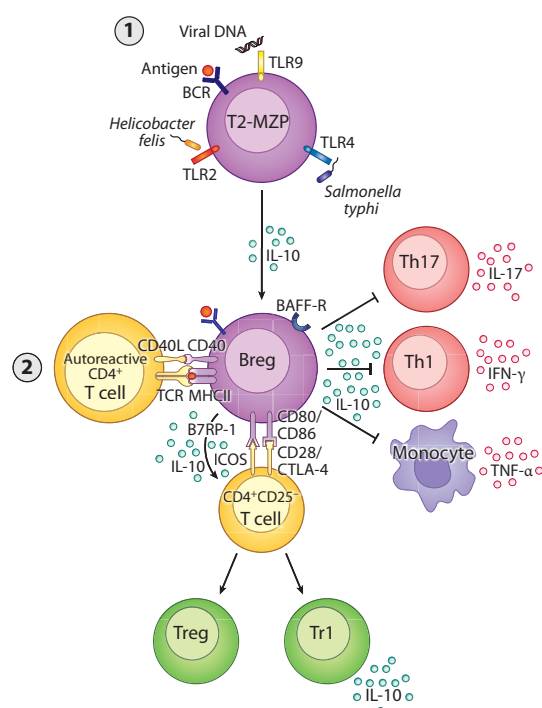
## II) Marginal Zone B cells

The MZ B cells reside in the spleen of a mouse and are characterized by the expression of  $CD1d^{high} CD21^{high} CD23^{-} CD24^{high} IgM^{high} IgD^{low}$  and are able to secrete the anti-inflammatory cytokine IL-10. The regulatory function of this subtype was firstly studied in a model of colitis where it has been shown to delay the development of the disease, even if the effect was short term. Similar protection can be performed also by MZ B cells derived from mesenteric lymph nodes, which trigger the expansion of  $CD4^{-} CD8^{-} CD3^{+} NK1.1^{+}$  T cells and  $CD4^{+} CD8\alpha^{+}$  T cells in the secondary lymphoid and intraepithelial compartment. The ability of these cells to produce IL-10 and to protect against other autoimmune models such as CIA or lupus-like disease is dependent on their ex-vivo stimulation with either anti-CD40 mAb or CpG (TLR9 ligand), whereas adoptive transfer of non-stimulated MZ cells failed to confer protection to these models. It has been shown that the mechanism by which IL-10 production by activated MZ B cells suppresses autoimmune responses can be also through the inhibition of IL-12 production by APCs<sup>372</sup>. A critical point for the regulatory function of this cell subset is the fact that during their isolation, it is not easy to get 100% purity, so many other B cells could mediate their suppressive effects<sup>373-375</sup>. The role of MZ B cells has been shown also in a model of infection with *Leishmania donovani*. In  $\mu$ MT-infected mice, CD8 T cells displayed a greater cytotoxic potential and generated more effector memory cells compared with infected WT mice. Authors showed that the MZ B cells were the subset that was able to capture parasites, associated with upregulation of surface IgM and MyD88-signaling-dependent IL-10 production, targeting CD8 T cells and suppressing their responses<sup>376</sup>.

## III) T2-MZP B cells

The less mature subtype of B cells displaying regulatory functions has been described by the group of Mauri C, they are precursors of MZ B cells with a phenotype of  $CD19^{+} CD21^{high} CD23^{high} CD24^{high} IgD^{high} IgM^{high} CD1d^{high}$ , called T2-MZP B cells. These T2-MZP cells are at an immature stage of development and are thought to be auto reactive after interaction with environmental triggers. These cells have been shown to increase during the remission phase of arthritis and their isolation for adoptive transfer protects against the development of CIA. Upon stimulation with anti-CD40, they are able to produce large amounts of IL-10 and to suppress autoimmune diseases such as antigen-induced arthritis and lupus-like disease<sup>373,377</sup>. As a result of inflammation, auto reactive T cells become active, and this results in Bregs becoming fully active by activating signals from CD40, BCR, CD80 and CD86. The fact that

these cells are still in an immature stage of differentiation makes more difficult their use as a master Breg cell type since it is unknown if they can display their in-vivo effects in that stage or upon differentiation into more mature B cells with regulatory functions. In 2009, Rafei M et al provided evidence for the existence of a new population of Bregs that shares phenotypic characteristics with the previously described T2-MZP cells. Authors showed that GM-CSF-IL-15 fusion protein can mediate the development of naïve splenic cells into IL-10-producing B cells, able to protect against EAE, upregulating the expression of MHC-II and STAT-6 in B cells<sup>378</sup>. Mauri C et al proposed that the T2-MZP cells need a first signal from TLRs (response to pathogens) in order to secrete the first amounts of IL-10, but during inflammation, the activation of autoreactive T cells provides signals (CD40, CD80, CD86) which enhance the production of IL-10, suppressing autoimmune responses either through the inhibition of DCs maturation and their APC function or through the conversion of CD4<sup>+</sup> effector T cells into Tregs<sup>362</sup> (**Figure 18**).



**Figure 18. Functional Breg differentiation and regulatory mechanism.** A common progenitor, namely autoreactive T2-MZP Bregs, produces IL-10 after TLR stimulation. Bregs are further activated and release an optimal amount of IL-10 in response to CD154<sup>+</sup>CD4<sup>+</sup> T cells. This step also requires antigen recognition. The chief mechanism of suppression is via the release of IL-10, leading to the suppression of Th1, Th17 responses, and TNF- $\alpha$  from monocytes. IL-10 released by Bregs is pivotal for the differentiation of Tr1 cells and Foxp3<sup>+</sup> Tregs, resulting in immune regulation at the site of inflammation such as the joint and central nervous system. Mauri and Bosma, *Ann Rev Immunol*, 2012

#### IV) Regulatory B plasmocytes

Teichmann et al have shown for the first time that in a mouse model of systemic lupus erythematosus, CD19<sup>+</sup>CD138<sup>+</sup> plasma cells can produce the anti-inflammatory cytokine IL-10, suggesting a link between plasma cell differentiation and induction of IL-10 expression in B cells<sup>379</sup>. More recently, the group of Fillatreau S, provided evidence for a new subset of mature B lymphocytes which is able to produce IL-10 after infection with *Salmonella*. The production of IL-10 was restricted to a population characterized by the expression of CD19<sup>+</sup>CD138<sup>+</sup>, where CD138 is a cell surface marker for plasmablasts and plasma cells. Intrinsic MyD88-signaling in B cells is essential for the development of this IL-10-producing CD19<sup>+</sup>CD138<sup>+</sup> cell population, even in a very early phase post-infection. Whether these cells are antibody-secreting plasmablasts or plasma cells is still unknown<sup>380</sup>. Interestingly, the subset of CD138<sup>+</sup> B cells was related with high expression of the transcription factors Blimp1 and Irf4, which instruct plasma cell differentiation. The fact that plasma cells were the main subtype of B cells expressing IL-10 might seem intriguing, but it has to be mentioned that the transcription factors controlling B cell differentiation into antibody secreting cells (APCs) are equally involved in the induction of IL-10 expression in suppressive T cells<sup>381–384</sup>. The capacity of B cells to secrete the anti-inflammatory cytokine IL-10 is somehow a surprise since plasma cells are known for their roles in antibody production.

#### V) B-1a B cells

The main characteristics concerning the development, phenotype and function of B-1a B cells have been already discussed in the first part of the introduction. This subtype of B-1 cells is known to be able to display regulatory functions. CD5<sup>+</sup>CD11b<sup>low</sup>IgD<sup>-</sup> B-1a B cells are found mainly in the peritoneum and are characterized by their strong ability to produce the anti-inflammatory cytokine IL-10 and has been already demonstrated to induce cell-death of CD4<sup>+</sup> T cells through a FasL/Fas-dependent mechanism. Their suppressive properties are not restricted to the production of IL-10, since the production of natural IgM antibodies can reduce the load of antigens and lead to an effective clearance of apoptotic cells. It was showed that the secretion of IgG and IgA against epithelial cells and enteric bacteria at the peak of inflammation is increased and their transfer to  $\alpha\mu$ -deficient mice can trigger the suppression of colitis with less apoptotic cells<sup>357,375</sup>. More recently, a study by Margry B et al showed that in vitro activation of peritoneal cavity derived B-1 cells with anti-CD40 and LPS induces these cells to secrete more IL-10, IL-6 and IgM as compared to splenic B cells. Only the

activated B-1 cells were able to suppress the production of TNF- $\alpha$  and IFN- $\gamma$  by CD4<sup>+</sup> T cells whereas non-activated B-1 cells augmented the production of these inflammatory cytokines<sup>386</sup>.

### **F3) Stimulatory signals for the induction of B regulatory cells**

#### **I) Toll-like Receptors Signaling**

Signaling through TLRs has been shown to be involved in autoimmunity by mediating either inflammatory or protective effects. For many years, scientists were trying to identify the cell types whose TLR signaling is responsible for displaying the above effects. It is known that murine B cells can express all TLRs (TLR1-9)<sup>387</sup> which induce the production of a large variety of cytokines, among them, the anti-inflammatory cytokine IL-10, whose production seems to be increased upon additional stimulation with CD40, a proof that TLR-activated B cells need to interact with CD4<sup>+</sup> T cells for an optimal production of IL-10, while only cross-linking of B cell receptor (BCR) for antigen or CD40 can't trigger the induction of similar responses<sup>388,389</sup>. Interactions of B10pro cells with TLR4 or TLR9 contributes to the production of IL-10 by these cells. Studies have shown that in mice the progenitors of B10 cells only mature after in vitro stimulation by LPS, while B10pro cells in human can be induced via ex vivo stimulation by LPS or CpG. Signaling through MyD88 by TLR4 and TLR9 results in the highest production of IL-10 and maturation of B10pro cells. However, it should be noted that MyD88 signaling is not required for Breg development because equivalent numbers of Bregs have been produced in MyD88-deficient mice in the presence of CD40 ligation and PMA plus ionomycin stimulation. Thus, MyD88 is crucial to induce the highest expression of IL-10 from Bregs and is required for LPS to induce the highest IL-10 expression. Similar to T cells, MyD88 is thought to be involved but is not critical for the development of Breg, but rather is thought to play a key role in helping to induce Breg to express IL-10 in the highest amount. Tian JI et al showed that the activation of B cells through a TLR-4 ligand (LPS) can induce a killer phenotype to B cells since their adoptive transfer protects NOD mice against T1D through the production of TGF- $\beta$  and expression of Fas Ligand, whereas Scott et al demonstrated that mouse B cells which have been engineered to express an antigen-protein, were able to suppress antigen-specific T-cell responses after an adoptive transfer in mice, upon stimulation with LPS<sup>390</sup>. It has been also shown that LPS-activated male B cells are more efficient to induce tolerance against antigens when transferred in female recipient mice than the naïve cells<sup>391</sup>. As it has been described already, the stimulation of TLRs is essential for the induction of the regulatory B10 cells. Lampropoulou

V et al provided evidence for the implication of the adaptive protein MyD88, a signaling molecule downstream of TLRs, in autoimmune diseases. It has been shown that MyD88 signaling is required for the induction of EAE since it drives the differentiation of Th17 cells. MyD88 signaling on B cells antagonizes the MyD88 signaling in other cells whereas mice deficient for MyD88 restricted to B cells do not recover from EAE in contrast with mice displaying global deficiency of MyD88 which are resistant to EAE. TLR activation of B cells induces the production of IL-10 inhibiting the maturation of dendritic cells<sup>388</sup>. In a case of infection by *Salmonella*, the lack of TLRs and MyD88 signaling restricted in B cells could increase the survival of infected mice, in comparison with wild type mice that were less resistant to the disease<sup>380</sup>. In the same study, authors showed that the inhibitory role of MyD88 was related with more efficient responses by NK and neutrophils and that the infection induced the production of IL-10 by a subset of plasma B cells expressing the molecule CD138. In accordance with these observations, Madan R et al proposed that in a model of viral infection by murin cytomegalovirus (MCMV), the accumulation of CD19<sup>+</sup>CD138<sup>+</sup> IL10-producing B cells in the spleen was responsible for inhibiting MCMV-specific CD8<sup>+</sup> T cell responses<sup>392</sup>.

## II) BCR Signaling

In addition to the role of TLRs, BCR signaling has been shown to be also required for the regulatory properties in B cells. The importance of BCR diversity is confirmed in experimental cases of mice deficient for the surface molecule CD19, a co-receptor that optimizes BCR signals, and in MD4 mice which express a BCR specific for the antigen hen egg lysozyme (HEL). These mice display an impairment in the ability of B cells to produce IL-10 and to suppress antigen-specific immune responses<sup>365,393</sup>. BCR stimulation is related with an increase of intracellular Ca<sup>2+</sup> whereas mice deficient for the Ca<sup>2+</sup> sensor stromal interaction molecules 1 and 2 are unable to activate NFAT (nuclear factor of activated T cells) and to induce the production of IL-10 by B cells<sup>368,394</sup>. Interestingly, mice which are deficient for STIM-1 and STIM-2 restricted in B cells display more severe EAE, similar to mice lacking expression of IL-10 in B cells. Moreover, in a model of colitis, it has been shown that mice lacking the p11a subunit of the enzyme phosphoinositide 3-kinase which mediates BCR signaling, develop spontaneously inflammatory bowel disease, suggesting the lack of a potential suppressive function from B cells due to the impairment of BCR signaling<sup>395-397</sup>.

### III) CD40

CD40 belongs to the family of tumor necrosis factor receptors (TNF) and is necessary for interaction between B and T cells through its CD40L (CD154). Mice deficient for the expression of CD40 restricted in B cells have been shown to be more susceptible to EAE development, displaying less IL-10 production by B cells<sup>359</sup>. In accordance with that observation, the fact that B cells isolated from an EAE recovery phase are good producers of IL-10 upon in vitro stimulation with BCR and CD40, suggests that the in-vivo regulatory effect of B cells is mediated by a process including TLRs and then BCR and CD40 activation<sup>395</sup>. However, Mauri C and Evans JG in two independent studies showed that stimulation of splenocytes with agonistic anti-CD40 antibodies can trigger the induction of Bregs with suppressive properties via the production of IL-10<sup>361,374</sup>. The maturation of B cells through cognate interactions with T cells through CD40/CD40L may be an essential step for the induction of suppressive Bregs.

### IV) Cytokines

The development as well as some of the functional properties of the B regulatory cells are dependent on the production of cytokines. Recently, Yoshizaki et al demonstrated that in a mouse model of Multiple Sclerosis, EAE, the B10-cell maturation into functional IL-10 secreting cells which can inhibit in vivo autoimmune responses requires the provision of IL-21, together with other factors such as CD40-dependent cognate interactions with T-cells<sup>252</sup>. In the same study it has been shown that IL-21 can promote the secretion of IL-10 in a manner similar to LPS stimulation, even in the absence of stimulation from ionomycin and phorbol esters. Interestingly, Holan V et al provided evidence for the role of other cytokines in the regulation of IL-10-producing B cells after activation through TLR4. In this study, authors showed that IL-10 production was enhanced after stimulation with IL-12 and IFN- $\gamma$  and on the other hand, IL-10<sup>+</sup> B cell development was inhibited in the presence of IL-21 and TGF- $\beta$ <sup>398</sup>. Concerning the opposite results regarding the role of IL-21, different factors may influence these results such as the model that had been used, the subtype of isolated B cells and the sensitivity of B cells to distinct stimulatory signals. On one side, the cytokines which affect the development of IL-10-producing B cells do not seem to display similar effects on the proliferation of B cells whereas on the other side, other cytokines, such as IL-4 and IL-13, have been shown to enhance B cell proliferation without any effect on the production of IL-10. Two independent studies, provided evidence for the autoregulatory effects of IL-10 in the

proliferation and development of IL-10-producing B cells. Sindhava et al showed that in a model of *Borellia* infection, the stimulation of B-1 cells through TLR activation triggered the production of IL-10 which in turn, mediated autoregulation of B-1 cells<sup>399</sup>. Holan et al reported that the inhibition of endogenous IL-10, either by the use of neutralising anti-IL-10 mAb or by blocking its effects with anti-IL-10R mAb enhanced B-cell proliferation and IL-10 production, suggesting that autoregulation of IL10 affects the development of IL-10<sup>+</sup> B cells<sup>398</sup>. Interestingly, it is obvious that the development of different regulatory populations is not affected by the same cytokine milieu so, every time that we do try to induce one regulatory cell type, we should take into account the fact that we may inhibit the development of another one. More recently, Klinker MW et al demonstrated that the B cells which display killer properties through the expression of FasL are enriched in the IgM<sup>hi</sup>CD5<sup>+</sup>CD1d<sup>hi</sup> B cell subset, previously reported to contain a higher frequency of IL-10-producing B cells. Most FasL<sup>+</sup> B cells did not produce IL-10. They showed that IL-5 is the main inducer of killer B cell function since this B cell subtype expressed high levels of IL-5 receptor and treating B cells with IL-5 and CD40L resulted in the expansion of a B cell population enriched for FasL<sup>+</sup> cells. IL-5 also enhanced the IL-10 secretion in B cells stimulated with CD40L suggesting that IL-5 can induce or enhance both killer and B cell activity and IL-10 secretion in B cells<sup>400</sup>.

#### **V) Transcription Factors**

There are also some transcription factors found to be related to IL-10 production. Maseda et al performed a study focusing on enriched splenic regulatory B cells in mice that were stimulated by LPS and measured the amount of the transcription factors blimp1, irf4, pax5, and bcl6. The expression of blimp1 and irf4 was two-to-six-fold higher in these enriched B cells. Conversely, in this study the expression of the transcription factors pax and bcl6 was significantly reduced. Despite this observation, definitive transcription factors related to Breg development remain to be found<sup>401</sup>.

#### **F4) IL-10-independent B regulatory mechanisms**

In addition to their capacity to display suppressive functions through the production of the anti-inflammatory cytokine IL-10, Bregs have been shown to be able to perform their regulatory properties in an IL-10 independent manner. B cells able to produce the anti-inflammatory cytokine TGF- $\beta$  have been shown in both CD5<sup>+</sup> and CD5<sup>-</sup> B cells. The suppressive effect of this cytokine may not be so important as that mediated by IL-10 and

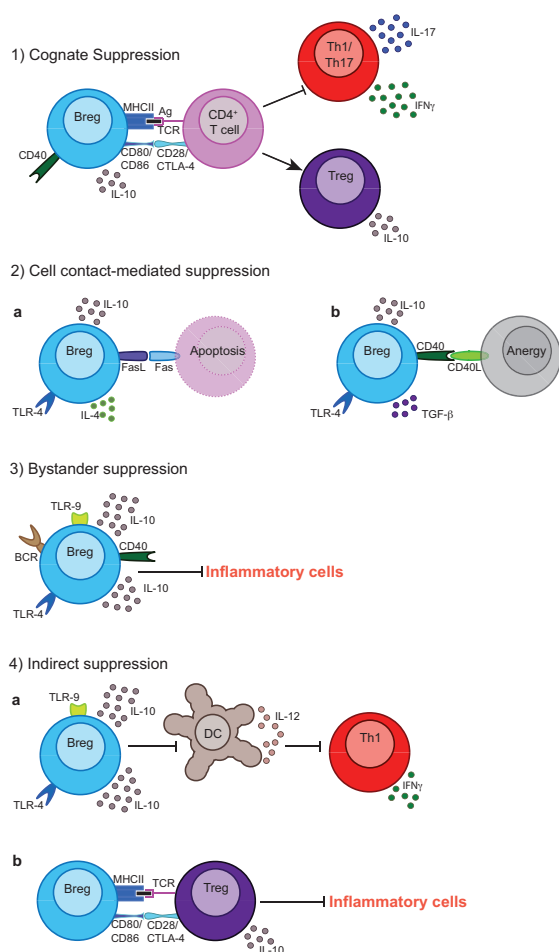


may provide help for a more efficient conversion of effector T cells into Tregs by IL-10-producing B cells. It has been reported that the TGF- $\beta$  displays a negative autocrine loop leading CD5<sup>+</sup> B cells to apoptosis, but at the same time it is known to promote directly the expansion of Tregs<sup>402</sup>.

The induction of death-inducing ligands such as Fas Ligand, TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand) and PD-L1 is an additional mechanism which makes B cells able to activate programmed cell-death after interaction with the ligation on their cell surface receptor suggesting that not only soluble factors are responsible for the immune suppression by B cells<sup>403</sup>. Granzyme B has been shown also to induce the apoptosis of infected cells<sup>404</sup>. The first evidence for the expression of FasL had been reported by Hahne et al. in 1996, showing that mouse splenocytes can express high levels of FasL upon stimulation with PMA/ionomycin<sup>405</sup>. About 25% of naive B220<sup>+</sup> mouse splenocytes have been shown to express TRAIL but after activation, almost all the purified B220<sup>+</sup> cells were able to express both FasL and TRAIL, and a stronger proof for the role of activating signals at inducing the expression of TAIL had been demonstrated by Kemp TJ, showing that B cells stimulated through a TLR9 signal, could trigger tumor cell lysis through the participation of TRAIL-R2<sup>406</sup>. PD-L1 and PD-L2 belong to the family of B7 co-stimulatory molecules and are known to induce cell death to target cells expressing the PD-L1 receptor. They are expressed on B cells and have been shown to play a role in the protection from autoimmune diseases. An interesting point regarding the role of Bregs is their ability to induce CD4<sup>+</sup>CD25<sup>+</sup>Treg cell expansion and proliferation. In the case of EAE, Mann MK et al showed that upregulation in the expression of B7 co-stimulatory molecules plays an important role for the expansion of Tregs resulting in the suppression of CNS inflammation<sup>323</sup>. TGF- $\beta$  has been also shown to support the same regulatory mechanism leading to the expansion of Foxp3<sup>+</sup> Tregs and/or the conversion of effector T cells into CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs. Interestingly, studies in  $\mu$ MT mice (lack of B cells) seem to display a reduction in terms of the number of Tregs compared with wild-type, suggesting a role of B cells for Treg expansion and for protection against EAE since these mice develop exacerbated EAE symptoms<sup>377</sup>. Several studies with the use of different animal models, have shown the relationship between Bregs and the development of Tregs. In *Schistosoma mansoni* worms infection in mice triggers the production of IL-10 by T2-MZP Bregs, which then, induce the expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs. The indirect ability of IL-10 to induce Tregs expansion suggest the need of cognate interactions mediated by co-stimulatory molecules CD80 and CD86<sup>407</sup>. In addition to CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs,

Bregs have been shown also to participate in the expansion of Tr1 cells through the production of IL-10. This hypothesis has been confirmed by two independent studies, the first one using B cells from MRL/lpr mice that when activated with agonistic anti-CD40 managed to trigger the conversion of effector T cells into Tr1 and the second one showing that regulatory apoptotic cells which stimulate B cells through TLR signals, induce the differentiation of Tr1 cells through the production of IL-10<sup>362,371,373</sup>. Another study from Ray et al suggested that the expression of the glucocorticoid-induced TNF ligand on B cells is required for their protective effect in the context of EAE through the expansion and/or proliferation of Tregs which have been shown to express the glucocorticoid-induced TNFR ligand (GITRL)<sup>321</sup>. The complete mechanism of how Bregs regulate the expansion of Tregs remains to be investigated in more details. Finally, B cells can also perform regulatory functions through the secretion of immunosuppressive factors that can either reduce the APC capacity of DCs inhibiting their maturation or more specifically through the production of Ig displaying salic acid activity<sup>334,408</sup> (**Figure 19**).

More recently, Shen P et al provided evidence for the existence of a new type of B cells, which can perform suppressive functions through the production of IL-35. This study showed that mice deficient for IL-35 restricted in B cells could not recover from T-cell mediated EAE disease. Additionally, the same mice (IL-35<sup>-/-</sup> in B cells) have been shown to be resistant to infection with Salmonella Typhimurium, characterized by increased immunity with higher activation of macrophages, inflammatory T cells and increased APC function of B cells. During both EAE and infection with Salmonella, these IL-35-producing-B cells could also produce IL-10 and were found to be enriched in the CD138<sup>+</sup> plasma cells expressing the transcription factor Blimp1, suggesting a novel role of activated B cells with potential therapeutic target for autoimmune and infectious diseases through the production of IL-35<sup>409</sup>. In addition to their role to produce the anti-inflammatory cytokines IL-10 and IL-35, the regulatory subset of plasma cells should be investigated in more details to identify its stability and the exact cytokine expression profile upon exposure to various environments. The observation that plasma cells can also produce cytokines and not only antibodies is in accordance with other studies showing that either B-1 cells can differentiate into granulocyte-macrophage colony stimulating factor (GM-CSF) CD19<sup>+</sup>CD138<sup>+</sup> plasma cells in a mouse model of sepsis induced by caecal ligation puncture or that plasma cells can also express both inducible NO synthase (iNOS) and tumor necrosis factor (TNF) in the lamina propria of the small intestine in mouse<sup>411,412</sup>.



**Figure 19. Mechanisms of regulatory B cell (Breg)-mediated suppression.** (1) *Cognate suppression.* Interaction of Bregs with CD4<sup>+</sup> T cells via MHC class II (MHCII) presenting antigen (Ag) and the T cell receptor (TCR), leads to the suppression of T helper 17 cells (Th17) and T helper 1 cells (Th1) and the induction of Foxp3<sup>+</sup> Tregs and Tr1 cells. An interaction between CD80/CD86 and CD28 may stabilize the contact between Bregs and CD4<sup>+</sup> T cells. (2) *Cell contact.* (a) Stimulation of toll-like receptor (TLR)-4 leads to production of IL-10 and IL-4 by FasL-expressing Bregs, which interact with Fas-expressing cells to induce cell death. (b) Stimulation of CD40 or TLR-4 on B cells leads to production of IL-10 or TGF- $\beta$  by Bregs, that can target other cells to induce anergy. (3) *Bystander suppression.* Stimulation of the B cell receptor (BCR), TLR-4, TLR-9, and CD40 leads to production of IL-10 by Bregs, which, in turn, suppresses a large variety of inflammatory cells. (4) *Indirect suppression.* (a) Stimulation of TLR-4 and TLR-9 leads to IL-10 production by Bregs, which suppresses IL-12 production by dendritic cells (DCs) and consequently Th1 priming. (b) Bregs induce the differentiation of Tregs, which, in turn, suppress a large variety of inflammatory cells<sup>413</sup>. Rosser et al, 2014

### **F5) B cells-producing IFN- $\gamma$ and IL-17**

Interestingly, in 2005, Harris et al performed a study where they demonstrated that B cells can produce IFN $\gamma$  in response to IL-12 and IL-18 when primed by Th1 cells. The development of these IFN- $\gamma$ -producing B cells (Be1) is dependent on the expression of the IFN- $\gamma$ R and the T-box transcription factor, T-bet. The Be1 cells do not secrete IL-4 or IL-13 but can secrete IL-10, TNF- $\alpha$  and IL-6 and it was suggested that Be1 cells not only amplify Th1 responses but also imprint a type 1 phenotype on B cells themselves and in cases of responses to bacterial or viral pathogens, this B-cell driven autocrine feedback loop may be beneficial<sup>414</sup>. In a recent paper published in *Cell Research*, Yan Bao and colleagues characterized a new population of INF- $\gamma$ -producing innate-like B cells that promotes innate immune responses and contributes to early pathogen control following intracellular bacterial infection. This population is characterized by the expression of CD11a<sup>hi</sup> CD16/32<sup>hi</sup>, low levels of MHC-II, CD80 and CD86 and increased levels of CD40 and CD40L compared to conventional follicular B cells. Upon infection, these activated innate B cells can produce huge amounts of IFN- $\gamma$  and induce infected macrophages to produce TNF- $\alpha$  and NO (nitric oxide) and can provide protection against infections<sup>415</sup>.

More recently, in 2013, Bernejo et al showed that B cells can produce IL-17 after an infection with the extracellular parasite *Trypanosoma cruzi* in a RoR $\gamma$ t-independent way. Direct exposure to *T.cruzi* modifies glycoproteins on the B cell membrane and induces a signaling pathway dependent on the tyrosine phosphatase CD45 and the kinases Src and Btk-tec that culminates in the expression of IL-17A and IL-17F. The main producers of IL-17 are found within the IgM<sup>+</sup>CD138<sup>+</sup> plasmablasts and interestingly the production of IL-17 is not related with stimulation of TLRs since mice deficient for the MyD88 restricted in B cells still produce IL-17. Authors demonstrated these IL-17-producing B cells are required for the protection from infections. It should be noted that IL-17 and B cells are highly associated with immunopathology in chronic autoimmune diseases, so more work should be performed in that field to clarify if these cells sustain or exacerbate inflammation<sup>416,417</sup>.

## F6) Human B regulatory cells

During the last 10 years, the therapeutic potential of B regulatory cells has been also investigated in humans. Today the analysis of human B cell populations is based on the expression of the surface molecules CD19, CD24, CD38 and CD27 and according to the expression of these markers, three different subtypes can be distinguished: Naïve B cells characterized by the expression of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>int</sup>, immature transitional B cells with a phenotype of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> and a memory B cell type with CD19<sup>+</sup>CD27<sup>+</sup> expression. Bouazis et al provided evidence for the B cell subtypes, which are able to produce the largest quantities of IL-10. The authors reported that the IL-10 production can't be restricted to a specific B cell type, either to memory (IgD<sup>+</sup>CD27<sup>+</sup>) or transitional B (CD24<sup>hi</sup>CD38<sup>hi</sup>) cell compartments<sup>363,418,419</sup>. Additionally, two independent studies showed that different B cell types can be considered as the best IL-10 producers. Blair et al suggested that the majority of IL-10 producing B cells are included within the CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> immature transitional B cell compartment, suppressing CD4<sup>+</sup> T cell cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , requiring the production of IL-10, expression of CD80-CD86 but not TGF- $\beta$ , whereas Iwata et al proposed that a hybrid of CD24<sup>hi</sup>CD27<sup>+</sup> is the most IL-10 competent cell type<sup>420,421</sup>, displaying their regulatory effect by influencing the function of monocytes (decrease of TNF- $\alpha$ ). Compared with the B10 cell type found in mice, in humans, B10 blood B cells are found in the CD24<sup>hi</sup>CD27<sup>+</sup>CD10<sup>-</sup> compartment, a phenotype between those of memory and transitional B cells<sup>421</sup>. More recently, the group of Mauri C described a population of B regulatory cells in peripheral blood, characterized by the expression of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup>CD5<sup>+</sup>CD1d<sup>hi</sup>, which are strong producers of IL-10 upon stimulation with CD40 in vitro. These regulatory cells have been shown to be able to suppress Th1 immune responses in an IL-10-dependent manner with the need of interaction with CD4<sup>+</sup> T cells through CD80-CD86 signaling, enhancing also the differentiation of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs<sup>420,421</sup>. Lemoine et al have shown that the emergence of human Bregs is associated with activation through CD40 signaling. They provided evidence for the existence of a Breg population characterized by the expression of IgD<sup>+</sup>CD38<sup>hi</sup>CD24<sup>hi</sup>CD5<sup>hi</sup>, a phenotype which doesn't belong to one of the previous described human Breg subtypes. This regulatory subset of CD5<sup>+</sup> B cells required a direct B-T cell interaction for the suppression of Th1 cells proliferation in vitro, and at the same time these activated-B cells could trigger the expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs which may participate in the suppressive effect against T-cell proliferation<sup>422</sup>. Despite the fact that in mice, Ding et al reported that TIM-1 may be one common functional marker for all Breg

subtypes, in human there is no a similar marker so, it is possible that only the production of IL-10 represents the stimulated-B cells which can display regulatory functions<sup>418</sup>. Interestingly, until today, human Bregs have been shown to display suppressive functions only against Th1 cells. An additional marker for the identification of human Bregs is also the CD48 and CD148. CD148 is considered to be a marker for memory B cells (together with CD27), whereas CD48 is a marker for B cell activation. This has contributed to the theory that the CD24<sup>hi</sup>CD148<sup>+</sup> phenotype found on Bregs may shed light on their development from the memory B-cell pool or perhaps reflects the shared markers between these 2 B-cell populations<sup>423</sup>. In the case of MS and SLE patients, IL-10 producing-B cells have been shown to decrease and to lose their suppressive functions even after stimulation in vitro with CD40, unlike with healthy individuals<sup>424</sup>. MS patients with helminth infections display high numbers of CD19<sup>+</sup>CD1d<sup>hi</sup>IL-10<sup>+</sup> B cells with suppressive effects of T cell responses in vitro and contributing to Th2 responses in vivo. Unlike MS, in SLE patients, the number of IL-10-producing B cells is higher than in healthy individuals<sup>425</sup>. This may be explained by the increased overexpression of CD154 on T cells which leads to the exhaustion of Bregs that lose their suppressive function and induce effector B cells to produce IL-10 together with other pro-inflammatory cytokines such as TNF- $\alpha$ . Additional studies propose that immature B regulatory cells seem to be necessary for inducing a protective effect even after a deletion of total B cells with the use of the anti-CD20 treatment, rituximab. Recently, a new study by Silva HM et al suggested that the preservation of a B cell compartment including Bregs can induce tolerance in human renal transplantation, enhancing the therapeutic potential of these cells for clinical treatments of inflammatory diseases and providing for immune tolerance in case of transplantations<sup>418,426</sup>.

Marker	Expression level <sup>a</sup>	Function	Limitation in their use	Mouse	Human
CD1d	High	Prevalently expressed on APC, presents lipid-antigen	Constitutively expressed on all B cell subsets; subject to variation in response to environmental stimuli	Yes	Yes
CD5	+	Major ligand of CD72, found on thymocytes, T lymphocytes, thymic NKT cells, and a subset of B lymphocytes	Upregulated upon activation; present on B1 cells	Yes	Yes
CD10	Variable	Zinc metalloprotease enzyme degrading small secreted peptide, marker for follicular lymphoma			Yes
CD19	+	BCR activation		Yes	Yes
CD21	+	BCR activation		Yes	
CD23	Variable	Low-affinity IgE receptor; acts as a negative regulator of IgE production	Downregulated upon activation	Yes	
CD24	High	Heat-stable antigen, expressed on several cells. On B cells, CD24 is expressed at high levels on B cell progenitors on mature resting B cells. CD24 provides costimulation for T cell proliferation.	Upregulated upon activation with LPS	Yes	Yes
CD27	Variable	Marker for memory B cells; binds to CD70; induces T-cell costimulation and B cell activation	Upregulated upon activation		Yes
CD38	Variable	Marker for activation; monitors intracellular calcium levels			Yes
CD62L	-	Cell adhesion molecule involved in lymphocyte homing to peripheral lymph nodes	Downregulated upon migration?	Yes	
CD138	Variable	Marker for plasma cells; mediates B cell-matrix interactions			Yes
CD93	Variable	Intercellular adhesion and clearance of apoptotic cells, expressed on B cells at early stage of maturation		Yes	
IgD	Variable	Provides activation signal for B cells		Yes	
IgM	High	Role in complement activation		Yes	Yes
IL-10	+	Anti-inflammatory cytokine		Yes	Yes
FasL	Variable	Induces apoptosis		Yes	
Hes-1		Transcription factor downstream Notch-1	Also expressed in “conventional” T2 B cells	Yes	
DTX1		Transcription factor downstream Notch-1	Also expressed in “conventional” T2 B cells	Yes	
STIM1		Calcium sensor, triggers SOCE	Ubiquitously expressed	Yes	
STIM2		Calcium sensor, stabilizes cytosolic calcium levels	Ubiquitously expressed	Yes	
NFATc1		Component of DNA-binding transcription complex	Ubiquitously expressed	Yes	

<sup>a</sup>Variable indicates that the expression level of the referred marker has been reported differently in various publications.

**Table 3 Phenotypical and functional characterization of B regulatory cells in mice and humans, Mauri and Bosma, *Annual Review Immunology*, 2012**

### III-3-5 Innate Immune Cells

#### A) Macrophages/Microglia

Macrophages are bone marrow derived cells, which first differentiate from hematopoietic stem cells into circulating blood monocytes in the peripheral vasculature and reside in different tissues such as the lungs, liver and CNS. Once the blood brain barrier is opened, these BM-derived macrophages can migrate inside the CNS where they have been shown to participate in the pathogenesis of EAE. In the CNS, there is a population of resident immune cells, which are called microglia. Their differentiation program suggests that they come from erythromyeloid precursors in the embryonic yolk sac and later they invade the developing neural tube<sup>427</sup>. The pool of microglia in a healthy CNS tissue is supported by local progenitors and these cells seem to reside in the CNS parenchyma controlling for possible threats to neurons. In the context of EAE, it is difficult to distinguish the BM-derived activated macrophages and the microglia since both cell populations get an amoeboid shape and display very similar functions (phagocytosis)<sup>428,429</sup>. Many studies propose that in the inflammatory lesions there are two types of activated macrophage/microglia cells, the classically activated ‘M1’ and the alternative activated ‘M2’<sup>430,431</sup>. The effector properties of the first type has been shown by their production of proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  and their ability to secrete nitric oxide via (iNOS) which is a prominent mediator of inflammation induced by several cytokines such as IFN- $\gamma$ , IL-2 and TNF- $\alpha$  and is related to many pathologies. Since the observation that activated macrophages can infiltrate inside the CNS and that microglia cells can induce neural death through the elaboration of free radicals and proteases, many studies tried to focus on these cells for targeted therapies. Treatments either with clodronate liposomes for the depletion of macrophages or with the antibiotic minocycline which inhibits the production of proinflammatory cytokines and matrix metalloproteinases by activated macrophages, showed an improved neurological recovery<sup>432</sup>. More specifically in the case of EAE, many groups have confirmed the determinant role of macrophages/microglia either using transgenic mice in these cells providing evidence for their resistance to EAE induction, blocking the secretion of their effector mediators such as MPP-2, MPP-9 or showing the action of these cells as MHC-II APCs activating CNS-pathogenic T cells<sup>245,433,434</sup>. Additionally, some other studies reported that the mechanism of these cells is mainly through the degradation of synaptic proteins, phosphorylation of neurofilaments and other proteins secreted by astrocytes (CNS resident cells) to activate M1 macrophages/microglia cells<sup>435,436</sup>. On the other hand, more recently, some studies have shown that the same cells can also



display beneficial role in the case of EAE. Denney L et al showed recently that the activation of iNKT cells can modify the balance between M1 and M2 cells inside the CNS and result in an increase of M2 macrophages associated with improvement in the neurological symptoms<sup>437</sup>. The role of iNKT cells has been shown to be through the IL-4 and CD1d, which can promote the expansion of M2 macrophages. Additionally, an interesting study from Jiang HR et al suggests an important role of the IL-33 at a polarization toward alternative activated M2 macrophages with increased production of IL-5 and IL-13, frequency of PD-L2 expression, with reduction in the production of inflammatory cytokines such as IL-17 and IFN- $\gamma$ <sup>438</sup>. Finally, another inducer of a M2 phenotype cells seems to be the neutralization of the CCL22 which results in the decrease of TNF and can trigger the production of the anti-inflammatory cytokine IL-10<sup>439</sup>.

### **B) Dendritic Cells (DCs)**

Dendritic cells are a type of cells whose importance at inducing immune responses has been shown in many different contexts. They derive from bone-marrow myeloid and lymphoid progenitors and they are considered to be the professional antigen-presenting cells (APCs). They reside in lymphoid and non-lymphoid tissues where they are waiting for a signal to activate them, which can be either through TLR stimulation or encounter with antigen (Ag). Activated DCs are able to prime Ag to naïve T cells, which will differentiate into helper T cells. This process requires a number of modifications on DCs such as expression of costimulatory molecules (CD80, CD86, CD40) and MHC-II molecule. DCs are categorized in two groups. Conventional DCs (cDCs) which are characterized by the expression of markers CD11c, CD11b, CD45, can be either CD8<sup>+</sup> or CD8<sup>-</sup> and reside in the marginal zone of the spleen where they act as APCs to activate naïve T cells by priming the Ag. The second type is the plasmacytoid DCs (pDCs) which are B220<sup>+</sup>, CD11c<sup>int</sup>, PDCA1<sup>+</sup> and are characterized by their ability to produce Type I IFN upon viral infection and can be mainly found to the LNs in an activated form. In the context of EAE, it has been shown that DCs are able also to migrate to the CNS. Zozulya AL et al provided evidence for the implication of the chemokine MIP-1 $\alpha$  (CCL3) and the secretion of MMPs by DCs which facilitate their migration inside the CNS where they get more mature, expressing higher levels of costimulatory molecules, making them much more efficient APCs<sup>440</sup>. Different subsets of DCs have been found in inflammatory regions of the CNS with myeloid DCs (CD11c<sup>+</sup>CD11b<sup>+</sup>CD8<sup>-</sup>) to migrate in the perivascular foci, cerebellum and spinal cord in comparison with the pDCs that are found mainly in the cerebellar meningeal area<sup>441</sup>. Except for their ability to act as APCs, DCs upon

stimulation through their TLRs, can start secreting cytokines affecting the adaptive immune responses. They can produce IL-12 which enhances Th1 differentiation, and recently they have been shown to produce also GM-CSF and IL-23 via their CCR4 and CCR7 receptor which is required for the induction of EAE disease and TGF- $\beta$  in an  $\alpha$ -integrin dependent manner<sup>442-444</sup>. The function of antigen-presentation cells is more than necessary to induce T-cell dependent autoimmunity. A higher number of DCs has been shown to enhance the induction of EAE since mice treated with Flt3L (binds to a DCs receptor and leads to their expansion) display more severe disease. The infiltration of DCs inside the CNS is also an important factor as they can present the local Ag to T cells even without exogenous myelin peptides demonstrating the capacity of DCs to migrate and to recognize the large variety of myelin Ags which reside in that tissue playing a crucial role in the propagation of the disease<sup>246,445</sup>. On the other hand, DCs have been shown to display some regulatory properties making them able to protect against CNS inflammation. In the context of EAE, pDCs are known to induce T cell anergy through the production of IFN- $\alpha$ , IL-10 and TGF- $\beta$  but more recently, Balley-Bucktrout et al demonstrated that the main mechanism through which pDCs can eliminate T-cell responses inside the CNS is in indolamine 2,3 dioxygenase (IDO)-dependent manner<sup>446</sup>. Additionally, a study of Yan Y et al showed that IDO can trigger the generation of Tregs via tryptophan metabolites which enhance production of TGF- $\beta$  from DCs promoting Treg expansion<sup>438</sup>. Interestingly, Mascanfroni ID et al provided evidence for the important regulatory role of IL-27 signaling on DCs since they showed that IL-27 induces the expression of the immunoregulatory molecule CD39 in DCs which is responsible for decreasing the concentration of ATP and downregulating nucleotide-dependent activation of the NLRP3 inflammasome, protecting against RR-EAE<sup>448</sup>. Potential targeted therapies on DCs implicate the expression on TIM-1 (T-cell immunoglobulin 1) which upregulate co-stimulatory molecules, production of pro-inflammatory cytokines and inhibition of T regulatory responses, enhancing EAE incidence and/or molecules which can inhibit the function of antigen-presentation such as chloroquine<sup>449</sup>. Finally, the production of pro-inflammatory cytokines by DCs is also an other field of interest and the inhibition of signaling pathways like NF- $\kappa$ B which can induce antigen-specific tolerance increasingly attracts the attention of scientists<sup>450</sup>. The role of costimulatory molecules in the interactions between DCs and T cells, is very important. We know that except for MHC:TCR-CD3-CD4/CD8 complex, CD80 and CD86 molecules expressed by DCs can interact with either CD28 or CTL-4 on T cells to determine either effector or suppressive functions in T cells<sup>451</sup>. More specifically, the B7/CD28 pathway (PDL1 and PDL2) has been shown to participate in the maintenance of

immune tolerance since mice deficient for the homologue B7-H1 display earlier neuroinflammatory responses characterized by the production of IFN- $\gamma$  and IL-17<sup>104,452,453</sup>.

### C) Natural Killer cells (NK)

NK cells are cells of the innate immune system which are known to display several important functions and among them we distinguish, their ability to interact with DCs, their participation in the lysis of tumor cells and regulation of the adaptive immune system through the production of cytokines, being a kind of defense against viral infections. In humans, this cell population consists of two subtypes according to the expression of a cell surface molecule, the CD56<sup>454</sup>. We distinguish the CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells. The first ones are characterized by the expression of high levels of CD16, perforin and inhibitory killer immunoglobulin receptors (KIRs) whereas the CD56<sup>bright</sup> are high for the expression of several cytokine receptors and CD94/NKG2A. They mainly reside inside the secondary lymphoid organs where they perform their cytokine and chemokine production while CD56<sup>dim</sup> are known to display cytolytic functions<sup>455</sup>. The role of NK cells in the context of MS/EAE has been investigated by many groups with some suggesting effector functions and others focusing on a potential regulatory role for the amelioration of the disease. Segal et al showed that the depletion of NK cells before the induction of the MOG<sub>35-55</sub>-induced EAE can aggravate the disease demonstrating an increased capacity of T cells to proliferate and to produce inflammatory cytokines<sup>456,457</sup>. Hammarmber et al performing a study in EAE mice induced with the use of MBP, showed that both T and NK cells are potent producers of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) whereas at the same time they can also participate in the damage of neurons through the production of pro-inflammatory cytokines such as TNF- $\alpha$  and IFN- $\gamma$ <sup>458</sup>. Some other studies suggest that different cytokines can facilitate the development of NK cells with deleterious effects such as IL-12 produced by astrocytes. IL-2 by NK cells enhance their perforin-like killer properties against motoneurons whereas IL-18 during neuroinflammation can trigger enhanced production of IFN- $\gamma$  by NK cells promoting the development of Th1 cells<sup>459-461</sup>. NK cells are known to be able to produce large quantities of IFN- $\gamma$ . Lunemann et al showed that CD56<sup>+</sup> NK cells lose their capacity to produce IFN- $\gamma$  in MS patients and that their CD16<sup>+</sup> display no cytolytic properties<sup>462</sup>. The importance of the migration of NK cells to the CNS had been reported by different studies either showing that mice deficient for the chemokine CX3CR1, which is known to be involved in that process of NK cells, are more susceptible to EAE or by depleting already CNS-residing NK cells with the use of antiasialo

GM1 exacerbating the clinical score of the disease<sup>463,464</sup>. The killing properties of NK cells against pathogenic T cells have been shown both in vitro and in vivo assays. Blocking the interaction of Qa-1b<sup>+</sup> T cells with NKG2A/CD95 NK cells can ameliorate EAE, proposing an inhibitory role for the Qa-1b molecule on T cells for the regulatory function of NK cells<sup>465</sup>.

#### **D) Natural Killer T cells (NKT)/Invariant Natural Killer T cells (iNKT)**

NK T cells are known to recognize lipid antigens in the context of MHC-1-like CD1d molecule and are characterized by the expression of signature cytokines such as IFN- $\gamma$  and IL-4. Jhang et al have demonstrated that activation of NK T cells with the glycolipid alpha-galactosylceramide (alpha-Galcer) can play a role in the pathogenesis of EAE enhancing the profile of Th1 cells producing more IFN- $\gamma$ , a similar cytokine profile can be induced also by administration of the cytokine IL-21<sup>257</sup> whereas activation prior to EAE induction can protect against EAE through the secretion of IL-4. At the same time Miyamoto et al showed that a synthetic glycolipid which is analogue of alpha-GC ligand for CD1d-restricted NK T cells, but not alpha-GC, is able to induce a switch of the pathogenic T cells into a Th2 profile through the production of IL-4 by NK T cells<sup>466</sup>. This kind of therapy can also be effective for other models of human diseases since the lack of polymorphisms of the CD1d molecule allows to target NK T cells in other pathological contexts. The important expression of CD1d by NK T cells for displaying a therapeutic effect upon stimulation with a specific ligand has been shown also by a study of Singh et al demonstrating that an injection of alpha-GC in CD1d<sup>-/-</sup> or beta-GC in WT mice, which binds to CD1d but can't be recognized by NK T cells did not confer protection to EAE mice<sup>467</sup>. In addition to their ability to display regulatory functions through the production of IL-4, NK T cells have been shown to be protective in other concepts and to be the target of already existing treatments. Recently, it has been shown that Natalizumab, which is used today as a treatment for MS patients because of its ability to inhibit the migration of leukocytes inside the CNS by blocking the alpha-4 subunit of the Very Late antigen (VLA-4), has a direct effect on NK T cells since these cells, either naïve or activated, express high levels of VLA-4 and the administration of anti-VLA-4 impairs the function and migratory ability of NK T cells inside the CNS<sup>468</sup>. NK T cells, expressing high levels of activating Fc $\gamma$  receptors have been shown to be the targeted cells in the case of IgG-treatment, which protects against EAE. IgG acts on NK T cells to trigger the induction of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg from naïve CD4<sup>+</sup> T cells through the production of IL-2, a cytokine whose signaling via IL-2R has been demonstrated to be responsible for the generation of CD56<sup>bright</sup> NK T cells, conferring protection in MS patients<sup>469,470</sup>. NK T cells are also able to

kill pathogenic T cells through a Fas/FasL mechanism upon their activation with an endogenous peptide–epitope from mouse collagen type II, recognized by CD1d NK T cells<sup>471</sup>. An additional cell population which is CD1d-restricted to glycolipid antigens is the invariant-NK T cells (iNKT). iNKT cells can also confer protection against EAE in a CD1d-dependent manner since the infiltration of NK cells inside the CNS keep them able to recognize glycolipid Ags activating them and inducing the production of IFN- $\gamma$ , IL-4, IL-10 and granzyme B<sup>472</sup>. Instead, Oh et al suggested that IL-4 and IL-10 producing-iNKT cells inhibit the Th1 cell response, but not the Th17. Moreover, iNKT-derived-IFN- $\gamma$  has a minimal effect in the regulation of the Th1 and Th17 responses in EAE<sup>473</sup>. Interestingly, more recently, a study from Parekh et al provided evidence for an additional regulatory mechanism of iNKT cells. They showed that the activation of these cells through administration of their CD1d-restricted ligand  $\alpha$ -GalCer triggered the expansion of Myeloid-Derived Suppressive Cells (MDSCs) with suppressive properties even inside the CNS. The immunoregulatory effect in this setting relies on IFN- $\gamma$  and IL-4 from NK T cells and through the induction of NO synthase, arginase 1 and IL-10 by MDSCs<sup>107</sup>. A newly described type of innate cells which share phenotypical characteristics of both NK T and DCs and are referred as Interferon-producing Killer DCs (IKDCs) have been shown to display regulatory properties in an EAE model, genetically induced with the MOG peptide fused to reovirus protein  $\sigma 1$ . These cells are characterized by the expression of CD11c<sup>+</sup> NK1.1<sup>+</sup> MHC-II<sup>high</sup> and upon activation by HVEM can express CD107a, PDL-1 and granzyme B and resolve CNS-autoimmunity<sup>474</sup>.

### **III-3-6 Stem cell therapies of EAE**

Several types of stem cells have been shown to be able to display potential therapeutic effects for the treatment of autoimmune diseases. Among them, we mainly distinguish, neural, mesenchymal and hematopoietic cells which increase our hope for more efficient cell therapies taking advantage of the unique properties of these stem cells.

#### **A) Neural Stem cells**

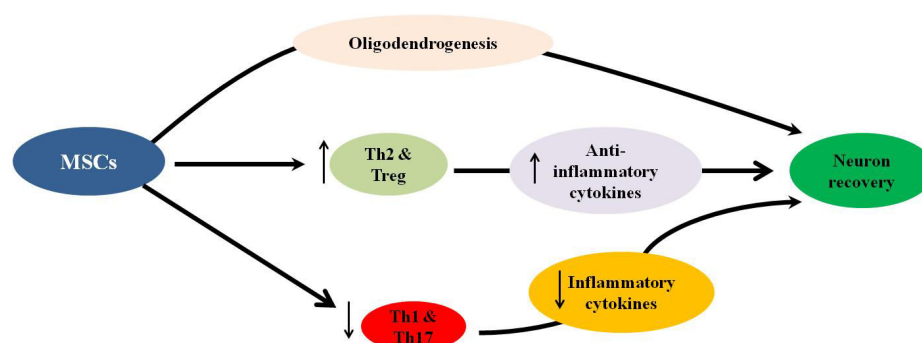
The main region for the isolation of neural stem cells is considered the subventricular zone of the lateral ventricle wall inside the adult central nervous system. Based on the properties of the Neural Stem Cells (NSCs) as self-renewing, multipotent cells with large migratory properties, they can easily give rise to adult oligodendrocytes and their use attracted the interest of many investigators. A neuroprotective or immunomodulatory effect of NSCs has been shown in different models of neurological diseases such as Parkinson, Huntington,

Stroke and spinal cord injuries. Today, many studies have shown the ability of NSCs and/or Neural Progenitor Cells (NPCs) to differentiate into mature oligodendrocytes and neurons, conferring protection to MS. Interestingly, Einstein et al have demonstrated that injection (i.v) of neural progenitor cells can protect against EAE even without being able to migrate inside the CNS by inhibiting encephalitogenic immune responses by CD4<sup>+</sup> T cells in the lymph nodes<sup>496</sup>. Plutchino et al provided more detailed evidence about the mechanism of action of these cells showing that they can upregulate the expression of a developmental stem cell factor, BMP-4, hampering the activation of myeloid dendritic cells (mDCs) and restraining the expansion of antigen-specific CD4<sup>+</sup> T cells<sup>497</sup>. Additionally, Hackett et al showed that the protective effect of NPCs is not dependent on FasL pathways since, injection of NPCs from *lpr* mice (Fas-deficient) can confer protection to EAE in a similar way as wild-type cells, inducing apoptosis of both Th1 and Th17 cells, enhancing further the hypothesis that the protective effect of these injected cells is due to their peripheral immunomodulation<sup>498</sup>. NPCs were also shown to be able to suppress Th17 responses in an EAE model through the expression of the Leukemia inhibitory factor (LIF) by blocking the phosphorylation of IL-6-induced signal transducer and activator of transcription 3 (STAT3) and triggering a signaling cascade that activated signal-regulated MAP kinase (ERK), up regulating the expression of the SOCS3 (suppressor of cytokine signaling 3)<sup>499</sup>. More recently, Deboux C et al showed that the injected NPCs enhanced their migratory capacities into cerebral endothelial cells and facilitated invasion of perivascular sites, through the upregulation of the expression of the molecule CD44 but with no effect on the proliferation and differentiation of the injected NPCs in EAE mice<sup>500</sup>. All the observations suggest that these progenitor cells could display a high therapeutic potential for the treatment of MS, finding the best balance in order to take advantage of all their regulatory properties.

## **B) Mesenchymal Stem Cells**

The therapeutic potential of stem cells has been confirmed also by the use of the mesenchymal stem cells. They have been shown to express MHC-I co-stimulatory molecules and display immune suppressive properties against the proliferation of allogeneic lymphocytes both in vitro and in vivo. This immunosuppressive function can be performed by a large variety of MSCs derived from different sources and tissues (mostly BM-derived) mainly through the production of IFN- $\gamma$ <sup>333,501–504</sup>. MSCs have been shown to be able to modulate immune responses in different ways. They can inhibit the production of pro-inflammatory cytokines such as TNF- $\alpha$  and IFN- $\gamma$  by both B and T cells or to upregulate the

expression of IL-10, IL-4 and IL-5 in stimulated T cells<sup>505,506</sup>. In addition to their cytokine modulation, Bone-marrow derived MSCs can suppress the proliferation of both T and B (even of stimulated B cells by anti-CD40) cells via a mechanism dependent of PD-1/PD-L1, PD-L2 interactions<sup>507,508</sup>. Some limitations on the use of these stem cells come from the fact that several immune cells have been shown to trigger the lysis of MSCs such as cytotoxic CD8<sup>+</sup> T cells or NK cells. It is a crucial point because in order for MSCs to perform long-lasting functions, their effectiveness should be not diminished if they are lysed soon after infusion. Additionally, in a case of graft-versus host disease (GVHD), it has been shown that MSCs can display their regulatory effects by triggering the activation and/or expansion of other immune cells, such as Tregs<sup>509-511</sup>. Their ability to exert immune-inhibitory functions, differentiate into neuron-like cells and migrate to the CNS as well, promote their use for a treatment of MS disease. Several studies have proposed the differentiation potential of MSCs to give rise to neuron-like cells, both in vitro and in vivo, since they express a large variety of neuron-specific markers like neurofilament M, synaptophysin and neuron specific nuclear proteins. More specifically, in EAE, BM-derived MSCs were able to ameliorate EAE recovery through stimulation of oligodendrogenesis, reducing the inflammatory infiltrates and demyelination, as well as modulating the production of inflammatory cytokines such as IL-17 and IFN- $\gamma$  by Th1 and Th17 cells in both lymph nodes and CNS, triggering a switch of T cell differentiation towards a Th2 profile and finally increasing the number of oligodendrocytes in the CNS tissues of EAE mice<sup>378,512-515</sup>. Additionally, the neuroprotective effect of the injected MSCs in EAE mice is associated with peripheral immunomodulatory properties in the lymph nodes, resulting in a decrease in the number of immune cells infiltrate in the CNS, by up regulating the expression of cytokines IL-10, IL-27 and IDO (indoleamine-2,3-dioxygenase), triggering also the expansion of Treg cells<sup>516</sup> (**Figure 20**).



**Figure 20.** Mesenchymal stem cells can induce neuron recovery in multiple sclerosis via a mechanism that stimulates oligodendrogenesis and decreases the numbers of Th1 and Th17 cells and their secretion of inflammatory cytokines while increasing the numbers of Th2 and Treg cells and their secretion of anti-inflammatory cytokines<sup>333</sup>. Al Jumah et al, Intern J Mol Scien, 2012

Apart from the protective effect of BM-derived MSCs, it has been shown that the intravenous administration of adipose-derived MSCs to EAE mice either before or after disease onset can ameliorate EAE incidence either by homing in the lymph nodes and migrating to the CNS where they decrease spinal cord inflammation or by inducing Th-2 type cytokine production and differentiation to neuron-like cells<sup>505</sup>. Additional mechanisms by which human BM-derived MSCs can display their suppressive effects include the secretion of PGE2, which is dependent on IDO, promoting the development of oligodendrocytes and neurons via the implication of several soluble factors. Additionally, it is known that MSCs can display either anti-apoptotic functions preventing neurons from apoptosis as well as mediate the differentiation and proliferation of endogenous NPCs via the secretion of NGF (neural-growth factor), VEGF (vascular endothelial growth factor), basic fibroblast growth factor and BMI-1 and inhibit CNS cells such as microglia to secrete pro-inflammatory factors such as nitric oxide (NO), TNF- $\alpha$ , IL-1 and reactive oxygen species<sup>517-520</sup>. The use of MSCs for treatment of MS patients should be investigated in more details since many questions remain still unclear including, how long the injected MSCs can keep their protective functions and if the age of recipients can influence their capacity of differentiation, migration and proliferation.

### C) Hematopoietic stem/progenitor cells (HSCs/HPCs)

The potential of HSCs in the field of cellular therapy for MS has been investigated by many groups, taking advantage of their differentiation capacities to give rise to all myeloid and lymphoid cell lineages. Until today, hematopoietic stem cell transplantation (HSCT) or bone marrow transplantation (BMT) is widely used for treatment of hematological malignancies but during last years, the same therapies have been also applied for autoimmune diseases such



as MS. The concept of HSCT includes the idea of destroying the pool of the aberrant T-cells of a MS patient, injecting hematopoietic stem/progenitor cells which can regenerate the patient's immune system with non-autoreactive T-cells. For that process, naive cells should replace the defective immune cells. For the mobilisation and proliferation of blood cells, granulocyte-colony stimulating factor (G-CSF) and/or cyclophosphamide (CY) are mainly used followed by the positive selection of CD34<sup>+</sup> cells, since CD34 is expressed by hematopoietic progenitor cells<sup>521</sup>. Mobilized grafts upon administration of cyclophosphamide, display a lower number of immune T-cell load which results in reduction of the inducible auto-reactive cells into the recipients. A common low-intensity regimen includes administration of cyclophosphamide together with a lymphocyte depleting antibody such as antithymocyte globulin (ATG) in comparison with more intensive regimens which include BEAM (BCNU-etoposide-cytarabine-melphalan) with ATG or busulfan with cyclophosphamide and ATG<sup>522-525</sup>. Treatments of Multiple Sclerosis has two main aspects : The first one includes immunomodulatory therapy for the immune nature of the disorder whereas the second one is to modify and ameliorate the symptoms. Since 1997, when the first complete study of MS treatment with the use of HSCT began, many others have followed with continuous changes in the treatment protocols in order to ameliorate the efficacy of the therapy<sup>526</sup>. After many trials, the protocols aiming at the elimination of only self-reactive T cells and not all of a patient's hematopoietic cells gained more supporters since it has been shown that HSCT display not only immunosuppressive but also immunomodulatory properties permitting that the patient's remaining hematopoietic cells would not differentiate into self-reactive T-cells<sup>527</sup>. In 2001, Carreras performed a phase II clinical trial in order to provide an additional evaluation for the efficiency of HSCT in MS patients. Only 1 out of 16 patients did not display hematological reconstitution after 2 HSCT attempts while the others either presented transient engraftment syndrome (3/16) and neurological deterioration (1/16) or high fever associated with myeloablative regimen. Because of the side effects such as loss of hair, premature menopause, higher risk of infections and drug reactions, the clinical trials of HSCT were restricted to MS patients with severe symptoms who had not responded to other immunotherapies. Another subject of discussion had been also the intensity of the conditioning regimen used. Several observations suggested that the use of low intensity HSCT regimens are less myeloablative with much less associated toxicity and mortality risk<sup>528,529</sup>. The majority of clinical trials that have been performed today are phase I/II safety with limited clinical applicability. Based on the fact that a big number of rapidly progressing MS patients do not respond to usual treatments, more and more clinical trials for these

patients should be implemented<sup>530</sup>. Although we have got encouraging results concerning the efficacy of HSCT in MS patients, more long-term observations should be evaluated. A new question has been opened in the last years, mainly if we can take advantage of the mobilisation conditions directly in vivo to induce an immune tolerance by the CD34<sup>+</sup> cells. In our laboratory, it has been demonstrated that the administration of G-CSF can confer protection to NOD mice against Type I Diabetes (T1D) through the involvement of dendritic cells which are responsible for recruiting T regulatory cells<sup>531,532</sup>. Parker MJ et al have shown that the prevention of T1D disease requires a combination of G-CSF + ATG whereas ATG or G-CSF alone seem to be unable to perform similar protective properties<sup>533</sup>. In humans, it has been shown that administration of G-CSF can also trigger the expansion of Tregs. Moreover, it has been demonstrated that bone-marrow derived hematopoietic progenitor cells can acquire regulatory properties through either the expression of FasL, production of TNF- $\alpha$  or inhibition of allogenic responses by inducing apoptosis in a caspase-dependent way<sup>534</sup>. Maki et collaborators have reported the emergence, within the bone marrow of NOD mice upon administration of IL-3 (hematopoietic growth factor), of an immature population of Thy-1<sup>+</sup>CD3<sup>low</sup>CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>-</sup> which is characterized by the ability to protect NOD mice from T1D<sup>535</sup>.

The main scientific interest of our laboratory is to identify new populations of hematopoietic progenitor cells with tolerating properties for a cell therapy of autoimmune diseases. The main animal model that has been used was the NOD and EAE mice. Today, it is widely known that the transplantations of hematopoietic progenitor cells occur using progenitors mobilized to the periphery upon administration mainly of G-CSF. In our laboratory, the idea was to verify if the mobilized progenitor cells can acquire tolerating properties in order to confer protection to NOD and EAE mice. In 2006, Kared H et al demonstrated that the injection of human recombinant G-CSF (200 $\mu$ g/Kg/day) together with Flt3L (20 $\mu$ g/Kg/day) for 4 days can trigger the mobilization of progenitor cells from the bone marrow to the spleen. These mobilized progenitors are characterized by the expression of Lin<sup>-</sup>c-kit<sup>high</sup>Sca-1<sup>high</sup>CD34<sup>+</sup>Flt3<sup>+</sup>CD106<sup>+</sup>CD127<sup>-</sup>, a phenotype which corresponds to a MPP2 stage of differentiation<sup>110</sup>. In comparison with the mobilized HPCs, the non-mobilized cells isolated from the bone marrow, displayed the phenotype of Lin<sup>-</sup>c-kit<sup>high</sup>Sca-1<sup>high</sup>CD34<sup>-</sup>Flt3<sup>-</sup>CD106<sup>int</sup>CD127<sup>-</sup>. Taking advantage of congenic markers and following the in vivo differentiation of the injected progenitor cells, it has been shown that these mobilized cells were indeed multipotent progenitor cells being able to give rise to several cell lines of immune system

such as CD4, CD8, B220, Gr1, CD11b, CD11c, PDCA-1 upon transfer into irradiated NOD mice. The injection of mobilized progenitor cells in NOD mice at the age of 6 weeks displayed a protective effect whereas the adoptive transfer of only  $10^4$  cells could prevent T1D for more than 5 months post-injection, even if injected in a later phase in 16-weeks-old NOD mice. Ex-vivo analysis of the injected and protected recipient mice provided evidence for an increase in the number of T regulatory cells, expressing high levels of Foxp3. After an isolation of these expanded Treg cells from recipient mice, it has been shown that they were able to suppress the proliferation of  $CD4^+CD25^-$  effector T cells in vitro and inhibit the transfer of diabetes in NOD-Scid recipient mice from diabetic splenocytes. Additionally, only the mobilized HPCs could increase the proliferation of T regulatory cells in vitro in the presence of anti-CD3, anti-CD28 and  $10\mu\text{g/ml}$  of IL-2. The presence of mobilized HPCs was necessary for the expression of high levels of Foxp3 and acquisition of suppressive capacities from Tregs. Interestingly, it has been shown that the mechanism, responsible for the increase of Tregs was not dependent on TGF- $\beta$  but a cell contact was needed for the stimulation of the Tregs proliferation from mobilized HPCs. This cell contact included Notch3 expression at the surface of T regulatory cells and the expression of Jagged2, which is known to be a Notch ligand, at HPCs. It has been confirmed that only the subtype of Jagged2<sup>+</sup> HPCs could confer protection to NOD mice, emphasizing the important role of the expression of Jagged2 by HPCs. In addition to this mechanism, the participation of the soluble factor GM-CSF secreted by mobilized HPCs and recognized by the receptor CD116 on T regulatory cells had been shown to be involved also in the expansion of Tregs. Interestingly, Gopissety et al showed that the protective effect of low doses of GM-CSF against T1D is mediated through the induction of tolerogenic CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> dendritic cells (DCs) and consequent expansion of Foxp3<sup>+</sup> regulatory T cells, showing that GM-CSF acted specifically on bone marrow precursor cells facilitating their differentiation into tolerogenic dendritic cells which directed Treg expansion in a contact-dependent manner. This mechanism required exogenous IL-2 and co-signaling from DC-bound OX40L and Jagged1<sup>536</sup>. We also recently demonstrated that CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup> DCs in G-CSF recipients, produced enhanced levels of the chemokine CCL22 which attracted CCR4<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (See Paper 4) in the pancreatic islets<sup>532</sup>.

### III-3-7 Current Therapies for Multiple Sclerosis

Despite the fact that the knowledge we have acquired on the pathogenic and potential therapeutic mechanisms of Multiple Sclerosis has been advanced considerably, Multiple Sclerosis remains an incurable autoimmune disease. Several treatments have been validated and are available in the commerce, which can improve the clinical outcome of MS patients.

Among them, we can distinguish treatments with IFN- $\beta$  which has been shown to display anti-inflammatory effects on the endothelial cells of the blood brain barrier together with its ability to modulate the activity of co-stimulatory molecules on dendritic cells which trigger the production of the anti-inflammatory cytokine IL-10, inducing a more anti-inflammatory environment inhibiting the proliferation of autoreactive T-cells<sup>475-478</sup>. Concerning the effect of IFN- $\beta$  on the endothelial cells of the BBB, it has been shown that it can block the migration of activated leukocytes inside the CNS by decreasing the expression of plasma endothelial microparticles, protecting endothelial cells from apoptosis and reducing as well the expression of matrix metalloproteinases which are known to be implicated in the disruption of the subendothelial matrix<sup>479-481</sup>. The inhibition of T-cell homing into the CNS is one of the main targeted therapeutic mechanisms in the case of MS. Many already existing treatments focus on that process in order to prevent from the local inflammation triggered by the activated T cells. The use of Natalizumab (humanized monoclonal antibody) is an anti-adhesion antibody, which targets the  $\alpha$ -chain of  $\alpha\beta$ -integrin, an adhesion-molecule, which is also recognized by the very late activating antigen (VLA-4). The interaction between the leukocytes which express VLA-4 and the vascular adhesion molecule-1 (VCAM-1) expressed by activated cerebral endothelial cells is a crucial step for the migration of pathogenic cells inside the CNS where they induce inflammation<sup>486,487</sup>. The concept of targeting an adhesion molecule as a potential therapy for MS, has been demonstrated for the first time by a study of Yednock et al in 1992, showing that the use of antibodies against the integrin molecule alpha 4 beta 1, prevented the entry of leukocytes in the central nervous system and the development of EAE disease<sup>488</sup>. The first clinical trial of natalizumab in MS patients showed about 70% reduction in the clinical relapse rate at 1 year and together with IFN- $\beta$ , the results were associated with less annualized relapse rate and much fewer new or expanding lesions in the brain<sup>489</sup>. In the same mechanistic concept is also the Fingolimod, which is a modulator of the action of sphingosine-1-phosphate (S1P) receptor.

Other drugs aim either at inhibiting the activation of T cells by making them unable to recognize through their TCRs the myelin-like antigens such as the Glatiramer Acetate which can bind to MHC-II molecules, inhibiting the binding of myelin-like proteins, which interact with TCRs and lead to the activation of auto-reactive pathogenic T cells. Clinical trials in MS patients have demonstrated that this treatment can reduce the frequency of relapses and contrast-enhancing lesions<sup>481-484</sup>. Interestingly, Begum-Haque S et al demonstrated that glatiramer-acetate could also affect the role of B cells, providing them with immunoregulatory properties in the context of EAE. They showed that GA-conditioned B cells are characterized by an increased production of suppressive cytokines whereas they can reduce CNS inflammation, decreasing the expression of the chemokine receptor CXCR5 and elevating the expression of BDNF in the CNS<sup>485</sup>.

The pathogenic role of B cells led scientists to design treatments targeting selective B cells. Rituximab is a chimeric (Human/mouse) monoclonal antibody with IgG1-heavy-chain and kappa light chain constant region sequences and mouse variable region sequences which targets specifically the CD20<sup>+</sup> B lymphocytes and it has been firstly shown to display therapeutic functions against non-Hodking lymphomas. CD20 is a surface molecule expressed by normal B lymphocytes and its precursors except plasma cells. Given that T lymphocytes and hematopoietic stem cells do not express this antigen, its administration targets the CD20<sup>+</sup> B lymphocytes in the peripheral circulation.

## Chapter IV: Type 1 Diabetes

### IV1 Generalities

Diabetes of type 1 and type 2 are two chronic pathologies characterized by an increase in the level of glucose in the blood (hyperglycemia) associated with serious consequences such as ketoacidosis, renal failure, cardiac disorders, stroke and blindness. The main symptoms of these diseases are significant excretion of urine (polyuria), thirst (polydipsia), constant hunger, weight loss, vision changes and fatigue. However, they are less marked for T2D (Type 2 Diabetes), which makes it difficult to diagnose the disease. On the contrary for T1D (Type I Diabetes), the symptoms can appear more suddenly. T2D is often associated with obesity, physical inactivity or at an older age, is mainly the result of resistance or misuse of insulin by the body and muscle or fat cells do not respond any more to normal levels of insulin produced by intact  $\beta$  cells. The T1D, is mostly declared in people whose age is less than 30 years and that is why it is called juvenile diabetes. It can still occur at any age. It is characterized by insufficient production or no producing at all of insulin and therefore requires daily insulin administration. Type 1 diabetes is a chronic autoimmune disease that is precipitated by environmental factors in genetically susceptible individuals. The body's own immune system attacks the  $\beta$  cells of islets of Langerhans, destroying or damaging them sufficiently to reduce or eliminate the production of insulin. In the current state of knowledge, it is still unknown how we can prevent its development.

### IV2 Epidemiology of Diabetes

Type 1 diabetes accounts for 5% to 10% of cases of diabetes diagnosed in the world which puts it far behind the type 2 non-insulin dependent diabetes. The WHO (World Health Organization) estimates that, currently, about 356 million people in the world are diagnosed with diabetes (including 10 to 15 million for type 1 diabetes), with 1.1 million deaths in 2005. According to the WHO, the number of diabetes deaths will double between 2005 and 2030. According to the Institut *de Veille Sanitaire*, the prevalence of type 1 diabetes in children and adolescents in France is 13.5 per 100 000 people (in 2004) and is increasing steadily for twenty years (3, 7% per year). A similar increase is observed in other European countries. If such an effect continues, the number of new cases will double in children at the age of 5 years and between 2005 and 2020 will increase by nearly 70% in individuals less than 15 years<sup>537</sup>.

### **IV3 Animal Model of Diabetes**

Although there are well established animal models for the study of Diabetes, the human disease will be always much more heterogeneous in terms of genetical and environmental factors which affect the time of its development. Nevertheless, for almost 30 years, our knowledge of the genetics and physiology of T1D is constantly enriched by the study of animal models of diabetes. Among them, we must distinguish between spontaneous and models whose disease is experimentally induced (eg with a toxic molecule for  $\beta$  cells of the pancreas such as streptozotocin). Among the spontaneous models, the most widely used are the NOD mouse (Non Obese Diabetic), the BB-DP rat (Bio Breeding Diabetes Prone), the KDP rat (Komeda Diabetes Prone) and LEW rat-IDDM (Lew.1AR1/Ztm).

### **IV4 Pathophysiology of T1D in NOD mice**

Investigating the pathophysiology of T1D in NOD mice, aims at understanding the pathology of diabetes in humans. In humans, studying organs from patients with T1D, it has been shown that the infiltrates of cadaveric pancreata include  $CD8^+$  T cells, macrophages and  $CD4^+$  T helper cells<sup>538</sup>. Infiltrates affect insulin positive islets as opposed to insulin deficient endocrine clusters. Analysis of  $\beta$  and  $\alpha$  cells suggested that there was only significant decrease of  $\beta$  cells together with persisting insulinitis and increased expression of the molecules MHC-I and Fas<sup>539</sup>. According to these observations, it has been proposed that specific immune reactivity to  $\beta$  cell antigens is a critical point to diabetes progression. In the NOD mouse, the disease has been shown to develop after an infiltration of pathogenic leukocytes into the pancreatic insulin-producing  $\beta$  cells. The first leukocytes can be found within islets at the age of 3 weeks, characterized mainly by the presence of dendritic cells and macrophages followed by both  $CD4^+$  and  $CD8^+$  T cells and B cells as well. The attack of  $\beta$  cells can be mediated either by killer  $CD8^+$  T cells through cytotoxicity using Fas and perforin/granzyme or via indirect factors such as proinflammatory cytokines and free radical production<sup>540,541</sup>. Other cell types, which have been shown to play also critical roles in the destruction of  $\beta$  cells, are macrophages, DCs, T cell subsets and B cells. While antibodies do not seem to be implicated in T1D development in the NOD mouse, complement-fixing antibodies are present in patients with T1D and these antibodies are cytolytic to human  $\beta$  cells<sup>542</sup>.

In the NOD mouse, many studies have confirmed the major role of T cells as the main effectors of  $\beta$  cell destruction, associated with autoreactive  $CD4^+$  and  $CD8^+$  T cell subsets and altered function of regulatory T cells. DCs and macrophages that are found in early pancreatic

islet infiltrates drive early autoreactive immune responses<sup>543,544</sup> whereas complete depletion of macrophages, or depletion of T cells from a macrophage-deficient environment have been shown to be associated with less severe form of T1D<sup>545,546</sup>. Additionally, DCs have been shown also to be critical in the initiation of T cell responses or induction of tolerance, suggesting an importance of innate immune cells to activate adaptive immunity, promoting pathogenic or tolerogenic responses<sup>544-546</sup>. The necessity of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells for T1D induction has been shown by adoptive transfer experiments, showing that T cells can recognize specific peptide antigens in the context of MHC-complexes molecules such as HLA (Human Leukocyte Antigens) through the involvement of the TCR. The recognition of autoantigens in the context of MHC has been shown to be more than crucial in initiating an autoimmune response in human and NOD diabetes. An additional factor, which may be necessary for the development of T1D is the breakdown of the immune tolerance. This mechanism is affected by many factors such as location, time of exposure and the concentration of the antigen but also by the co-stimulation. Many studies aimed at identifying malfunctions, which cause diabetes in NOD mice pointing to defect in central tolerance. Among these studies, some of them described morphological abnormalities of thymic stroma<sup>550</sup> and a lack of migration of thymocytes<sup>551</sup> that may affect the process of thymic selection. Other studies put forward a comprehensive defect in the negative selection process. As already mentioned, there is a similarity between the genetic susceptibility locus in humans and in NOD mice. In NOD mice, the region the most susceptible to T1D is the locus containing the genes of the MHC (Idd1). The NOD mouse features a particular MHC haplotype called H-2g7. It contains among other conventional molecules, MHC class I (Kd and Db), and a single class II molecule I-Ag7. Comparing this unique I-Ag7 molecule to other autoimmune prone mouse strains reveals the presence of a substitution of an aspartic by a serine at position 57 of the  $\beta$  chain<sup>552</sup>. Thus, it has been shown that I-Ag7 has a larger repertoire of peptide but with a reduced affinity<sup>553,554</sup>, which probably affects the process of negative selection. Evidence for the involvement of this molecule in the development of diabetes in NOD mice has been provided by experiments showing that homozygosity for the haplotype H-2g7 was necessary. Other studies had demonstrated that the introduction of a functional molecule or replacement of IE allele I-Ag7 by another allelic variant helped to induce protection against diabetes<sup>555-557</sup>. Additional work emphasized the importance of ectopic expression of antigens in the thymus on the incidence of autoimmune diseases. Ectopic phenomenon was originally identified in patients with the syndrome APECED (Autoimmune Polyendocrinopathy, Ectodermal Dysplasia and Candidiasis) which is



characterized by the spontaneous development of autoimmunity in various endocrine organs (including T1D in most individuals). Deficient thymic stroma area is sufficient to transfer the disease in B6 mice with normal Nude Aire gene. The team of Fornari et al recently demonstrated a decrease in the thymus of the Aire gene expression in pre-diabetic NOD mice. Recent studies on insulin in NOD mice support the model of a controlled area of central tolerance<sup>558</sup>. In rodents, two independent insulin genes, (*ins1* and *ins2*) are located on different chromosomes. Apparently, *ins2* seems to be preferentially expressed in the dependent *ins1* region of the mouse thymus<sup>559</sup>. Two groups have examined the consequences of a *ins2* deletion in the NOD mice and due to the fact that the NOD *ins2*-KO mice still have a normal gene *ins1*, they continue to produce insulin in a glucose-dependent manner in the pancreas, but is very reduced in the thymus<sup>560,561</sup>. The mice deficient for *ins2* have been shown to develop diabetes earlier than wild-type NOD mice. Additionally, a high percentage of pathogenic T cells isolated from early pancreatic infiltrate in NOD mice recognize insulin. In fact, it seems that there is a unique relationship between the expression of certain TCR V region and recognition of peptide (B9-23) of the  $\alpha$  chain of insulin. However preclinical evidence suggests that it occurs a sequential loss of tolerance to multiple epitopes during the progression of the disease. For example, tolerance to proinsulin prevents the induction of T-cells reactive to IGRP, while mice tolerant for IGRP develop diabetes<sup>562</sup>. Thus the expression of insulin in the thymus protects against diabetes. To be noticed that in humans the insulin gene (*INS*) is located in the region of susceptibility to diabetes IDDM2. Some alleles in VNTR (variable number tandem repeat) region of the *INS* promoter are associated with decreased gene expression in the thymus and an increased risk of T1D. Kishimoto et al have shown that thymocytes from NOD mice had an altered sensitivity to induced negative selection with an anti-CD3 monoclonal antibody or a superantigen (which allows the binding of T-APC) in vitro and in vivo<sup>563</sup>. This effect involves Fas-dependent and Fas-independent pathways. Another study comparing transgenic mice with a TCR specific for a self-antigen, either in NOD or B6 background, showed a reduced ability to exert negative selection of autoreactive cells in mice with NOD background<sup>564</sup>. This lack of intrinsic apoptosis in T cells has been linked to susceptibility locus *idd6*. NOD mice display many defects in thymic selection that would allow filtering an abnormal amount of autoreactive T cells to the periphery. Interactions of TCR/MHC and peptide have been also involved in the development of immune responses in the periphery.

On the other hand, it is known that defects occur during the interaction of B cells with auto-

antigens of low affinity. The inability of NOD mice to make tolerant B cells that could recognize soluble self-Ags may allow the development of specific B cells that could act as diabetogenic APCs. B cells from NOD mice are characterized by a higher induction of inflammatory responses compared to non-autoimmune strains, mainly through the activation of the NF- $\kappa$ B signaling pathway and higher expression of costimulatory molecules such as CD40, CD72 and CD86<sup>565,566</sup>. In that context, follicular B cells are considered to be good candidates for APCs, mainly because of their location in the arteriolar lymphoid organs, which facilitate the interaction with T cells and also through their ability to flow inside the pancreatic lymphoid glands. Other subpopulations of B cells such as Transitional, B1 or MZ B cells can also display functions responsible for developing diabetes. The MZ B cells, which are associated with T-cell independent responses, can be powerful APCs for naïve T cells. Interestingly, it has been shown that the frequency and expansion of MZ B cells can be increased with autoreactive responses in several autoimmune diseases<sup>567</sup>. The MZ B cells, which are normally associated with independent antibody responses of T cells can be powerful APCs for naïve T cells. Compartment MZ-B cells are characterized by increased numbers of autoreactive specificities, expansion and aberrant migration in several autoimmune diseases.

### **A) T effector cells**

As already mentioned, it had been demonstrated by several experimental procedures, that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells display effector functions for the development of T1D. These cells infiltrate the peri pancreatic tissue where they modify the balance between immunity and tolerance. During thymic selection, T cells acquire a co-receptor that will influence their future role in the immune system. CD8<sup>+</sup> T cells exert cytotoxic functions (hence the name of cytotoxic T lymphocytes: CTL) and the CD4<sup>+</sup> T cells can differentiate in a large number of helper T cell types (T helper Th) with pathogenic features. CD8<sup>+</sup> T cells can directly exert a cytotoxic effect on  $\beta$  cells by releasing granzyme B, after recognizing MHC-I complexes expressed on the surface of  $\beta$  cells<sup>568</sup>. Similar to CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells can produce cytokines such as IFN- $\gamma$ , which induce expression of Fas and chemokine production by  $\beta$  cells. An activation of the Fas/FasL signaling is also responsible for inducing apoptosis of  $\beta$  cells. Under those circumstances, the interferon- $\gamma$  displays an active destructive role, triggering macrophages to produce pro-inflammatory cytokines such as IL-1 and TNF- $\alpha$ .

Numerous studies have demonstrated an intrinsic defect of the effector T cells in NOD mice. Generally, it is quite accepted that effector Th1 responses are associated with disease progression in NOD mice whereas the Th2 responses can inhibit the development of diabetes. One of the most potent inducers of Th1 response, the cytokine IL-12 accelerates the disease<sup>569</sup>. However, NOD IL-12 KO mice normally develop diabetes. The *idd4* susceptibility locus is associated with overexpression of IL12p40 by macrophages of the NOD mouse. In contrast, it appears that the orientation towards a Th2 response protects against T1D. For example, either injection of IL-4<sup>570</sup> and of other Th2-related cytokines or Th2 cytokines' overexpression has been shown to protect islets to become diabetogenic. Moreover, the incidence of diabetes is not altered in IL-4 KO NOD mice and transfer of Th2-oriented cells in the NOD Scid cells can still induce diabetes<sup>566</sup>. Nevertheless, the factors that disrupt the Th1/Th2 balance may directly affect susceptibility to T1D. CD28/B7 costimulation seems to regulate the balance between Th1 and Th2 orientation and this is why the more aggressive nature of diabetes in CD28 KO mice was attributed to Th1 differentiation because of the absence of Th2 differentiation<sup>168</sup>. Other studies have shown that diabetes in NOD mice is regulated also by the expression of transcription factors, which control Th1/Th2 differentiation. Inactivation of the STAT4 transcription factor that controls the IFN- $\gamma$  signaling reduced T1D, by compromising the pathogenic T cell responses<sup>572,573</sup>. Interestingly, inactivation of *Tbx21*, a gene that encodes the transcription factor, which controls the expression of *Tbet*, improves the incidence of the disease. However, this beneficial effect by *Tbet* may be related to the migration of Th1 cells and the function of DCs<sup>574</sup>. The identification of T-cells producing IL-17 (Th17) and IL-23, opened new questions about the pathogenic potential of these cells in the model of diabetes. Interestingly, it has been shown that Th17 cells purified from NOD BDC2.5 mice, can be converted into Th1 cells when transferred into NOD.Scid mice inducing the development of diabetes<sup>575</sup>.

## **B) T regulatory cells**

During a normal immune response, the body will develop mechanisms, which can negatively control autoimmune responses or limit the collateral damage that might be deleterious to him. Regulation of the immune response is a complex process, which undoubtedly requires the action of multiple regulators on a wide variety of effector cells. Indeed, at the onset of peripancreatic infiltrate a battle between the effector arm, including all cells able to produce pro-inflammatory cytokines, and the regulatory arm, including mainly a type of T regulatory cells, will drive the decision of the restoration of tolerance or not. This "battle" may be responsible

for the long prodromal phase that lasts several months until insulinitis and diabetes have been established. As it has been mentioned already in a previous part, T regulatory cells are known to display their protective properties either through the production of anti-inflammatory cytokines such as TGF- $\beta$  and IL-10 or by inducing the expression of enzymes which consume amino acid at the exposure of DCs, decreasing the expression of costimulatory molecules such as CD80 and CD86<sup>576,577</sup>. Activated regulatory T cells also express CD73 and 5'ectonucleotidase ATPase/ADPase CD39<sup>166</sup> which gives them the ability to hydrolyze extracellular ATP to adenosine, thus promoting a hypoxic environment and triggering the entry of effector T cells into anergy (which potentially retain the ability to produce IL-10).

The respective share of cell contact and suppressive cytokines in the action of Treg and Tr1 *in vivo* remains to be elucidated. In the NOD mouse, thymectomy leads to an acceleration of the development of diabetes<sup>578</sup>. This accelerated the disease when it is committed under the age of three weeks. Thus, in models of adoptive transfer after the selective depletion of regulatory populations based on markers CD4, CD25, CD62L and CD45RB, it was determined that CD4<sup>+</sup>CD62L<sup>+</sup> T cells control diabetes, the CD4<sup>+</sup>CD25<sup>+</sup> control gastritis and diabetes whereas the CD4<sup>+</sup>CD45RB<sup>low</sup> cells control colitis<sup>579</sup>. Several studies have shown that the number and function of Treg was altered during the disease in NOD mice. At the onset of the disease, the number and function of regulatory T cells, and more particularly the CD25<sup>+</sup> subpopulation in the pancreas was significantly reduced in inflammatory islets (not within the pancreatic lymph nodes). This problem was attributed to a failure to produce IL-2 and to a reduction of the expression of CD25 and Bcl-2<sup>580</sup>. Some subpopulations of Treg begin to lose their expression of FoxP3 and produce IFN- $\gamma$ . Upon an adoptive transfer of these cells, they generate pathogenic memory T cells that contribute to the onset of the disease<sup>581</sup>. The susceptibility locus *idd3* contains the gene coding for IL-2 expression, which is required to maintain the regulatory cells.

### C) Interleukin 21 (IL-21) in T1D

Genetic studies have suggested that IL-21 could be important in the development of type 1 diabetes in the NOD mouse, as the gene encoding this cytokine is within the *idd3* susceptibility locus<sup>582</sup>. Several anomalies related to this cytokine have been reported in NOD mice. Firstly, NOD mice which develop diabetes have been shown to overexpress IL-21<sup>583</sup>. Biswas et al have recently demonstrated that overexpression of IL-21 was related to aberrant

activation of the serine-threonine kinase ROCK2, which phosphorylated IRF4 and affected its ability to control the production of IL-17 and IL-21 by T cells of the NOD mouse. It has also been suggested that the overexpression of IL-21 promoted homeostatic proliferation of autoreactive CD8<sup>+</sup> T cells<sup>584,585</sup>. It has also recently been shown that IL-21 allowed CD8<sup>+</sup> T cells to recognize self-antigens of lower affinity<sup>586</sup>. It was demonstrated that the production of IL-21 increased during the development of diabetes and that a deficiency of the receptor for IL-21 (IL-21R<sup>-/-</sup>) conferred resistance to insulinitis in NOD mice by reducing the production of autoantibodies and the development of diabetes<sup>587,588</sup>. Although this data is controversial, the lymphoid compartment of NOD IL-21<sup>-/-</sup> mice seems normal and does not contain higher concentrations of regulatory T cells. However unlike wild-type autoreactive T cells, autoreactive T cells from the IL-21<sup>-/-</sup> mice are defective because they are unable to cause disease when transferred in NOD SCID mice. Although T1D is considered a Th1-oriented disease, these data and the fact that IL-21 is an amplifier factor of Th17 response, suggest that IL-21 and Th17 lineage play a central role in the NOD mouse.

#### **D) Dendritic Cells**

Similar to other cells of the innate immune system, dendritic cells play a crucial role in the immune defense of our body. In the context of diabetes, it has been suggested that they are involved in the pathogenesis acting as antigen-presenting cells, absorbing and presenting autoantigens from  $\beta$  cells. The cDCs of NOD mice can trigger higher activation of T cells through an increased production of IL-12 and expression of costimulatory molecules<sup>589</sup>. The initiation of diabetes in NOD mice is also characterized by an increased production of type I IFN by pDCs<sup>590</sup>. In the model of diabetes, the IL-27 seems to participate in a complex way leading to an increase of production of pro-inflammatory cytokines such as IFN- $\gamma$  and IL-17 and decrease of the anti-inflammatory agents such as cytokines IL-4, TGF- $\beta$  and IL-10 secreted by diabetic splenocytes. Interestingly, it is also known that IL-27 promotes the production of IL-10 in the absence of IFN- $\gamma$ . These effects are inconsistent with the results obtained in C57BL/6J mice in which IL-27 inhibits the differentiation of Th17 cells and induces T cells to produce IL-10. However, it has been also reported that the DCs can produce large amounts of IL-27 under the influence of IFN- $\gamma$  produced by CD4<sup>+</sup> T cells. Therefore, blocking of IL-27 results in the reduction of the incidence of diabetes in NOD mice<sup>591</sup>. However, DCs have been also shown to exert a protective function, since mice deficient for dendritic cells display a fatal development of autoimmune diseases, which highlights their important role in the mechanisms of peripheral tolerance<sup>592</sup>. Indeed, DCs may be able to

induce anergy or deletion of autoreactive T cells and promote the expansion of regulatory T cells. Additionally, it has been demonstrated that tolerogenic DCs can emerge *in vivo* since treatment of NOD mice with G-CSF mobilizes pDCs to the spleen and prevent the development of T1D<sup>531</sup>. These DCs induce tolerance by promoting the production of TGF- $\beta$  by CD4<sup>+</sup>CD25<sup>+</sup>. Recently, Layseca et al showed that only a subset of CD8 $\alpha$ <sup>-</sup> DCs can produce the chemokine CCL22 which attracts Treg cells, producing TGF- $\beta$ <sup>532</sup>. Similar protective effect has been attributed also to the Flt3L (FMS-like tyrosine kinase 3 ligand) which promotes the differentiation of DCs and protects against T1D when injected in young NOD mice. It has been shown to increase the frequency of CD4<sup>+</sup>CD25<sup>+</sup> Tregs in the pancreatic lymph nodes triggering a switch of cDCs to CD8<sup>+</sup> cDCs which exert tolerogenic functions<sup>593</sup>. On the other hand, Flt3L accelerates diabetes incidence in NOD mice at the age of 15 weeks<sup>594</sup>. Several molecules expressed by pDCs are involved in inducing tolerance, such as PDL-1 and IDO. Saxena et al have shown that during the transfer of naive T cells from NOD mice BDC2.5 in NOD-SCID mice, the pDCs are able to inhibit diabetogenic responses of CD4<sup>+</sup> T cells, inducing IDO expression in the pancreas and preventing the onset of diabetes<sup>595</sup>. IDO catalyzes the oxidative catabolism of tryptophan, an essential nutrient of T cells and thereby regulates the immune response of T cells. In young NOD mice, there is a lack of expression of IDO, arguing for a lack of control of immune responses in the animal<sup>596</sup>.

### **E) Macrophages**

Numerous studies have provided evidence for the presence of early macrophage infiltration in the pancreatic islets where inhibition of their flow to the pancreas by blocking their adhesion receptors can inhibit T1D in NOD mice. Macrophages are recruited to potential islets via the secretion of CCL2 (CC-chemokine ligand 2) by  $\beta$  cells and CCL1 by CD4<sup>+</sup> T cells. *In vitro* and *in vivo* studies have shown that the deleterious effects of macrophages on  $\beta$  cells could be induced by TNF- $\alpha$  and IL1- $\beta$ <sup>597</sup>. It is interesting to note that the inflammatory macrophages can be detected in pancreatic islets before infiltration of T cells<sup>598</sup>. The macrophages can produce IL-12 which promotes the differentiation of cytotoxic diabetogenic CD8<sup>+</sup> T cells (CTLs). Macrophages recruited to the pancreas produce IL-1 $\beta$ , TNF, and reactive oxygen species (ROS) such as nitric oxide, which can cause death of  $\beta$  cells.

## F) Natural Killer Cells (NK cells)

Natural killer (NK) cells are responsible for early protection against viruses and they induce the death of infected or cancer cells. They are endowed with cytotoxic activities and are also able to produce cytokines and particularly IFN- $\gamma$ . This cytokine is known to be capable of inducing the expression of FAS and chemokine production by  $\beta$  cells. Studies show a correlation between the frequency or activation of NK cells and the destructive potential of the infiltrates<sup>599,600</sup>. NK cells isolated from the pancreas of NOD mice display an active phenotype characterized by the expression of CD25, CD69, and PD1, KLRG1 (killer cell lectin -like receptor group phenotype G, member 1)<sup>601</sup>. These NK cells proliferate more and spontaneously produce high levels of IFN- $\gamma$ , expressing on their surface the exocytosis granulocyte marker CD107a which highlights their cytotoxic function<sup>602</sup>. Similar to macrophages, NK cells are present in the pancreas before infiltration of T cells. Numerous studies using wild-type and transgenic NOD mice showed that NK cell depletion prevented the onset of T1D<sup>603,604</sup>. NK cells are known to naturally express NKG2D (Natural killer group 2, member D) whose expression is also present on the surface of  $\gamma\delta$  T cells, activated CD8<sup>+</sup> T cells and NKT cells. The activated NK cells from NOD mice express both NKG2D and its ligand RAE1 (retinoic acid early transcript 1). This inappropriate overexpression of RAE-1 induces internalization of receptor-ligand complex, affecting the activity of NK cells and contributing to the observed defect in NOD mice. Note that NKG2D is also expressed on the surface of  $\beta$  cells in NOD mice and is associated with their damage. The engagement of NKp46, of another NK cell receptor, by an unknown ligand (NKp46L) at the surface of  $\beta$  cells causes degranulation of NK cells and the death of  $\beta$  cells<sup>602,605</sup>. Despite their pathogenic role in T1D, several studies suggest that NK cells may exert protective functions since NK cells from the blood of patients with T1D or lymphoid tissues of NOD mice have been shown to display impaired function<sup>606</sup>. Since this defect is observed in patients with long-term diabetes, it could be a consequence of the disease. However, injection of CFA (complete Freund 's adjuvant) in young NOD mice induces the production of INF- $\gamma$  by NK cells and prevents diabetes. The protective role of NK in this context has been demonstrated in adoptive transfer experiments<sup>607</sup>. Experiments of islets allografts have shown also the protective role of NK cells, which has been linked to their ability to kill DCs through perforin<sup>608</sup>.

### **G) Invariant Natural Killer T cells (iNKT)**

Although iNKT cells have been shown to promote immunity against pathogens, several studies have demonstrated their protective role in diabetes<sup>609</sup>. Indeed, the stimulation of these cells by the  $\alpha$ -galactosylceramide reduced the incidence of diabetes in NOD mice as well as increased their frequency by an adoptive transfer or by introduction of a transgene *V $\alpha$ 1-J $\alpha$  18*. This protection may be mediated by the induction of a Th2 response in the islets. Some studies also reported the inhibition of the differentiation of CD4<sup>+</sup> and CD8<sup>+</sup> T effector cells in the pancreatic lymph nodes by recruiting tolerogenic DCs<sup>609,610</sup>.

### **H) Effector B cells**

The pathogenetic role of B cells has been demonstrated in the NOD mouse model, since the mice which lack B cells either by a mutation of the gene encoding for  $\mu$ -chain of the immunoglobulin<sup>611</sup> or treated with anti-IgM Ab were strongly protected from type 1 diabetes<sup>612</sup>. The B cells have been also shown to act as APCs in the pathology of T1D<sup>566,613,614</sup> as well as through their autoantibody output<sup>615,616</sup> and their ability to alter the microenvironment<sup>617</sup>. Therefore, many experimental protocols have been reported, related with trials of removal of B cells in mice. Among them, those that target surface proteins with monoclonal antibodies against CD22 or CD20 of murine origin (mCD20) or human (hCD20) in a transgenic mouse model. Additionally, there are protocols, which aim at neutralizing signaling pathways of BAFF and/or APRIL, responsible for the development of B cells, in particular with anti-BAFF antibody, treatment with Fc-BCMA, or by blocking fusion proteins. Although these experimental protocols have shown that a potential removal of B cells can confer protection to NOD mice against T1D, it was interesting to clarify which are the targeted populations, the kinetics and detailed mechanisms of action. The short treatment with an anti-CD20 antibody is known to target immature populations of the bone marrow, transitional follicular cells of the spleen and lymph nodes and the memory cells as well. However, the anti-CD20 has been shown to suppress some of B-1 cells and also plasma cells, which are characterized by a low expression of CD20. Treatment with an anti-CD20 in NOD mice, whose B cells express human CD20, has been shown to delay the progression of diabetes. When anti-CD20 was injected into already diabetic mice, there has been a reduction of glycemia, suggesting that B cells are not involved in the initial stage of the T-cell responses<sup>618</sup>. More generally, it has been demonstrated that treatment with anti-CD20 antibodies in NOD mice at the age of 5 weeks, can efficiently prevent the onset of the disease



in over 60% of the treated mice<sup>619</sup>. On the other hand, depletion with the use of anti-CD22 Abs leads to a partial removal of B cells since it is not expressed on immature B cells. In NOD mice, it can significantly delay the onset of diabetes and 50% of treated mice remained protected for a long-time post-depletion. The efficacy of the depletion system by anti-CD22, can also be ameliorated by fixing the antibody with the cytotoxic agent calicheamicin (Cal). The injection of this fusion agent has been shown to restore normoglycemia in 100% of mice when injected in already diabetic mice<sup>620</sup>. The treatment with BCMA-Fc or anti-BAFF Ab has been shown to inhibit the access of B cells to survival signal by blocking both soluble and transmembrane signaling by BAFF and/or APRIL. These agents are not able to trigger the depletion of B cell function as fast as anti-CD20 or CD22 Abs treatment does. Generally, the use of anti-BAFF Ab is more effective on the MZ-B and that of BCMA-Fc on the FO-B. Although the B and B-1 memory cells express both BAFF-R and TACI, they are insensitive to treatment with the anti-BAFF or BCMA-Fc<sup>621</sup>. The long-term treatment of NOD mice with anti-BAFF Ab induced the depletion of marginal zone and follicular zone B cells, resulting in a significant delay in the initiation of diabetes. The anti-BAFF Ab restores the normal negative selection of B cells between the stages of transitional and follicular B cells which can efficiently reduce the number of auto-reactive cells<sup>622</sup>. A common characteristic of all experimental methods that are based on depletion of B cells is the inhibition of both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses. Some targeted-therapies based on the depletion of B cells have been already used clinically. Rituximab, for example is an antibody that recognizes the human CD20. This antibody significantly reduced the symptoms in many patients with rheumatoid arthritis (RA), it is used in the treatment of patients with systemic lupus erythematosus (SLE)<sup>623</sup> and it also preserves the loss of function of  $\beta$  cells in T1D patients<sup>624</sup>. The LymphoStat-B, an anti-BAFF monoclonal antibody, is currently in Phase III of clinical trials for the treatment of SLE<sup>625</sup> whereas the Atacicept, blocking BAFF and APRIL, is in clinical trials in patients with SLE or rheumatoid arthritis (RA)<sup>626,627</sup>.

### **I) T1D and B regulatory cells**

B cells have been implicated in the development of type 1 diabetes mellitus. Studies in nonobese diabetic (NOD) mice have shown that B cells are the first to penetrate pancreatic islet cells and interact with T cells in lymphoid tissues to form autoreactive B cells. Pancreatic islet cells in NOD mice have also been found to possess a population of CD1d<sup>+</sup> B cells, which is one of the cell markers found on Breg. However, it is not clear whether these CD1d cells have a suppressive role in the pancreas or contribute to the development of diabetes in mice.

Additionally, Jogannathan M et al showed that in patients with diabetes, activation of TLRs can regulate the production of cytokines by B cells whereas altered TLR function can lead to elevated expression of IL-8 and downregulation of the production of IL-10<sup>628</sup>. Other studies have shown that B cell-deficient mice failed to develop diabetes. This has led to the hypothesis that B cells could be the target of treatment against autoimmune attack of pancreatic beta cells by B cells<sup>629,630</sup>. Tian et al have demonstrated that LPS-activated B cells, but not control B cells, express Fas ligand and secrete TGF- $\beta$ , leading diabetogenic T cell to apoptosis, inhibiting Th1 autoimmunity and APC activity<sup>631</sup>. Interestingly, it has been shown that although bone marrow production is normal in NOD mice, transitional B cell production collapses at the age of 3 weeks, reflecting a lack of successful immature B cell migration to the periphery, delay the establishment of the follicular pool and a lack of selection at the transitional checkpoint<sup>632</sup>. In order to examine the relative importance of APC functions of B-cells, Wang FS et al generated B-cell-deficient nonobese diabetic (NOD) mice that express a transgene encoding a mutant heavy chain immunoglobulin transgene at the cell surface but cannot secrete immunoglobulins (mIgs). This allowed them to dissect the importance of the relative roles of antigen presentation, dissociated from antibody production, finding that the expression of the mIg transgene increased insulinitis and the incidence of diabetes compared with transgene-negative NOD B-cell-deficient mice, indicating that the ability to produce antibodies is not necessary for B-cells to have some effect on the development of diabetes. However, diabetes was not restored to the level seen in normal NOD mice<sup>571,633</sup>. Interestingly, Hu C et al reported that human CD20 transgenic mice treated with anti-CD20 mAb respond to the treatment and present a long-term response in mice with higher frequencies of T2-MZP B cells over MZ B cells<sup>618</sup>.

More recently, in our laboratory, we demonstrated that CpG-activated proB cells from the bone marrow can confer protection to NOD mice against T1D upon an adoptive transfer. In vitro, CpG-proB cells can lead effector T-cells to apoptosis through the activation of FasL signaling and it has been shown that the production of IFN- $\gamma$  by the pro-B cells plays a crucial role for this mechanism. Moreover, in vivo, the injection of pro-B cells can dramatically reduce the production of IL-21 by T effectors. The protective effect of these CpG-activated proB cells is also due to their progeny since the injected pro-B cells differentiated into more mature B cells, including T2-MZP, follicular and marginal zone B cells, which were characterized by the expression of high levels of FasL, comparing with that of host B cells, keeping the ability to induce apoptosis of activated effector T cells<sup>634</sup>.

## Results

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The aim of this thesis was to investigate a potential protective effect of activated hematopoietic progenitor cells against two different models of autoimmune diseases : Type I Diabetes and Experimental Autoimmune Encephalomyelitis.

It is known that various infections or other stimuli of the immune system can trigger changes to the hematopoietical process. We investigated whether the stimulation of TLRs can induce the emergence of selective progenitor cells within the bone marrow with immunoregulatory properties. A new population of CpG-activated pro-B cells emerged in both NOD and C57BL/6J mice. We addressed the question if these activated progenitor cells can protect NOD mice against T1D and C57BL/6J mice against MOG<sub>35-55</sub>-induced EAE. A comparative study regarding the mechanism of action of these progenitors in these two models has been performed (**Paper 1 and Paper 2**).

In our laboratory, it has been shown that a cocktail of hematopoietic factors (G-CSF and Flt3l) can induce the mobilisation of progenitor cells from the bone marrow to the spleen. These mobilized progenitors are at the stage of differentiation of MPP2 and are able to trigger the expansion of Foxp3<sup>+</sup> Tregs through a cell-to-cell contact mechanism including interaction of the ligand Jagged2 with its receptor Notch3 and of the soluble factor GM-CSF on CD116 expressed by T regulatory cells. The adoptive transfer of a low number of mobilized progenitors could confer protection to NOD mice against T1D.

We investigated if the same mobilized progenitor cells can be protective also against an animal of model of MS. An adoptive transfer of about 25,000 cells at day 0 of the disease was sufficient in order to control MOG<sub>35-55</sub>-induced EAE disease in C57BL/6J mice. In that project, we tried to dissect the mechanism of action and to compare with our findings in the case of T1D (**Paper 3**).

### Contribution to other papers

G-CSF has been shown to prevent type I diabetes in the NOD mouse by inducing dendritic cells to promote Treg expansion. Recently, in our laboratory, we demonstrated that the particular DC subset which mediates this effect is within the CD11c<sup>high</sup>CD8α<sup>-</sup> cells. Their adoptive transfer enhanced the pancreatic recruitment of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg by secreting the chemokine CCL22 which attracts CCR4<sup>+</sup> Tregs. (**Paper 4**)

## Scientific Papers

1) *Innate pro-B-cell progenitors protect against type 1 diabetes by regulating autoimmune effector T cells*

Ruddy Montandon\*, **Sarantis Korniotis**\*, Esther Layseca-Espinosa, Christophe Gras, Jerome Megret, Sophie Ezine, Michel Dy, and Flora Zavala \***contributed equally**

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2) *Innate CpG-activated pro-B cells protect against ongoing Experimental Autoimmune Encephalomyelitis by restraining T-cell homing to the CNS and reducing the production of pathogenic cytokines*

**Sarantis Korniotis**, Ruddy Montandon, Christophe Gras, Hélène Letscher, Jerome Megret, Stephanie Favre, Simon Fillatreau, Sanjiv Luther and Flora Zavala

***(in preparation)***

3) *Mobilized multipotent progenitor-derived IL-1 $\beta$  drives enhanced regulatory Foxp3<sup>+</sup> T cell expansion and protects against experimental autoimmune encephalomyelitis*

**Sarantis Korniotis**\*, Maud D'Aveni\*, Julien Rossignol, Christophe Gras, Guillemette Fouquet, Ruddy Montandon, Nicolas Cagnard, Marie-Thérèse Rubio, Olivier Hermine and Flora Zavala \***contributed equally**

***(in preparation)***

4) *CCL22-producing CD8 $\alpha$  myeloid dendritic cells mediate regulatory T cell recruitment in response to G-CSF treatment*

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## **Paper 1**

**Innate pro-B-cell progenitors protect against type 1 diabetes by regulating autoimmune effector T cells**

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# Innate pro-B-cell progenitors protect against type 1 diabetes by regulating autoimmune effector T cells

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**Diverse hematopoietic progenitors, including myeloid populations arising in inflammatory and tumoral conditions and multipotent cells, mobilized by hematopoietic growth factors or emerging during parasitic infections, display tolerogenic properties. Innate immune stimuli confer regulatory functions to various mature B-cell subsets but immature B-cell progenitors endowed with suppressive properties per se or after differentiating into more mature regulatory B cells remain to be characterized. Herein we provide evidence for innate pro-B cells (CpG-proBs) that emerged within the bone marrow both in vitro and in vivo upon Toll-like receptor-9 activation and whose adoptive transfer protected nonobese diabetic mice against type 1 diabetes (T1D). These cells responded to IFN- $\gamma$  released by activated effector T cells (Teffs), by up-regulating their Fas ligand (FasL) expression, which enabled them to kill Teffs through apoptosis. In turn, IFN- $\gamma$  derived from CpG-proBs enhanced IFN- $\gamma$  while dramatically reducing IL-21 production by Teffs. In keeping with the crucial pathogenic role played by IL-21 in T1D, adoptively transferred IFN- $\gamma$ -deficient CpG-proBs did not prevent T1D development. Additionally, CpG-proBs matured in vivo into diverse pancreatic and splenic suppressive FasL<sup>high</sup> B-cell subsets. CpG-proBs may become instrumental in cell therapy of autoimmune diseases either on their own or as graft complement in autologous stem cell transplantation.**

immune therapy | peripheral tolerance | MyD88 signaling | B lymphocytes | T lymphocytes

**A** growing body of evidence attests that immune cells with immunoregulatory functions do not exclusively belong to mature populations of diverse lineage, but also comprise several hematopoietic progenitor subsets. The first subset to be recognized comprised myeloid progenitors that acquired suppressive properties in tumoral and inflammatory environments (1) and played either detrimental or beneficial roles in different pathological situations. We have reported previously that mobilization with hematopoietic growth factors conferred tolerogenic properties to multipotent hematopoietic progenitors at the multipotent progenitor (MPP2) stage of differentiation that enabled them to promote the expansion of regulatory T cells (2, 3), thereby preventing spontaneous autoimmune type 1 diabetes (T1D) in the nonobese diabetic (NOD) mouse model. Moreover, parasitic infections were shown to stimulate via IL-25 the emergence of Th2 cytokine-secreting MPPs (MPP<sup>Th2</sup>) that ultimately differentiated into mature cell types with pro-Th2 functions, thus contributing to parasitic clearance (4).

Direct interactions between pathogens and hematopoietic stem cells occur through Toll-like receptor (TLR) activation, driving their differentiation along myeloid pathways to enforce anti-infectious defenses (5, 6). TLR agonists also promote hematopoiesis by enhancing the production of the hematopoietic growth factor G-CSF, with whom they synergize to mobilize hematopoietic stem cells from the bone marrow to the periphery (7).

TLR-mediated innate-type stimulation by infectious (8) and parasitic (9) agents also plays a major role in promoting the

emergence of regulatory B cells (Bregs), along with acquired-type stimulation, such as B-cell receptor (BCR) engagement concomitant or not with CD40 activation (10, 11). Such induced regulatory B-cell functions are believed to be more robust than those expressed by naive and resting B cells, which can nevertheless tolerate naive T cells and induce regulatory T cells (Tregs) (12, 13).

Bregs are a heterogeneous lymphocyte subset present among all major B-cell populations (14–17). The rare so-called B10 cells identified by their CD19<sup>+</sup>CD1d<sup>hi</sup>CD5<sup>+</sup> phenotype (18–20), peritoneal CD5<sup>+</sup> B1a cells (21, 22), large follicular B cells, and activated transitional, marginal zone (MZ) B cells can all acquire regulatory properties. The most immature Breg subset described so far is composed of B220<sup>+</sup>IgM<sup>+</sup>CD21<sup>low</sup>CD93<sup>+</sup>CD23<sup>+</sup> transitional T2 MZ precursor B (T2 MZP-B) cells, which are continuously produced in adult bone marrow and home to the MZ of the spleen, where they differentiate into IgM<sup>high</sup>CD1d<sup>high</sup>CD21<sup>high</sup>CD23<sup>low</sup> MZ B cells (23, 24).

The differentiation pathways of the various Breg subsets remain unknown. Only functional precursors, named “B10pro,” which are mature B cells requiring additional BCR-activating antigenic signals to produce immunosuppressive IL-10 but cannot be distinguished from B10 cells by phenotypic criteria, have hitherto been identified (25). It is unknown whether Bregs derive from one or several progenitors or solely from conventional B-cell subsets. Moreover, an immature B-cell progenitor population endowed with suppressive properties per se or after differentiation into more mature Bregs has not been demonstrated as yet.

Herein we describe a hematopoietic progenitor population that emerges transiently in vitro and in vivo in the bone marrow of

## Significance

**Immunoregulatory properties have been principally ascribed to various mature immune cell types, including regulatory B cells. An immature B-cell progenitor population endowed with suppressive properties per se or after differentiation into more mature regulatory B cells has not been demonstrated as yet. We now describe a pro-B-cell progenitor population that emerged upon stimulation with the Toll-like receptor-9 ligand CpG and prevented disease upon adoptive transfer into autoimmune type 1 diabetes-prone mice. Effector T cells were the target of immunoregulatory pro-B cells and of their mature progeny. Such protective pro-B cells could be instrumental for cell therapy of autoimmune diseases.**

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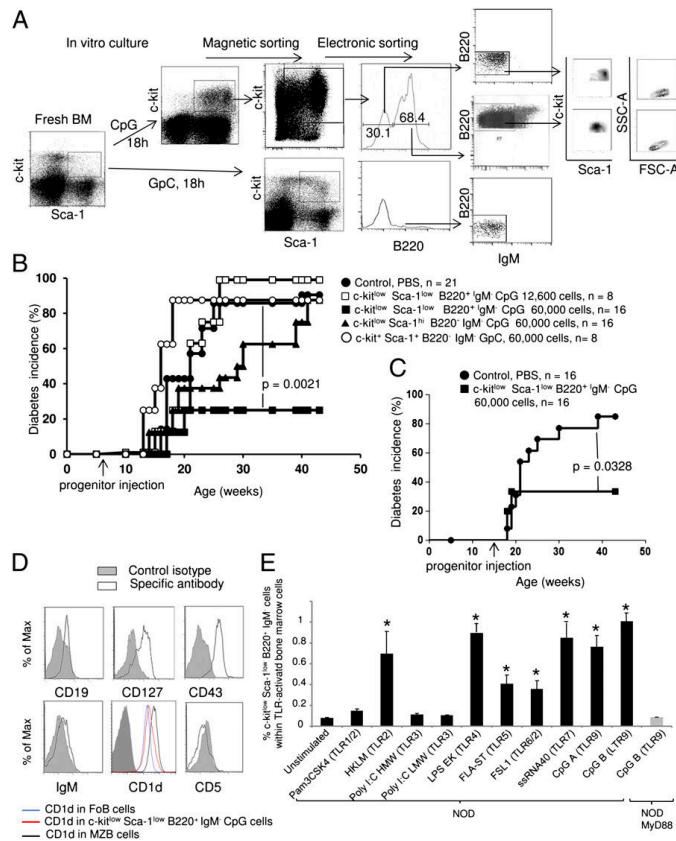
NOD mice, after activation with the TLR-9 agonist CpG and whose adoptive transfer into NOD mice prevents T1D onset. These cells were c-kit<sup>low</sup>Sca-1<sup>low</sup>CD127<sup>+</sup>B220<sup>+</sup>CD19<sup>+</sup>IgM<sup>-</sup>CD1d<sup>int</sup>CD43<sup>+</sup>, a phenotype consistent with a pro-B-cell stage of differentiation, except for their CD1d expression. The cells differentiated in vivo exclusively into B lymphocytes, at various stages of maturation.

Functionally, these TLR-induced hematopoietic progenitors suppressed pathogenic effector T cells (Teffs) by reducing their IL-21 production and by inducing their apoptosis via Fas ligand (FasL). Additionally, the B-cell progeny of CpG-induced proBs continued to express high levels of FasL and to suppress Teffs,

and may play a major role in the durable protection against T1D provided by the progenitors in vivo.

**Results**

**c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup> Bone Marrow Progenitors Emerging upon Exposure to CpG Prevent T1D.** Incubation of bone marrow cells from NOD mice with the oligonucleotide CpG 1668 (CpG-B), but not with control GpC oligonucleotide, led to the transient emergence of a c-kit<sup>low</sup>Sca-1<sup>+</sup> cell population within 18 h. These cells were heterogeneous in terms of size, Sca-1 and B220 expression (Fig. 1A), and could be further sorted into a small-size Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> and a large-size Sca-1<sup>high</sup>B220<sup>+</sup>IgM<sup>-</sup> fraction. Adoptive transfer to NOD mice at 6 wk of age revealed that only small-size CpG-



**Fig. 1.** Phenotypic characterization and prevention of T1D in vivo by a CpG-induced c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> bone marrow subset. (A) Bone marrow (BM) cells, incubated for 18 h with CpG-B (1 µg/mL), were magnetically selected for c-kit<sup>+</sup> cells, further stained for Sca-1, B220, and IgM and electronically sorted into large-size (FSC<sup>high</sup>SSC<sup>high</sup>) c-kit<sup>low</sup>Sca-1<sup>high</sup>B220<sup>+</sup>IgM<sup>-</sup> and small-size (FSC<sup>low</sup>SSC<sup>low</sup>) c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> cells. BM cells incubated with the control oligonucleotide GpC were electronically sorted as c-kit<sup>+</sup>Sca-1<sup>+</sup> cells that were B220<sup>+</sup>IgM<sup>-</sup>. (B) Diabetes incidence in NOD mice injected with PBS or after intravenous transfer of the above sorted subsets at 6-wk of age, *P* = 0.0021 by Kaplan–Meier estimates when comparing cumulative incidence curves for controls and for CpG-induced c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> progenitor recipients, not significant for other groups. (C) Incidence of T1D in NOD mice injected intravenously either with PBS or with 60 × 10<sup>3</sup> CpG-induced c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> progenitors at 16 wk of age, *P* = 0.0328. Results in B and C are pooled from two experiments. (D) Phenotypic characterization of the protective bone marrow progenitor subset by flow cytometry analysis of expression of CD19, CD127, CD43, IgM, CD1d, and CD5. Cells were stained with specific antibodies (open histograms) or control isotype antibodies (filled histograms) after FACS sorting. CD1d level on CpG-induced c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> progenitors (red histogram) was compared with that measured on splenic follicular B cells (FoB, blue histogram) and MZ B cells (MZB, black histogram). (E) Percentage of c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> cells emerging in the bone marrow of NOD mice after 18-h incubation with different TLR-agonists. CpG-B was tested in bone marrow cultures of both NOD and NOD MyD88<sup>-/-</sup> mice. Results are expressed as mean ± SEM of three experiments. \**P* < 0.05, when comparing BM incubated with different stimuli to unstimulated BM.

induced c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> cells protected against T1D in vivo, whereas their large-size c-kit<sup>low</sup>Sca-1<sup>high</sup>B220<sup>+</sup>IgM<sup>-</sup> counterpart had no such effect (Fig. 1B). c-kit<sup>+</sup>Sca-1<sup>+</sup> cells isolated from bone marrow cells incubated with the control oligonucleotide GpC that were negative for B220 and IgM expression, did not prevent the onset of T1D (Fig. 1B). Note that small-size c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> cells remained protective in 66% of mice when injected as late as at 16 wk of age, which corresponds to disease onset (Fig. 1C).

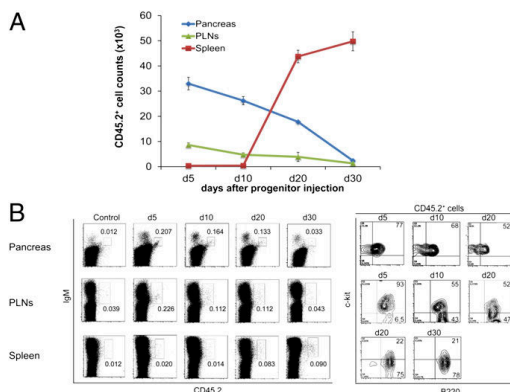
**Protective c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup> Cells Share the Characteristics of B-Cell Progenitors at the Pro-B Stage.** The protective c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup> subset expressed CD19 at low levels together with the IL-7R $\alpha$  chain CD127 and CD43 but no IgM, in keeping with an immature B-cell phenotype at a pro-B-cell stage of differentiation. CpG-induced cells were also positive for CD1d, at intermediate levels between CD1d<sup>low</sup> follicular B cells and CD1d<sup>high</sup> MZ B cells, but CD5 was not consistently detected (Fig. 1D). Among the TLR agonists tested, those activating TLR-2, -4, -5, -6, and -7 induced a progenitor population with the same phenotype as that induced by CpG, conversely to compounds targeting TLR-1 and -3 (Fig. 1E). CpG-A (1586) and -B (1826 or 1668) had a similar effect. The protective pro-B population did not arise in the bone marrow of myeloid differentiation primary response gene 88 (MyD88)-deficient NOD mice incubated with CpG-B (Fig. 1E).

At variance with the data of Welner et al. (26), who used C57BL/6 mice to establish that incubation with CpG-A for 48 h directed common lymphocyte progenitors toward differentiation into PDCA-1<sup>+</sup> plasmacytoid dendritic cells (PDCs) at the expense of the B-cell lineage, the protective pro-B subset induced in the NOD mouse after a 18-h incubation with CpG-B did not express the dendritic cell markers CD11c or PDCA-1.

**CpG-Induced Pro-B Cells Mature Exclusively into B Cells in Vivo.** Small-size c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> cells, isolated from congenic NOD CD45.2 bone marrow cells incubated with CpG-B were electronically sorted before transfer to NOD mice (70,000 progenitors per mouse). The protective progenitors were then analyzed in different tissues for migration and in vivo differentiation potential. Approximately 50% of injected CD45.2<sup>+</sup> cells were found as early as 5 d after adoptive transfer in the pancreas, whereas only 7% were located in the pancreatic lymph nodes (PLNs) at the same time point (Fig. 2A). CD45.2<sup>+</sup> cells gradually decreased over time in pancreas and PLNs, becoming undetectable at day 30 posttransfer. Conversely, between day 10 and day 20 posttransfer, a large proportion of CD45.2<sup>+</sup> cells, representing ~65% of injected cells, migrated to the spleen where their numbers remained stable afterward. CD45.2<sup>+</sup> cells remained detectable up to 3 mo posttransfer in the spleen. A minor percentage was present in the peritoneal cavity, but not in bone marrow, blood, or liver.

The differentiation status of injected CD45.2<sup>+</sup> cells was followed by their expression of IgM, c-kit, and B220 (Fig. 2B). CD45.2<sup>+</sup> cells have matured into IgM<sup>low</sup>c-kit<sup>+</sup>B220<sup>+</sup> cells in the pancreas from day 5 until day 20, and in PLNs as far as day 5. Thereafter in PLNs, between day 10 and day 20, CD45.2<sup>+</sup> cells gradually lost the expression of c-kit, while acquiring higher IgM expression. Finally, in the spleen, the bulk of CD45.2<sup>+</sup> cells were c-kit<sup>-</sup>B220<sup>+</sup>IgM<sup>+</sup>, corresponding to mature B cells as the final progeny.

The mature c-kit<sup>-</sup>IgM<sup>+</sup>B220<sup>+</sup>CD45.2<sup>+</sup> B-cell progeny was further analyzed by FACS (Fig. 3A) in the spleen and PLNs at day 30 and at day 20 posttransfer, respectively, where it consisted exclusively of B cells sharing the CD19<sup>+</sup>B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>+</sup>IgD<sup>+</sup> phenotype. Neither CD11b<sup>+</sup> nor CD11c<sup>+</sup> cells were found within CD45.2<sup>+</sup> cells (Fig. 3B). In the spleen, these CD45.2<sup>+</sup> cells represented ~0.1% of all cells and 0.3% of B cells and were CD1d<sup>+</sup>CD5<sup>-/low</sup> (Fig. 3B). These cells were mostly follicular (40%) and MZ (15%) B cells, as well as T2 MZ B precursors (15%) and



**Fig. 2.** Time-dependent evaluation of recovered CD45.2<sup>+</sup> cells in pancreas, PLNs, and spleen. (A) Absolute cell counts of total CD45.2<sup>+</sup> cells recovered from the pancreas, PLNs, and spleen of CD45.1<sup>+</sup> mice at different time points after injection of 70,000 CD45.2<sup>+</sup> protective progenitors per mouse. Results are expressed as the mean  $\pm$  SEM of two to four mice per point. (B) FACS analysis of the maturation status of CD45.2<sup>+</sup> cells in recipient mice over time. The proportion of IgM<sup>low</sup>/CD45.2<sup>+</sup> cells in pancreas, PLNs and spleen (Left) as well as their expression of c-kit and B220 (Right) at indicated time points after progenitor injection in CD45.1<sup>+</sup> NOD recipients are depicted. A representative experiment of two to four is shown.

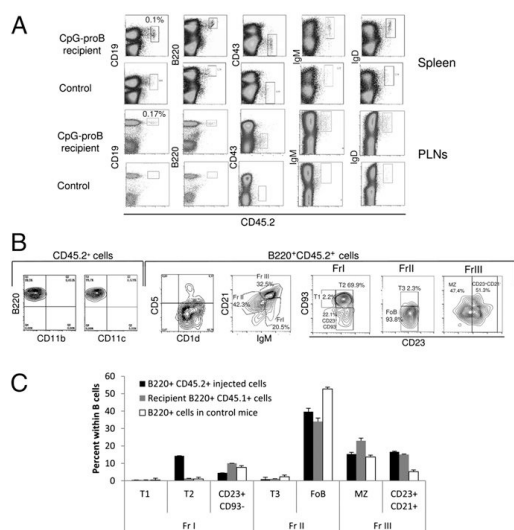
CD21<sup>+</sup>CD23<sup>high</sup> cells (15%) (Fig. 3B and C), as defined by their relative expression of CD93, IgM, CD21, and CD23 (27).

Furthermore, the adoptive transfer of the progenitors had no significant effect on the proportion of the various B-cell subsets in the spleen of the recipients, relative to age-matched noninjected controls (Fig. 3C).

#### CpG-proBs Suppress Teff Proliferation and Trigger Their Apoptosis.

To assess whether CpG-proBs modulated Treg or Teff proliferation, we cocultured c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>-</sup> CpG-proBs with either CD4<sup>+</sup>CD25<sup>high</sup> Tregs (all Foxp3<sup>+</sup>) or CD4<sup>+</sup>CD25<sup>-</sup> Tefs, each T-cell population electronically sorted from the spleen of NOD mice. CpG-proBs did not modify the proliferation of Tregs, but suppressed the expansion of Tefs, as measured by dilution of carboxyfluorescein diacetate succinimidyl ester (CFSE) staining (Fig. 4A) after 5 d of coculture at a Tef: CpG-proB ratio of 2:1. We assessed by FACS analysis among gated B220<sup>+</sup> or CD4<sup>+</sup> cells that during the 5 d of coculture, the CpG-proB cells remained essentially alive, excluding Topro III (Fig. 4B), whereas only 12% of T cells cultured alone but ~70% of cocultured T cells died, incorporating ToproIII (Fig. 4C). In contrast, neither the c-kit<sup>+</sup>Sca-1<sup>+</sup> subset sorted from bone marrow cells incubated with the control oligonucleotide GpC nor control pro-B cells purified ex vivo from bone marrow cells as a c-kit<sup>+</sup>Sca-1<sup>+</sup>B220<sup>+</sup>CD24<sup>hi</sup>CD43<sup>hi</sup> population shared this suppressive function (Fig. 4D). This result occurred independently from IL-2 (Fig. 4E) and coincided with apoptosis of Tefs evidenced both by annexin-V staining and To-ProIII incorporation (Fig. 4F and G). CpG-proBs expressed a variety of molecules inducing death and tolerance, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and programmed death ligand (PDL)-1, PDL-2, respectively. However, none of the corresponding neutralizing antibodies restored Teff proliferation, apart from anti-FasL antibody, which allowed a recovery of cell divisions (Fig. 4H and I). Notably, FasL was expressed not only on CpG-proB cells derived from bone marrow cultures with CpG (Fig. 5C) but also on the pancreatic CD45.2<sup>+</sup>IgM<sup>low</sup>B220<sup>+</sup>c-kit<sup>+</sup> cells recovered between day 5 and





**Fig. 3.** Phenotypic characterization of the IgM<sup>+</sup>CD45.2<sup>+</sup> progeny of CpG-induced pro-B-cell progenitors. (A) Flow cytometry analysis of the IgM<sup>+</sup>CD45.2<sup>+</sup> progeny in CpG-proB-injected recipients compared with untreated CD45.1<sup>+</sup> controls, for cell-surface expression of the B-cell markers CD19, B220, CD43, IgM, and IgD, performed in spleen at day 30 and in PLNs at day 20 after injection of the CD45.2<sup>+</sup> progenitors. (B) Flow cytometry analysis of the expression of CD11b and CD11c within splenic CD45.2<sup>+</sup> cells and of the frequency of the B220<sup>+</sup>CD45.2<sup>+</sup> splenic progeny of the CpG-induced proB cell progenitors, recovered 1 mo after progenitor injection, in diverse B-cell subfractions according to the expression of CD5 and CD1d, or CD21, IgM, CD93, and CD23. (C) Histogram representation of the percentages of the different B-cell subsets as defined in B, among injected CD45.2<sup>+</sup> cells, CD45.1<sup>+</sup> cells of the recipients and cells from noninjected age-matched control mice. Data from A and B are from one representative experiment of at least three. Data in C are expressed as mean  $\pm$  SEM of three experiments.

day 20 after adoptive transfer of the progenitors and on the splenic mature IgM<sup>+</sup>B220<sup>+</sup>CD45.2<sup>+</sup> B-cell progeny recovered 30 d after progenitor injection. Notably, FasL levels of the CpG-proB progeny were even higher than those exhibited by the recipient's IgM<sup>+</sup>CD45.2<sup>+</sup> cells analyzed at the same time in the corresponding tissues (Fig. 4J).

Moreover, when electronically sorted 1 mo after adoptive transfer of the progenitors, the splenic CD45.2<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup> B-cell progeny triggered Teff apoptosis during coculture (Fig. 4K) and suppressed Teff proliferation (Fig. 4L).

**Teffs Control Their Own Expansion by Producing IFN- $\gamma$ , Which Confers Suppressive Properties to CpG-proBs.** CpG-proBs required 5 d of coculture with Teffs to express optimal suppressive activity. On day 3, the cells had no effect on their target cells, emphasizing the requirement of prior conditioning by activated Teffs (Fig. 5A). In addition, even after 5 d the cells failed to inhibit the proliferation of Teffs sorted from IFN- $\gamma$ -deficient NOD mice (Fig. 5B). This lack of effect coincided with a significantly reduced up-regulation of FasL on the surface of CpG-proBs (Fig. 5C), in support of the conclusion that IFN- $\gamma$  derived from activated inflammatory Teffs renders CpG-proBs suppressive by up-regulating their FasL expression.

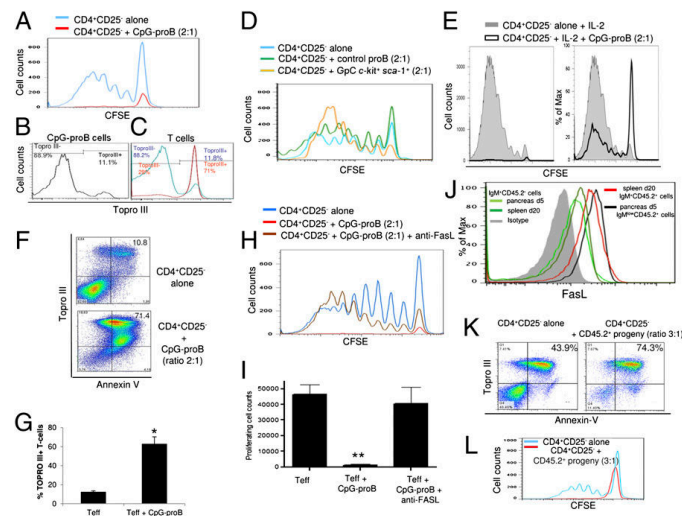
**Progenitor-Derived IFN- $\gamma$  Reduces IL-21 Production by Teffs During Coculture with CpG-proBs and Is Required in Vivo for Protection Against T1D by CpG-proBs.** After 5 d of coculture with CpG-proBs, spared Teffs were electronically sorted and their mRNA

was extracted. Real-time RT-PCR analysis revealed that over 30 genes of 89 were modulated in the Lonza Th17 pathway-oriented transcripts (Table 1). In keeping with the enhanced FasL expression on the surface of CpG-proBs, FasL mRNA levels were increased 55-fold in Teffs, suggesting enhanced suicide. Transcripts for cytotoxicity-related molecules, such as granzyme B and the transcription factor coesodermin, were likewise amplified. The latter is known for inhibiting Th17 differentiation (28), which is further hampered in our experimental setup by the down-regulation of IL-12R $\beta$ 1, the binding site of IL-23 (29). Instead, Th1-oriented gene expression was strongly up-regulated, including IFN- $\gamma$ , t-Bet (Tbx21), and IL-12R $\beta$ 2, the target of IL-12 p70, whereas transcription of genes associated with a Th2 profile, such as GATA-3 and IL-13, was reduced. In addition, up-regulation of coesodermin, which causes the repression of IL-21 production (28), occurred together with decreased transcription of inducible costimulator (ICOS), a positive regulator of IL-21 (30). Both effects culminated in a dramatic 77-fold reduction of mRNA encoding IL-21, an autocrine factor abundantly produced by Th17 cells that promotes or sustains Th17 lineage commitment (31). Given that IL-21 does also inhibit IFN- $\gamma$  production in developing Th1 cells by repressing coesodermin expression (32), the regulatory loop initiated by CpG-proBs clearly favors Th1 over Th17 differentiation of Teffs.

Cytokine assays in supernatants from cocultures set up with Teffs and CpG-proBs from wild-type or IFN- $\gamma$ -deficient mice (Fig. 5D) suggested that optimal production of IFN- $\gamma$  depended on or originated from both cellular sources. Intracytoplasmic flow cytometry revealed that CpG-proBs already produced IFN- $\gamma$  when sorted from CpG-treated bone marrow cells, a production that was further enhanced during coculture with activated Teffs, but declined in CpG-proBs cultured alone (Fig. 5E). Taking advantage of the fact that similar progenitors emerged in bone marrow cells from IFN- $\gamma$ -deficient NOD mice cultured with CpG-B (Fig. 5F), we evaluated how progenitor-derived IFN- $\gamma$  affected IL-21 production by Teffs by coculturing wild-type target cells with CpG-proBs recovered from mice that were either competent or deficient for the production of IFN- $\gamma$ . We found that this cytokine was essential both for increased coesodermin expression (Fig. 5G) and enhanced IFN- $\gamma$  transcription in Teffs themselves, as well as for the decrease of IL-21 mRNA levels (Fig. 5H). The importance in vivo as well of IFN- $\gamma$  production by CpG-proBs was further emphasized by the failure of CpG-proBs prepared from IFN- $\gamma$ -deficient NOD mice to provide long-lasting protection against T1D in NOD mice (Fig. 5I). Hence, IFN- $\gamma$ , generated by both Teffs and CpG-proBs, plays a key role in the regulatory cross-talk between the two cell populations.

Finally, sphingosine 1-phosphate receptor, which is required for Teff egress from secondary lymphoid organs, was reduced fivefold upon coculture (Table 1), suggesting that exposure to CpG-proBs not only killed Teffs, and hampered their Th17 differentiation, but eventually prevented their recruitment to target tissues.

**CpG-proB-Induced Protection Against T1D Is Associated with Decreased IL-21 Production and Effector Memory T-Cell Loss but Not with Modulation of IL-10 Production.** IL-21 has recently been reported for its deleterious role in both early and late phases of T1D in the NOD mouse (33–35). Pancreatic IL-21 levels were significantly reduced in NOD mice having received CpG-proBs (Fig. 6A). Plasmatic levels of IL-21 were below detection limits. These data suggested that the CpG-proBs and their progeny, which prevented T1D onset, also targeted IL-21-producing Teffs in vivo in the NOD mouse. This finding could result from their killing Teffs or their reducing the capacity of T-cells to produce IL-21. We found that activated CD4<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup> memory effectors were significantly diminished in the spleen, PLNs, and



**Fig. 4.** Mechanisms underlying the suppressive properties of CpG-proBs. (A) Proliferation of sorted CFSE-loaded CD4<sup>+</sup>CD25<sup>+</sup> T cells 5 d after activation with anti-CD3 + anti-CD28 when cocultured at a 2:1 ratio with CpG-proBs. (B and C) Viability of gated B220<sup>+</sup> CpG-proBs (B) and gated CD4<sup>+</sup> T-cells (C) in the coculture. Dead and live cells are distinguished by their capacity to take up or exclude Topro III, respectively. (D) Proliferation of sorted CFSE-loaded CD4<sup>+</sup>CD25<sup>+</sup> T cells 5 d after activation with anti-CD3 + anti-CD28 when cocultured at a 2:1 ratio with control proBs or c-kit<sup>+</sup>Sca-1<sup>+</sup> cells from GpC-treated bone marrow. (E) Teff proliferation in coculture with CpG-proBs with or without 10 U/mL of IL-2. (F and G) Apoptosis measured by annexin V staining of phosphatidylserine and Topro III incorporation among gated Teffs cocultured with CpG-proBs (ratio 2:1) at day 5 after anti-CD3 + anti-CD28 activation. Numbers in the *Upper Right* quadrants represent the percentage of dead cells. Histograms in G represent the mean  $\pm$  SEM of four different experiments, \* $P = 0.0286$ . (H and I) Reversal of the antiproliferative effect of CpG-proBs in coculture with Teffs by a neutralizing anti-FasL antibody (5  $\mu$ g/mL). T-cell proliferation assessed at day 5 by the numbers of cells in which the CFSE content is diluted in response to anti-CD3 + anti-CD28, either alone or in coculture with CpG-proBs (Teff:CpG-proB ratio at 2:1) with or without neutralizing anti-FasL antibody. Data in I are expressed as means  $\pm$  SEM of three to five experiments. \*\* $P = 0.008$ . (J) FasL expression by the c-kit<sup>+</sup>B220<sup>+</sup>IgM<sup>low</sup>CD45.2<sup>+</sup> cells found in the pancreas 5 d after transfer, compared with that of the c-kit<sup>+</sup>B220<sup>+</sup>IgM<sup>low</sup>CD45.2<sup>+</sup> splenic progeny sorted after 1 mo, and to that of the recipient's IgM<sup>+</sup>CD45.2<sup>+</sup> cells found in pancreas and spleen at the same timings, respectively, relative to staining with isotype control antibodies (K and L) Ability of the splenic CD45.2<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup> progeny in coculture with Teffs (ratio Teff:CpG-proB progeny of 3:1) to induce Teff apoptosis (K), assessed by Topro III incorporation and Annexin V staining (numbers in the *Upper Right* quadrant represent percentages of dead cells) and to suppress the proliferation of CFSE-loaded Teffs (L) at day 5 of coculture. Results shown in A-F, H, and J-L are from one representative experiment of two to five.

particularly in the pancreas of the CpG-proB recipients (Fig. 6B) as soon as 2 wk after progenitor transfer, without a significant modification of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs [14.01  $\pm$  0.49 vs. 10.92  $\pm$  1.5% of spleen CD4<sup>+</sup> cells (mean  $\pm$  SEM, n.s.) and 12.4  $\pm$  1.55 vs. 8.84  $\pm$  1.03% of pancreatic lymph node CD4<sup>+</sup> cells (mean  $\pm$  SEM, n.s.)], in control mice relative to CpG-proB recipients. Moreover, pancreatic CD4<sup>+</sup> cells, 2 wk after progenitor transfer, became more efficient IFN- $\gamma$  producers, while decreasing their secretion of IL-21 (Fig. 6C). Hence, both Teff numbers and their capacity to produce IL-21 were similarly reduced in vitro in CpG-proB:Teff cocultures and in vivo in CpG-proB recipients.

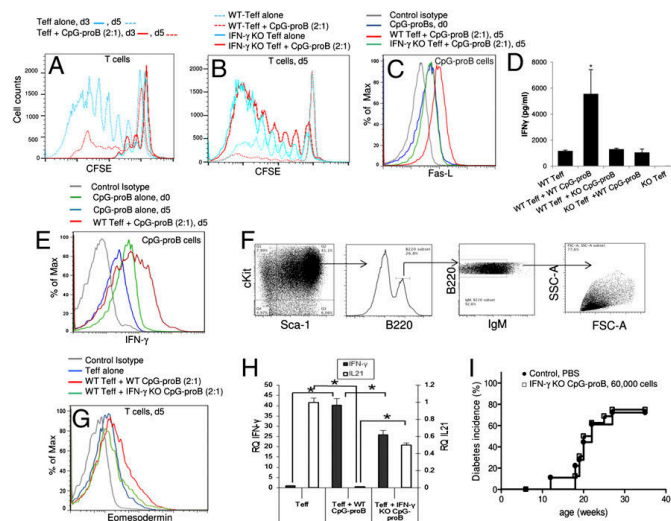
Conversely, no significant modulation of IL-10 production was observed either in spleen or PLN CD4<sup>+</sup> T cells after activation ex vivo with phorbol 12-myristate 13-acetate (PMA) + ionomycin or among CD19<sup>+</sup> cells gated from spleen cells activated for 48 h with LPS followed by PMA + ionomycin (Fig. 6D).

**Suppressive ProB-Cell Progenitors Emerge in Vivo in CpG-Treated Mice.** Finally, we examined whether suppressive B-cell progenitors were also generated in vivo in mice having received TLR-9 activation. As shown in Fig. 7A, c-kit<sup>+</sup>Sca-1<sup>low</sup>B220<sup>+</sup>IgM<sup>+</sup> small-size cells were effectively detected in the bone marrow of NOD mice 18 h after injection of CpG-B (30  $\mu$ g/mouse, i.p.), similarly to in vitro kinetics. Electronically sorted cells shared the same phenotype and suppressive effect on Teffs (Fig. 7B) as their culture-derived counterpart.

## Discussion

We have shown herein that bone marrow cells respond to the TLR-9 agonist CpG by giving rise to a transient population of c-kit<sup>+</sup>Sca-1<sup>low</sup> progenitors at the pro-B stage that, conversely to control Sca-1<sup>+</sup> proBs, provide protection against T1D upon adoptive transfer into NOD mice. We refer to this unique protective B-cell progenitor population as CpG-proBs. Progenitor populations with similar phenotypes, of which the suppressive properties remain to be established, similarly emerge upon activation of TLR-2, -4, -5, -6, and -7, but compounds targeting TLR-1 and -3 as well as GpC had no such effect. The fact that CpG-A, a poor direct activator of B cells, triggers the emergence of a similar progenitor population, may suggest that other non-B-cells may be the primary target of CpG in the bone marrow, leading to the emergence of the CpG-proB cells. CpG-proBs are highly efficient for immunotherapy because a single adoptive transfer of less than 100,000 could prevent T1D in the NOD mouse, at both early and prediabetes stages.

The immunosuppressive functions of CpG-proBs develop in vitro through a cross-talk with activated T cells, which produce IFN- $\gamma$  and thereby up-regulate FasL expression on CpG-proBs. As a result, CpG-proBs become capable of inducing apoptosis of target T cells, including diabetogenic Teffs. In addition, CpG-proBs induced a shift of Teff functions toward cytotoxicity and a cytokine profile dominated by a clear-cut enhancement of IFN- $\gamma$  and its target genes IL-12R $\beta$ 2, granzyme B, and the t-Box



**Fig. 5.** Contribution of Teff- and CpG-proB-derived IFN- $\gamma$  to suppression, cytokine switching and protection against T1D induced by CpG-proBs. (A) CpG-proBs cocultured with CFSE-loaded Teffs (Teff:CpG-proB ratio at 2:1) derived from NOD mice do not suppress Teff proliferation when measured at day 3 but suppress Teff proliferation measured at day 5. (B) CpG-proBs suppress the proliferation of CFSE-loaded Teffs in cocultures on day 5 only when derived from WT-NOD mice, not when derived from IFN- $\gamma^{-/-}$  NOD mice. (C) FasL expression by gated B220 $^{+}$  CpG-proBs cocultured with Teffs from either WT or IFN- $\gamma^{-/-}$  mice on days 0 and 5. (D) IFN- $\gamma$  assays in supernatants at day 5 of cocultures set up with CpG-proBs combined either with IFN- $\gamma$ -competent or IFN- $\gamma$ -deficient Teffs. \* $P < 0.05$ . (E) Intracytoplasmic levels of IFN- $\gamma$  measured by FACS in gated B220 $^{+}$  CpG-proB cells before (d0) and after culture for 5 d (d5) alone or together with Teff at a cell ratio of 2 Teff for 1 CpG-proB. (F) Cell-sorting procedure and phenotype analysis by flow cytometry of CpG-proBs prepared from bone marrow cells of IFN- $\gamma^{-/-}$  NOD mice incubated for 18 h with CpG-B. (G) Nuclear eomesodermin expression measured by FACS at day 5 in gated CD4 $^{+}$  Teffs sorted from WT-NOD donors and cultured alone or cocultured (Teff:CpG-proB ratio at 2:1) with CpG-proBs derived either from WT or from IFN- $\gamma^{-/-}$  NOD mice. (H) qRT-PCR determination of IFN- $\gamma$  (Left axis) and IL-21 (Right axis) mRNA levels relative to 18S in Teffs cultured for 5 d alone or together with CpG-proBs isolated from WT or IFN- $\gamma$ -deficient NOD mice. Data are expressed as means  $\pm$  SEM of three experiments. \* $P < 0.05$ . (I) Diabetes incidence in control NOD mice injected at 6 wk of age with PBS ( $\bullet$ ,  $n = 18$  mice) or with CpG-proBs (60,000 cells per mouse) prepared from IFN- $\gamma$ -deficient NOD mice ( $\square$ ,  $n = 16$  mice). n.s., not significant. Data are pooled from two experiments.

transcription factors t-Bet and eomesodermin. Up-regulation of IFN- $\gamma$  and of eomesodermin (32), a negative regulator of IL-21 production, together with down-regulation of ICOS, a positive regulator of IL-21 (30) in target Teffs, led to their dramatically reduced IL-21 production. CpG-proBs from IFN- $\gamma$ -deficient NOD mice were far less potent in shifting the cytokine profile of Teffs in vitro and similarly unable to provide protection against T1D when transferred to NOD recipients. IFN- $\gamma$  clearly plays a key role in vitro in the cross-talk between CpG-proBs and Teff, inasmuch as Teff-derived IFN- $\gamma$  promotes FasL-dependent cytotoxicity of CpG-proBs toward Teffs; CpG-proB-derived IFN- $\gamma$  accounts for the cytokine switch within the Teff population. However, whether IFN- $\gamma$  is actually playing such a key role in vivo remains to be fully demonstrated at this stage. Indeed, the CpG-proB cells that emerge from cultures set up with bone marrow cells from IFN- $\gamma$ -deficient mice might also be modulated by various IFN- $\gamma$ -dependent factors through mechanisms that remain to be identified. Hence, a definitive proof of the key role of T-cell-derived IFN- $\gamma$  in the differentiation of pro-B cells with suppressive functions would require experiments with CpG-proBs generated from mice in which only B cells would lack IFN- $\gamma$ -R (chimeric mice or mice with conditional knock-out).

Nevertheless, both the reduction of Teffs and the cytokine regulation, with enhanced IFN- $\gamma$  and reduced IL-21 production, could be evidenced in vivo within pancreatic CD4 $^{+}$  T-cells from CpG-proB recipients relative to age-matched noninjected controls.

The role of IFN- $\gamma$  in T1D developed by NOD mice is complex. Genetic absence of IFN- $\gamma$  delays but does not prevent diabetes in NOD mice (36). Although IFN- $\gamma$  is cytotoxic for pancreatic

$\beta$ -cells, its production in the spleen leads to a paradoxical protective effect against T1D in bacillus Calmette–Guérin-treated mice (37, 38). Furthermore, IFN- $\gamma$  restores normoglycemia in Ig-GAD<sub>206–220</sub>-treated NOD mice (39) because of its capacity to control the balance between Th1 and Th17 differentiation and particularly to reduce IL-21 production.

IL-21 is a cytokine (40) whose receptor belongs to the common  $\gamma$ -chain receptor family. It is produced by Th17 cells (as well as follicular helper T cells and NKT cells) and thereby serves as an autocrine amplification factor for IL-23R-expressing T cells favoring Th17 differentiation. IL-21's role remains debated (41, 42) in the resistance of Teffs to regulation by Tregs, which has been reported in the NOD mouse during progression of T1D. IL-21 plays a pathogenic role in various autoimmune diseases, such as colitis, gastritis, experimental autoimmune encephalomyelitis, and rheumatoid arthritis. It is encoded by *idd3*, a gene locus of predisposition to diabetes in the NOD mouse (43). Polymorphisms for IL-21 and its receptor are also linked to prevalence of T1D in humans (44).

Recent data from several laboratories have demonstrated a key role for IL-21 in the pathogenesis of T1D. This cytokine is produced at high levels in NOD mice (45) and accelerates disease onset upon transgenesis, but IL-21R deficiency protects against T1D (34, 35). Furthermore, neutralization of IL-21 with either IL21R-Fc or anti-IL-21 prevents disease development both at early and late prediabetes stages (33). In this line of evidence it has been reported that the IL-21 receptor controls both antigen transport and expansion of autoreactive CD4 $^{+}$

**Table 1. Modulation of gene expression in Teffs after coculture with CpG-proBs**

Gene name	Fold-change	Statistical significance
<i>FasL</i>	55.676342	0.000699
<i>Eomes</i>	6.314982	0.003092
<i>Gzmb</i>	4.599565	0.006441
<i>S1pr1</i>	-5.795792	0.007901
<i>Irfng</i>	14.098450	0.008533
<i>Ccr4</i>	-21.421090	0.008623
<i>Gata3</i>	-5.284992	0.011812
<i>Socs3</i>	-7.477339	0.015402
<i>Il21</i>	-77.062769	0.016882
<i>Il12rb2</i>	2.388677	0.017140
<i>Ccl5</i>	4.384641	0.018714
<i>Il17rd</i>	-3.359100	0.019037
<i>Cxcl10</i>	1.806543	0.020413
<i>Csf2</i>	2.887744	0.020796
<i>Stat3</i>	1.579430	0.021611
<i>S100a9</i>	2.726780	0.022745
<i>Fas</i>	-3.538830	0.023138
<i>Il12rb1</i>	-2.424316	0.023798
<i>Il13</i>	-11.022945	0.023816
<i>Rn18s</i>	1.052367	0.025393
<i>Cd4</i>	-1.403812	0.026754
<i>Itgal</i>	1.129510	0.027668
<i>Cd28</i>	1.244431	0.030131
<i>Il6</i>	1.899298	0.030779
<i>Tgfb1</i>	1.081882	0.031073
<i>Jak2</i>	-2.067066	0.031177
<i>Prkcg</i>	-1.883761	0.034156
<i>Icos</i>	-1.975810	0.035781
<i>Il2</i>	2.716978	0.035822
<i>Tbx21</i>	1.766007	0.036636
<i>Smad7</i>	-1.555453	0.038206
<i>Il10</i>	1.849752	0.039904
<i>Lif</i>	-2.066163	0.042685
<i>Stat5a</i>	-9.092846	0.045780

After 5 d of culture with or without CpG-proBs (ratio 2:1), live Teffs were electronically sorted, their mRNA was extracted, and reverse transcription performed followed by qPCR analysis. Global Pattern Recognition online analysis provided the ratio of gene modulation and their statistical significance.  $n = 5$  samples from different experiments per cell culture condition. *FasL*, fas ligand (TNF superfamily, member 6); *Eomes*, eomesodermin; *Gzmb*, granzyme B; *S1pr1*, sphingosine-1-phosphate receptor 1; *Irfng*, interferon gamma; *Ccr4*, C-C chemokine receptor type 4; *Gata3*, gata binding protein 3; *Socs3*, suppressor of cytokine signaling 3; *Il21*, interleukin 21; *Il12rb2*, interleukin 12 receptor, beta 2 subunit; *Ccl5*, chemokine (C-C motif) ligand 5; *Il17rd*, interleukin 17 receptor D; *Cxcl10*, chemokine (C-X-C motif) ligand 10; *Csf2*, colony Stimulating Factor 2; *Stat3*, signal transducer and activator of transcription 3; *S100a9*: S100 calcium binding protein A9; *Fas*, Fas (TNF receptor superfamily member 6); *Il12rb1*, interleukin 12 receptor, beta 1 subunit; *Il13*, interleukin 13; *Rn18s*, 18S ribosomal RNA; *Cd4*, CD4 antigen; *Itga1*, integrin, alpha 1; *Cd28*, CD28 antigen; *Il6*, interleukin 6; *Tgfb1*, transforming growth factor, beta 1; *Jak2*, Janus Kinase 2; *Prkcg*, protein Kinase C, theta; *ICOS*, inducible T-cell co-stimulator; *IL-2*, interleukin 2; *Tbx21*, T-box transcription factor 21; *IL-10*, interleukin-10; *LIF*, leukemia inhibiting factor; *Stat5a*, signal transducer and activator of transcription 5a.

T cells as well as their ability to facilitate autoreactive CD8<sup>+</sup> T-cell recruitment to pancreatic islets (46).

In contrast, several observations indicate that IL-10 does not play a significant role in the protective properties of CpG-proBs. Immediately after their recovery from bone marrow cultures, CpG-induced pro-B cells produced IFN- $\gamma$  but no IL-10, which was generated only in response to PMA + ionomycin. IL-10 receptor blocking by a specific antibody had no effect on the suppression of Teffs promoted by CpG-proBs. Moreover, the

B-cell progeny of CpG-proBs did not produce IL-10, even after PMA + ionomycin activation alone or in combination with LPS or anti-CD40 or CpG. The percentage of IL-10-producing cells was also not increased among host-derived B-cell populations in CpG-proB-recipients relative to control mice. This finding is in clear contrast with the IL-10-driven regulatory mechanisms often ascribed to Bregs (10). However, it has been reported that mature Bregs can also use regulatory pathways that do not depend on IL-10, such as cell-to-cell interactions involving MHC (47) or B7 molecules (48) and blockade of Teff expansion via FasL-induced apoptosis (49–51). Evidently, the inflammatory signals and cytokines they encounter in their microenvironment engender diverse immunosuppressive mechanisms. For example, LPS-activated mature B cells prevented T1D in NOD mice (52) by FasL-dependent apoptosis of diabetogenic Teffs, similarly to the mechanism of action reported here. In addition, these LPS-activated B cells down-regulated Th1 cytokines, possibly through TGF- $\beta$  production, but Th2 cytokines were not increased. This mechanism could not account for the action of CpG-proBs that was not associated with detectable TGF- $\beta$  production. However, other reports have shown that TLR activation of B cells could instead foster Th1 differentiation, through MyD88-dependent B-cell activation driving IFN- $\gamma$ -producing T-cell differentiation and leading to an IgG2c primary antibody response (53). MyD88-dependent activation can therefore induce similar activities in mature B cells and pro-B-cell progenitors.

Here we provide evidence for the rapid recruitment of the early progeny of CpG-proB-cell progenitors to the pancreas as FasL<sup>high</sup>-c-kit<sup>+</sup>B220<sup>+</sup>IgM<sup>low</sup> cells, leading to a reduction in the numbers of active IL-21-producing Teffs as early as 15–20 d after injection. In addition, the suppressive properties and high FasL expression are maintained throughout the differentiation process into several mature B-cell subsets in the spleen and the PLNs, thereby providing long-term protection against T1D.

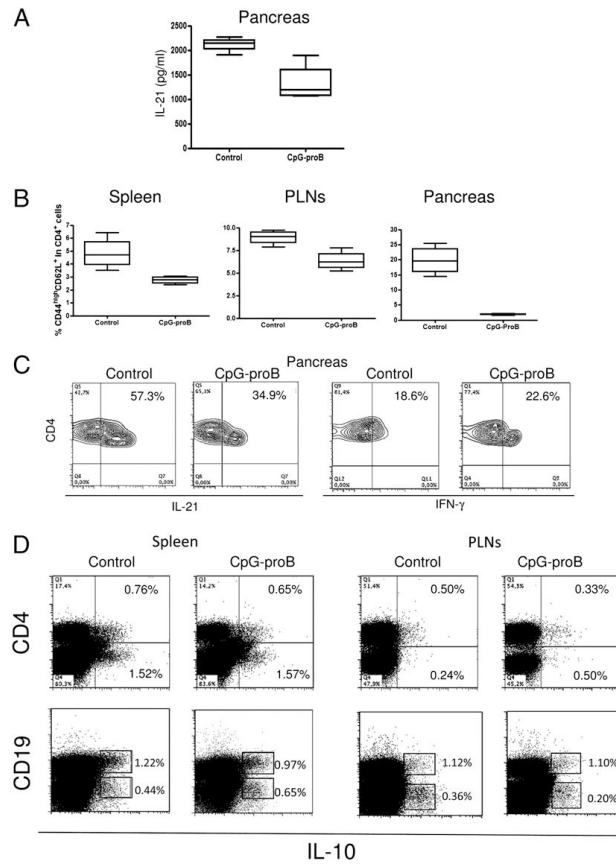
Indeed, CpG-proBs differentiated into various B-cell subsets, but not into CD1d<sup>hi</sup>CD5<sup>+</sup> B10 cells, suggesting that they are not the common precursors of this regulatory cell lineage. Surprisingly, follicular B cells, MZ B cells, and T2-MZP-Bs largely prevailed among the suppressive CpG-proB progeny in NOD recipients. Notably, tolerogenic properties of T2-MZP-Bs have been abundantly reported in several experimental models of autoimmune diseases (23, 24, 54–57).

Finally, because CpG-proBs appeared in the bone marrow of NOD mice in vivo upon injection of CpG, the possibility that they constitute early progenitors for different regulatory B-cell subsets linking innate and adaptive immune responses, capable of exerting the appropriate feedback control to prevent autoimmune and inflammatory responses might be worth examining. However, unless injected early and repeatedly (58, 59), an administration protocol that creates disorders of the lymphoid architecture (60) and mobilizes bone marrow cells to the periphery, causing aplasia, TLR agonists and particularly CpG are not protective against T1D in the NOD mouse but rather accelerate disease onset (61–63). Indeed, the immunotherapeutic influence of CpG-proBs is limited in vivo by their transient survival and by the proinflammatory properties of most myeloid cells that emerge concomitantly in CpG-treated mice. Therefore, the regulatory potential of CpG-proBs, like that of Tregs and Bregs, is most probably overwhelmed by the pathogenic process taking place during the accelerated development of T1D in NOD mice treated by CpG. For this reason, the protective properties of this CpG-proB population in the spontaneous T1D model are only revealed after cell-sorting and adoptive transfer.

## Conclusion

This unique induced B-cell progenitor subset adds to the ever expanding list of hematopoietic progenitors that can emerge in pathophysiological settings, such as infections, inflammation, and cancer to exert immunoregulatory and therapeutic functions,





**Fig. 6.** In vivo effects of CpG-proBs on pancreatic IL-21 levels in NOD recipients and on the incidence of effector memory T cells. (A) IL-21 levels (pg/ml) were quantified by ELISA in homogenates of whole pancreas, collected from control mice vs. CpG-proB recipients, 9 wk after progenitor transfer [i.e., in 15-wk-old animals ( $n = 4$  mice per group,  $P = 0.0286$ )]. (B) Proportions of effector memory T cells in the spleen (Left), PLNs (Center), and pancreas (Right) of NOD controls and CpG-proB-recipients were assessed 2 wk after progenitor transfer and expressed as percent CD44<sup>hi</sup>CD62L<sup>+</sup> cells in CD4<sup>+</sup> cells ( $n = 8$  mice per group,  $P = 0.0286$  in all three sites). (C) Intracytoplasmic IL-21 and IFN- $\gamma$  production was measured 2 wk after progenitor transfer by FACS in pancreatic CD4<sup>+</sup> cells after PMA + ionomycin stimulation for 5 h in controls and CpG-proB recipients. (D) Intracytoplasmic IL-10 production was measured by FACS in spleen and PLN CD4<sup>+</sup> cells 2 wk after progenitor transfer after PMA + ionomycin stimulation for 5 h and in spleen and PLN CD19<sup>+</sup> cells after 48 h stimulation with LPS (10  $\mu$ g/ml) followed by PMA + ionomycin stimulation for 5 h. Results in C and D are from one representative experiment of three.

either directly or indirectly by differentiating into regulatory mature cell types (1–4). Such tolerogenic progenitors could become instrumental in cell therapy of autoimmune diseases, injected either on their own or in combination with stem cell grafts in the context of autologous stem cell transplantation that is already implemented in patients with autoimmune diseases (64–66).

#### Materials and Methods

**Mice.** Wild-type CD45.1 and congenic CD45.2 NOD mice were bred in our animal facility under specific pathogen-free conditions. MyD88<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> NOD mice were gifts from N. Thiebaut (CNRS UMR8147, Paris) and from L. Chatenoud (INSERM U1013, Paris) and C. Benoist (Harvard Medical School, Boston), respectively, and bred in the same animal facility. Live animal experiments were approved by the Necker Animal Experimentation and Ethics Committee. Female CD45.1 NOD mice were used as a model of spontaneous diabetes and received intravenous progenitor cell transfers at 6- or 16-wk of age. Mice were screened for glycosuria (Glucotest; Boehringer-Mannheim)

twice a week or glycemia (Haemoglukotest and Reflolux F; Boehringer-Mannheim) and were considered diabetic when nonfasting blood glucose levels were >250 mg/dL in two consecutive readings.

**Isolation of TLR-Activated Bone Marrow Progenitors.** Bone marrow cells isolated from tibiae and femurs of 8- to 12-wk-old NOD mice were incubated in RPMI-1640 medium (PAA) supplemented with 10% (vol/vol) FCS and 1% antibiotics (penicillin and streptomycin) for 18 h with 1  $\mu$ g/ml of the oligodeoxynucleotides CpG 1585 (CpG-A) (InvivoGen), CpG 1668 (CpG-B) (Eurogentec), and its negative GpC control (InvivoGen), or with the respective agonists of TLR1-9 supplied in a commercial kit (InvivoGen): Pam3CSK4 (0.5  $\mu$ g/ml), FSL1 (1  $\mu$ g/ml), HKLM ( $2 \times 10^6$  cells/ml), Poly I:C (HMV) (5  $\mu$ g/ml), Poly I:C (LMV) (5  $\mu$ g/ml), LPS-EK standard (1  $\mu$ g/ml), FLA-ST standard (1  $\mu$ g/ml), and ssRNA40/LyoVec (2  $\mu$ g/ml) as well as CpG 1826 (CpG-B) (1  $\mu$ g/ml). c-kit<sup>+</sup> bone marrow cells were sorted by c-kit<sup>+</sup> immunomagnetic separation using a RoboSep automaton (StemCell Technologies). Sorted cells were further stained with appropriate fluorochrome-conjugated mAbs against B220, IgM, Sca-1, and electronically sorted into small-size,

## **Paper 2**

**Innate CpG-activated pro-B cells protect against ongoing Experimental Autoimmune Encephalomyelitis by restraining T-cell homing to the CNS and reducing the production of pathogenic cytokines**

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*(in preparation)***

**Innate CpG-activated pro-B cells protect against ongoing Experimental Autoimmune Encephalomyelitis by restraining T-cell homing to the CNS and reducing the production of pathogenic cytokines (In preparation)**

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**ABSTRACT**

Therapy with tolerogenic hematopoietic progenitor populations could represent a promising treatment for multiple sclerosis. Here, we showed that innate pro-B cells that emerged within bone marrow upon Toll-like receptor-9 activation with CpG-B (CpG-proBs) protected recipient mice against Experimental Autoimmune Encephalomyelitis (EAE) when adoptively transferred at the onset of clinical signs. CpG-proBs restrained T-cell homing to the spinal cord by reducing CCR7 expression, a critical effect of the CCR7 ligand CCL19, produced IFN- $\gamma$ -dependently by CpG-proBs. CpG-proBs differentiated into marginal zone precursor (T2-MZP) B cells, follicular B cells and CD11b<sup>+</sup>CD5<sup>+/-</sup> B cells in the lymph nodes but exclusively into IL-10-producing CD1d<sup>+</sup>CD5<sup>+</sup>CD11b<sup>+</sup> B cells in the spinal cord. Their B-cell progeny played a key therapeutic role since recipients of Rag2<sup>-/-</sup> and CD11b<sup>-/-</sup> CpG-proBs developed EAE like control mice. Thus, TLR-activated B-cell progenitors that give rise to regulatory B cells may represent novel cell therapy tools for the treatment of autoimmune diseases.

**Key words:** Immune therapy / Cell therapy / peripheral tolerance / MyD88 signaling / B regulatory lymphocytes / T lymphocytes

## Introduction

B lymphocytes are central to the pathogenesis of many autoimmune diseases, including multiple sclerosis (MS), through their ability to produce antigen specific antibodies (Chan et al., 1999). In addition to antibody mediated mechanisms, B cells participate in T-cell mediated inflammation via antigen presentation, costimulation and cytokine production. Several clinical studies have already shown the efficacy of B-cell depletion therapies in autoimmune diseases such as multiple sclerosis and SLE (Allman and Pillai, 2008; Lund and Randall, 2010).

In contrast, it has been shown that several subsets of B cells can also display regulatory functions in T-cell driven autoimmune responses, suggesting a strong therapeutic potential of these cells. The regulatory B cells are not restricted to a specific phenotype. In humans, they include either memory (IgD<sup>+</sup>CD27<sup>+</sup>) or transitional B (CD24<sup>hi</sup>CD38<sup>hi</sup>) cell compartments acting mainly through the production of IL-10 (Blair et al., 2010; Chesneau et al., 2013; Mauri and Bosma, 2012; Yanaba et al., 2008a). Their *in vivo* expansion requires stimulation with anti-CD40 or interaction with CD4<sup>+</sup> T cells through CD80/CD86. In MS, IL-10 producing-B cells have been shown to decrease and to lose their suppressive functions even after stimulation *in vitro* with CD40, unlike with healthy individuals (Duddy et al., 2007), whereas Michel et al reported that there is no lack of B regulatory cells (Michel et al., 2012). In MS patients, helminth infections increase the numbers of CD19<sup>+</sup>CD1d<sup>hi</sup>IL-10<sup>+</sup> B cells with suppressive effects of T cell responses that contribute to enhanced Th2 responses *in vivo* (Correale et al., 2012) and reduce disease scores. Among the already existing treatments for MS patients, some of them such as IFN- $\beta$  (Hedegaard et al., 2011; Krumbholz et al., 2007; Ramgolam and Markovic-Plese, 2010; Ramgolam et al., 2011), and glatiramer acetate (Begum-Haque et al., 2010, 2011; Kala et al., 2010) have been shown to act on the function of B cells, enhancing their ability to secrete IL-10 or promoting a potent B-cell survival factor, conferring to B cells suppressive properties.

In mice, various subtypes of B cells have been shown to suppress autoimmune responses through the production of IL-10, among them B10 cells characterized by the phenotype CD5<sup>+</sup>CD1d<sup>high</sup>, T2-MZP, CD5<sup>+</sup> B-1 as well as CD138<sup>+</sup> plasmacytes (Fillatreau et al., 2002; Mauri et al., 2003; Shen et al., 2014; Shimomura et al., 2008).



In experimental autoimmune encephalomyelitis, an animal model of MS, it has been shown that  $\mu$ MT mice (lack of B cells) failed to recover from EAE (Wolf et al., 1996)). Interestingly, B cells have been demonstrated to display regulatory properties during the phase of induction of the disease since the depletion of B cells prior to immunization exacerbated the clinical score (Matsushita et al., 2008; Weber et al., 2010). In mice, regulatory B cells suppress autoimmune inflammation mostly IL-10 dependently (Bettelli et al., 1998; Fillatreau et al., 2002). Several stimuli are necessary for making protective B cells IL-10-competent.  $CD5^+CD1d^{high}IL-10^+$  B10 cells arise from a progenitor cell population ( $B10_{pro}$ ) which shares the same phenotype but needs to be activated by TLR-4 or -9 agonists plus stimulation with PMA + ionomycin in order to produce IL-10 (Yanaba et al., 2008b). Additionally, TLR signaling through MyD88 (Lampropoulou et al., 2008), CD40 ligation (Evans et al., 2007; Mauri and Bosma, 2012; Mauri et al., 2003), BCR stimulation (Feske, 2007; Matsumoto et al., 2011; Matsushita et al., 2006; Sato et al., 1996), costimulation molecules (Mann et al., 2007) and presence of specific cytokines (Holan et al., 2014; Yoshizaki et al., 2012) such as IL-21 are some of the signals which can confer regulatory properties to several B cell subsets. The absence of IL-10 restricted in B cells is enough to make B regulatory cells, including  $CD5^+CD1d^{hi}$  B10 cells as well as  $CD138^+$  plasmacytes, unable to perform their protective effects in EAE (Fillatreau et al., 2002; Matsushita et al., 2008; Shen et al., 2014).

Alternative IL-10-independent immunoregulatory mechanisms used by B cells have been also described, including skewing of the immune responses towards a Th2 response (Wilson et al., 2010), production of other anti-inflammatory cytokines such as IL-35 (Shen et al., 2014) and expansion of T regulatory cells (Mann et al., 2007; Salomon et al., 2000; Zhong et al., 2007). The in vivo role of B cells in Treg induction/expansion has been supported by several groups showing that  $\mu$ MT mice have reduced percentages of Tregs (Shah and Qiao, 2008) whereas other studies show that the activation of  $CD4^+CD25^+Foxp3^+$  Treg occurs normally in the absence of B cells in EAE (Hoehlig et al., 2012). The existence of many different B cells subsets, able to display regulatory properties support the idea that different inflammatory signals can lead them to acquire suppressive functions. More or less mature B cells are able to regulate autoimmune responses in different ways.

We recently demonstrated that innate pro-B (CpG-proBs) cells, which emerge within bone marrow after stimulation with CpG, can acquire the capacity to prevent T1D in NOD mice (Montandon et al., 2013). The mechanism of action of these activated progenitor cells was IL-10-independent since their production of IFN- $\gamma$  was crucial for reducing the production of the pathogenic cytokine IL-21 by T cells. Additionally, the inflammatory environment because of activated autoreactive T cells was necessary in order to induce the upregulation of FasL at the surface of CpG-proBs resulting in apoptosis of T effector cells (Montandon et al., 2013). Moreover, the CpG-proB cells differentiated into various subsets of more mature B cells that exhibited high FasL expression and were able to induce T-cell apoptosis, thereby prolonging the *in vivo* efficacy of the adoptively transferred progenitors.

We addressed the question whether the same population can also emerge in the B6 mouse strain and whether its adoptive transfer can confer protection against the MOG<sub>35-55</sub>-induced EAE disease. Herein, we show that one single injection of 60,000 activated CpG-proB cells at the onset of clinical signs could control the development of EAE disease. The production of IFN- $\gamma$  by CpG-proB cells plays an important role for their protective effect blocking the homing process of CD4<sup>+</sup> T cells into the spinal cord. IFN- $\gamma$ -competent, CpG-proB cells showed enhanced production of the chemokine ligand CCL19, which was responsible for the internalization of CCR7, a receptor necessary for the extravasation of effector T cells inside the CNS. Additionally, CpG-proB cells reduced the production of pathogenic cytokines such as IL-17 and GM-CSF and enhanced the secretion of IL-10 by CD4<sup>+</sup> T and B220<sup>+</sup> B cells in the recipients. Finally, the B-cell progeny of CpG-induced proBs recovered inside the spinal cord was able to produce IL-10 and played a key role in maintaining the long-term protection against EAE.

## Results

### **Activated bone marrow proB cells emerging upon exposure to CpG, unlike control proB cells, confer protection to EAE mice when injected at the onset of clinical signs.**

Using the same conditions as previously described in NOD mice (Montandon et al, 2013) we incubated bone marrow cells of C57BL/6J mice with the oligonucleotide CpG 1668 (CpG-B) for 18h, and investigated whether a cell population purified with a c-kit<sup>+</sup> Sca-1<sup>+</sup> B220<sup>+</sup> IgM<sup>-</sup> phenotype emerged and could display protective effects in a mouse model of EAE. The population was heterogeneous for PDCA-1 and we sorted the PDCA-1<sup>-</sup> cells in order to concentrate on cells of the B-cell lineage (Figure 1A). More detailed analysis of the phenotype of this transient population showed that these cells are close to the pro-B cell stage of differentiation since they are positive for the B-cell markers CD19 and CD24, do not express IgM, in keeping with an immature B-cell phenotype, and are also negative for CD11b and CD11c. This population expressed also the IL-7R $\alpha$  chain CD127 and CD43 as well as CD5 and CD1d (Figure 1B). Among the TLR agonists tested, not only CpG-B but also those activating TLR-2,-4,-5,-6, and -7 were efficient to induce the emergence of the same population, conversely to ligands targeting TLR-1 and -3 (Figure 1C). Similar to NOD mice, the emergence of CpG-proB cells in C57BL/6J mice was dependent on MyD88 signaling since this population did not arise in the bone marrow of MyD88-deficient mice incubated with CpG-B for 18h (Figure 1C). A potential protective role of this population was investigated in a mouse model of MOG<sub>35-55</sub>-induced EAE (Figure 1E) in which the adoptive transfer of only 60,000 of CpG-proBs at the onset of clinical signs (d12) conferred protection to mice against EAE disease whereas mice that have been injected with control pro-B cells, isolated from non-stimulated bone marrow cells as c-kit<sup>+</sup>Sca1<sup>-</sup>B220<sup>+</sup>CD24<sup>hi</sup>CD43<sup>hi</sup> (Figure 1D), displayed the same clinical score as control mice (Figure 1F).

### **Functional properties of CpG-proB cells: Important role of IFN- $\gamma$ in the protective effect.**

To investigate how CpG-proB cells display their protective effects, the cytokine profile of this population was analysed. Immediately after their isolation, CpG-proB cells could produce the Th1-like cytokines IL12p40 and IFN- $\gamma$  but neither IL23p19 nor the anti-inflammatory cytokine IL-10 as assessed by FACS analysis (Figure 2A). The CpG-proB cells were stimulated for 4h with PMA plus ionomycin and their supernatant was analyzed for the production of cytokines using a multiplex array for Th1, Th2, Th17 like cytokines. Among the cytokines studied, the level of production of IFN- $\gamma$  was about 30 times higher than the others (Figure 2B). Whether this massive production of IFN- $\gamma$  by CpG-proB cells was necessary for their protective effect against EAE was investigated by preparing CpG-proB cells from mice deficient for IFN- $\gamma$  after stimulation with CpG-B for 18h (Figure 2C). IFN- $\gamma$ -deficient CpG-proB cells, adoptively transferred at day 12 post-immunization appeared unable to protect against MOG<sub>35-55</sub>-induced EAE mice whereas injection of wild type CpG-proB cells could prevent the progression of the disease (Figure 2D).

### **CpG-proB cells reduce the production of inflammatory cytokines and enhance the production of the cytokine IL-10 and the expansion of Tregs.**

The protection established by the injection of wild type, IFN- $\gamma$  competent, CpG-proB cells at day 12 post-immunization was confirmed by ex-vivo analysis of the autoimmune responses at the peak of the disease (day 18-21). In the periphery (lymph nodes) of recipient mice of WT CpG-proB cells, the production of the inflammatory cytokine GM-CSF by CD4<sup>+</sup> T cells was reduced comparing with control mice or mice that have been injected with IFN- $\gamma$  KO CpG-proB cells in which the production of GM-CSF was at comparable levels to control mice, as analyzed intracellularly by FACS (Figure 3A). CpG-proB cells had no effect on the production of the pathogenic cytokines IL-17 and IFN- $\gamma$  by CD4<sup>+</sup> T cells within the lymph nodes (Figure 3A). In the spinal cord, site of inflammation in EAE, the injection of WT but not IFN- $\gamma$  KO CpG-proB cells, reduced the production of all pathogenic cytokines studied including IL-17, GM-CSF and IFN- $\gamma$  by the infiltrated CD4<sup>+</sup> T cells (Figure 3B). Additionally, the reduction in the pathogenic cytokine profile was associated with an enhanced production of the anti-inflammatory cytokine IL-10 by host B220<sup>+</sup> cells and CD4<sup>+</sup> T cells in both tissues (Figure 3C and 3D), only in mice that have been injected with WT CpG-proB cells. Additionally to the shift in the cytokine production, an increase of the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells was observed exclusively in recipient mice of WT CpG-proB cells (Figure 3E and 3F)

together with an enhanced level of Foxp3 expression in the CD4<sup>+</sup> T cells of the same mice in both lymph nodes and spinal cord (Figure 3G and 3H).

### **CpG-proBs restrain T-cell trafficking to the spinal cord: role of IFN- $\gamma$**

Together with the reduced production of inflammatory cytokines and enhanced production of IL-10 by both B and T cells, the recipient mice of WT CpG-proB cells were characterized by higher numbers of total cells accumulating in the lymph nodes and spleen comparing to control mice with EAE. At the same time, the total number of infiltrated cells found in the spinal cord was reduced in WT CpG-proB recipients suggesting a possible restriction in their migration process from the secondary lymphoid organs to the CNS (Figure 4A). This restricted homing process also affected the CD4<sup>+</sup> T cells since in WT CpG-proB recipient mice, there was an accumulation of CD4<sup>+</sup> T cells in the periphery and much fewer cells inside the spinal cord (Figure 4B). Interestingly, this phenomenon was only observed in mice that had been injected with WT CpG-proB cells. In MS, the trafficking of the pathogenic T cells from the periphery to the CNS is an essential step for the induction of the disease. We addressed the question if WT CpG-proB cells could affect that process by down-regulating the expression of some chemokine receptors known to be involved in the migration of T cells. CCR7 is well known to be necessary for the extravasation of T cells inside the CNS in neuroinflammatory settings. FACS analysis of the extracellular expression of CCR7 on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the lymph nodes revealed a down regulation in its expression taking place only in mice that had been injected with WT CpG-proB cells (Figure 4C). IFN- $\gamma$  by CpG-proB cells seems to be highly involved in that process since only WT CpG-proB cells but not IFN- $\gamma$  KO CpG-proB cells could produce the chemokine ligand CCL19 which is one of the two specific ligands of CCR7 (Figure 4D). Conversely, no production of CCL21 could be detected. IFN- $\gamma$  may modulate the CCR7 expression indirectly by promoting the production of CCL19, which can lead to the internalisation of CCR7.

### **Role of CCL19 production by CpG-proBs in protection against EAE and T-cell trafficking restriction.**

Whether the production of the chemokine ligand CCL19, is necessary for the protective effect of CpG-proB by blocking CD4<sup>+</sup> T cells to the periphery, was investigated in vivo by injecting CpG-proB cells from CCL19 KO mice in MOG<sub>35-55</sub>-induced EAE mice. CCL19 KO CpG-proB cells were unable to prevent EAE disease comparing to WT CpG-proB cells (Figure 5A). The deficiency of CpG-proB cells to produce CCL19 also prevented them from restraining CD4<sup>+</sup> T cells in the periphery since in CCL19 KO CpG-proB recipient mice, there was no accumulation of either total or CD4<sup>+</sup> T cells in the periphery associated with fewer cells in the spinal cord as happened in WT CpG-proB-recipient mice (Figure 5B and 5C). Analysis of the extracellular and intracellular expression of CCR7 at CD4<sup>+</sup> T cells within the lymph nodes of control mice and WT or CCL19 KO CpG-proB recipients was carried out by FACS in order to investigate whether CCL19 displays its effect by occupying CCR7 and leading to its internalization. Only in WT CpG-proB recipient mice, the extracellular expression of CCR7 was downregulated (Figure 5D) whereas the internalization of CCR7 was shown by its enhanced intracellular expression. Therefore, the production of CCL19 by IFN- $\gamma$  competent CpG-proBs can induce the internalization of CCR7 in T-cells (Figure 5E)

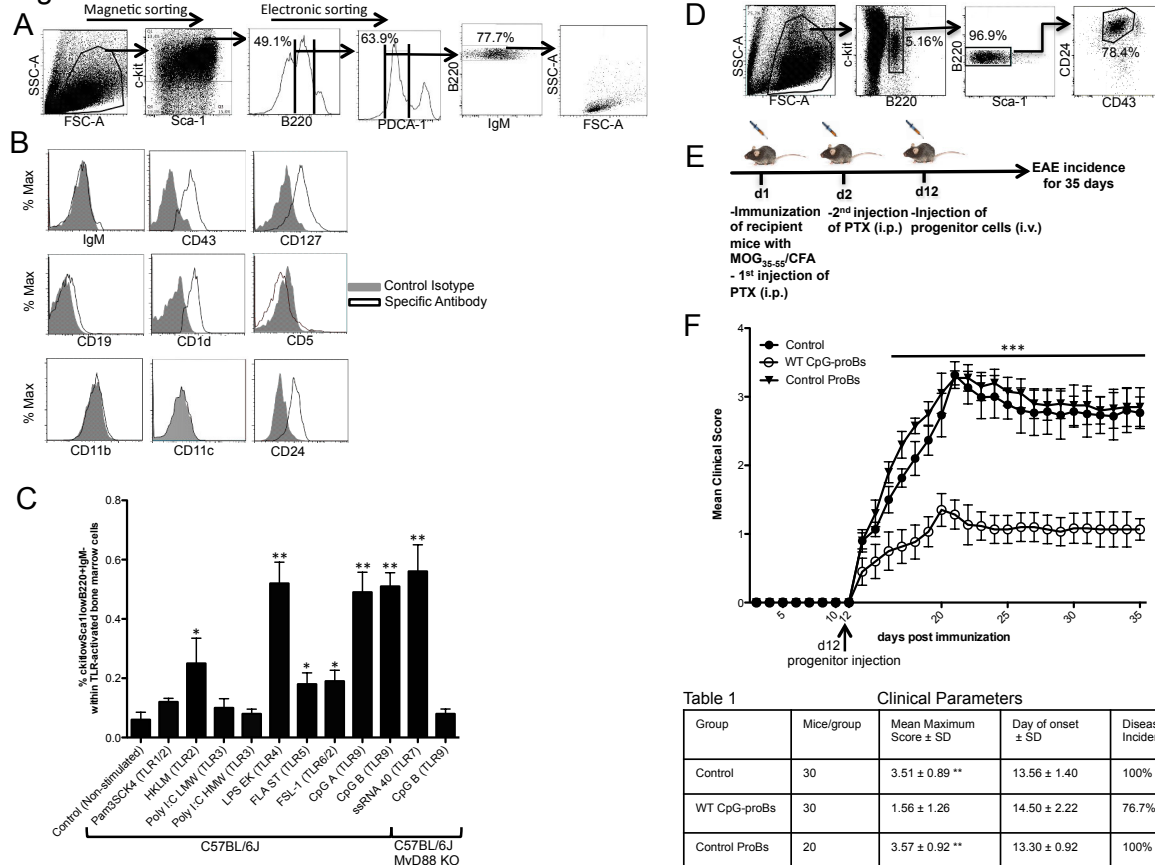
### **CpG-proB cells differentiate into more mature B regulatory cells.**

CpG-proB cells were prepared from the bone marrow of congenic CD45.1 C57BL/6J mice and injected at day 12 in MOG<sub>35-55</sub>-induced EAE CD45.2 recipient mice (60,000 cells per mouse). The protective population of injected CpG-proB cells was analyzed at the peak of the disease (d21) in different tissues for its migration and in vivo differentiation potential. The injected CD45.1<sup>+</sup> cells could be recovered mainly within the lymph nodes (approximately 80,000 cells) and spinal cord (38,000 cells) (Figure 6A). Thus, the total number of recovered cells in both organs was about twice the number of injected cells suggesting that they may have proliferated in vivo. The differentiation status of the injected CD45.1<sup>+</sup> cells was followed by the use of different markers either for B or non-B cell lineages. All the CD45.1<sup>+</sup> cells that could be recovered in the lymph nodes represented about 0.1% of the total cells, and displayed a phenotype positive for the B-cell markers B220, CD19 and IgM, suggesting that these cells were already mature B cells (Figure 6B). These mature B cells included CD11b<sup>+</sup> but CD11c<sup>-</sup> cells and a small proportion of the cells with the phenotype of CD5<sup>+</sup>CD1d<sup>hi</sup> (Figure 6C). For more detailed analysis regarding the different subsets of conventional B cells, a staining performed with the use of markers CD21, CD23 and CD93 revealed that the

recovered mature B cells included transitional 2, transitional 3, follicular B cells and CD93<sup>hi</sup>CD23<sup>hi</sup> cells (Figure 6D). On the other hand, in the spinal cord, the recovered CD45.1<sup>+</sup> cells displayed an exclusive phenotype of B cells characterized as B220<sup>+</sup> CD19<sup>+</sup> IgM<sup>+</sup> CD5<sup>+</sup> CD1d<sup>+</sup> CD11b<sup>+</sup> (Figure 6E). This population of differentiated B cells in the spinal cord was also able to produce the anti-inflammatory cytokine IL-10 (Figure 6F).

**The progeny of injected CpG-proB cells contributes to the long-term protection against EAE.** The respective role in vivo of the injected CD45.1<sup>+</sup> CpG-proB cell progenitors in the protection against EAE was evaluated by adoptively transferring at d12 to MOG<sub>35-55</sub> immunized mice progenitor cells derived either from Rag2<sup>-/-</sup> mice (that could not give rise to mature lymphocytes) or from CD11b<sup>-/-</sup> mice (that could not differentiate into the recovered phenotype of CD11b<sup>+</sup> B cells in the spinal cord). Neither CD11b KO CpG-proB cells nor Rag2 KO CpG-proB cells were able to prevent the development of EAE disease (Figure 7A) and to restrain T-cell homing to the CNS (Figure 7B).

Figure 1

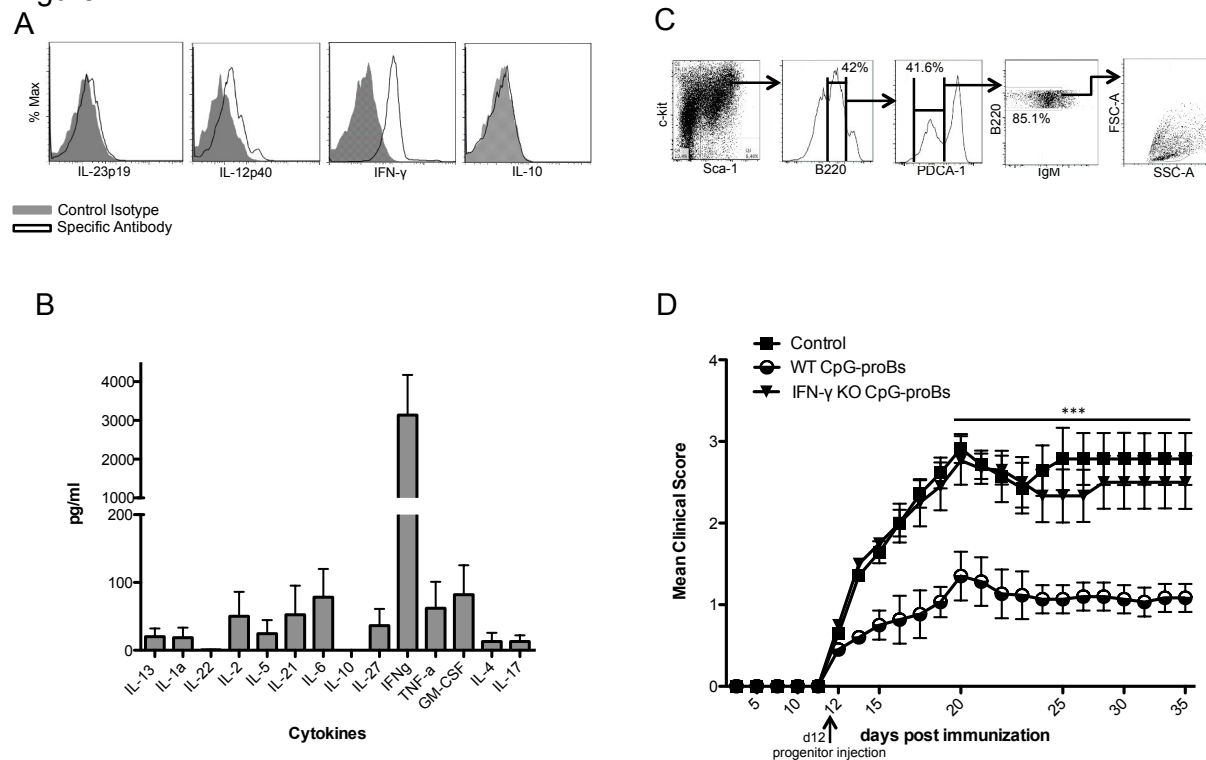


**Fig 1. Phenotypic characterization and protection of ongoing EAE disease by CpG-induced  $c\text{-kit}^{\text{low}}\text{Sca-1}^{\text{low}}\text{B220}^{\text{+}}\text{PDCA-1}^{\text{-}}\text{IgM}^{\text{-}}$  bone marrow cell subset.** (A) Bone marrow (BM) cells from C57BL/6J mice, incubated for 18 h with CpG-B (1  $\mu\text{g}/\text{mL}$ ), were magnetically selected for  $c\text{-kit}^{\text{+}}$  cells, further stained for Sca-1, B220, PDCA-1 and IgM and electronically sorted into small-size ( $\text{FSC}^{\text{low}}\text{SSC}^{\text{low}}$ )  $c\text{-kit}^{\text{low}}\text{Sca-1}^{\text{low}}\text{B220}^{\text{+}}\text{PDCA-1}^{\text{-}}\text{IgM}^{\text{-}}$  cells. (B) Detailed characterization of the phenotype of CpG-activated-proB cells by flow cytometry analysis of expression of IgM, CD43, CD127, CD19, CD1d, CD5, CD11b, CD11c and CD24. Cells were stained with specific antibodies (open histograms) or control isotype antibodies (filled histograms) after FACS sorting. (C) Percentage of  $c\text{-kit}^{\text{low}}\text{Sca-1}^{\text{low}}\text{B220}^{\text{+}}\text{PDCA-1}^{\text{-}}\text{IgM}^{\text{-}}$  cells emerging in the bone marrow of C57BL/6J mice after 18h-incubation with different TLR-agonists. CpG-B was tested in bone marrow cultures of both WT and  $\text{MyD88}^{-/-}$  C57BL/6J mice. Results are expressed as mean  $\pm$  SD of three experiments. \* $P < 0.05$ , \*\*  $P < 0.005$  when comparing BM cells incubated with different stimuli to unstimulated BM cells, using non-parametric Mann-Whitney t test. (D) Control pro-B cells, from non-stimulated BM cells were magnetically sorted for  $c\text{-kit}^{\text{+}}$  cells, further stained from Sca-1, B220, CD24 and CD43 and electronically sorted in  $c\text{-kit}^{\text{low}}\text{B220}^{\text{+}}\text{Sca-1}^{\text{-}}$



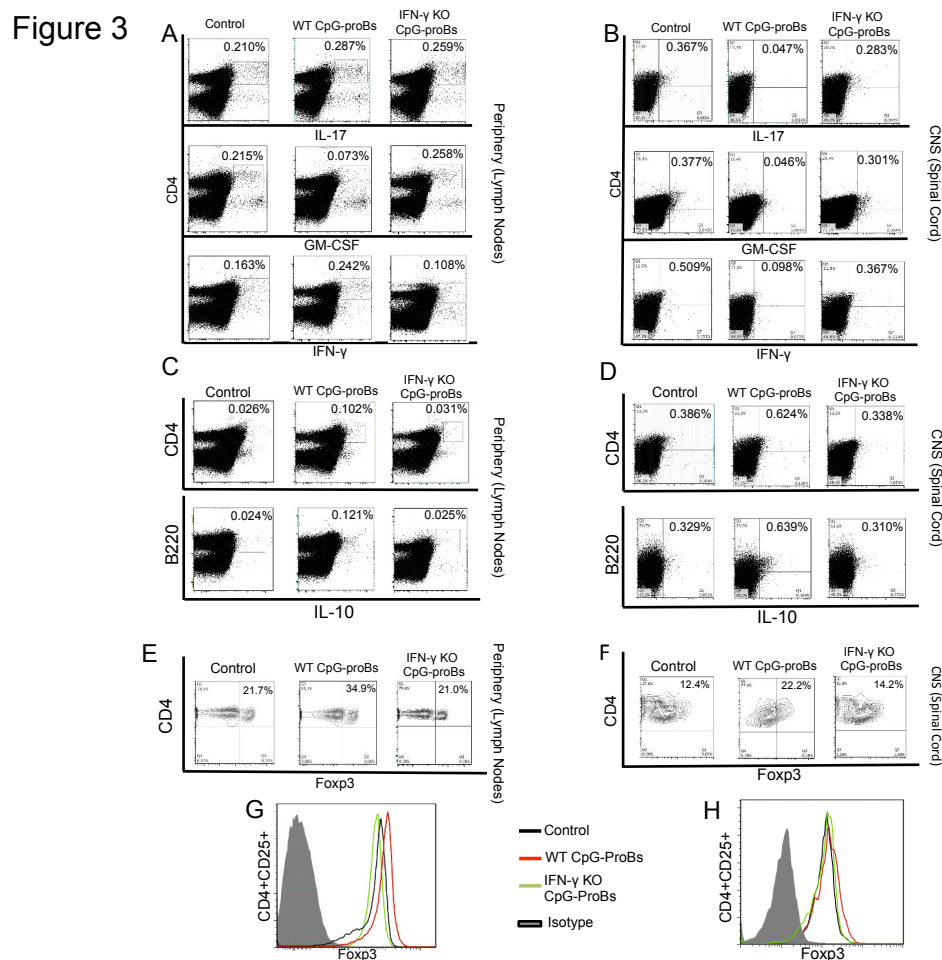
CD24<sup>high</sup>CD43<sup>high</sup> cells. (E) Experimental protocol for MOG<sub>35-55</sub> EAE disease induction in C57BL/6J mice after immunization with MOG<sub>35-55</sub>/CFA at day 0, and injections of pertussis toxin at day 0 and 1. Mice received intravenous progenitor cell transfer (60.000 cells per mouse) at day 12 post-immunization and the incidence of the disease was followed for 35 days. (F) EAE mean clinical score ( $\pm$  SD) in C57BL/6J mice after an adoptive transfer of the above-sorted subsets at day 12 of the disease. \*\*\*P < 0.001 when comparing mean clinical score curves for controls (and control pro-B) with CpG-induced c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>PDCA-1<sup>-</sup>IgM<sup>-</sup> progenitor recipients, by two-way repeated measures ANOVA test.

Figure 2



**Fig 2. Cytokine production by CpG-proB cells : Role of IFN- $\gamma$  in their protective effect.**

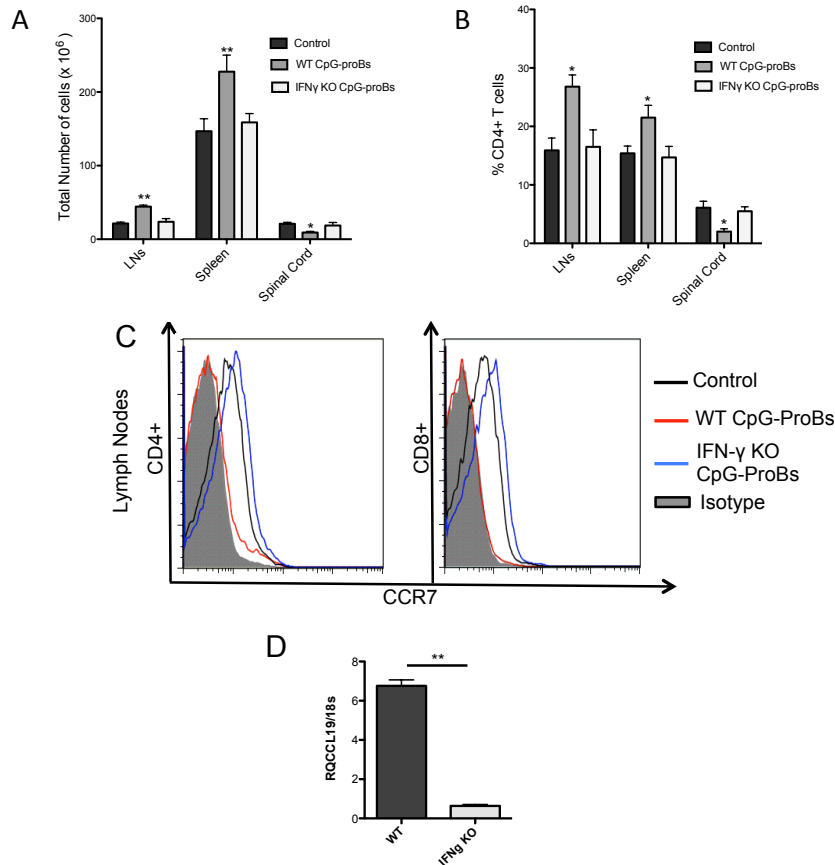
(A) Constitutive cytokine production by CpG-proB cells: immediately after their isolation by FACS sorting, CpG-proB cells were stained for the intracytoplasmic production of cytokines IL-23p19, IL-12p40, IFN- $\gamma$  and IL-10 with the specific antibodies (open histograms) or control isotype antibodies (filled histograms). (B) CpG-proB cells (50,000 cells/200 $\mu$ l) were stimulated for 5h with PMA + ionomycin and their supernatants collected and measured by multiplex ELISA for their concentrations (expressed in pg/ml) of Th1/Th2/Th17 cytokines. (C) Cell-sorting procedure and phenotype analysis by flow cytometry of CpG-proBs prepared from bone marrow cells of IFN- $\gamma$ <sup>-/-</sup> mice incubated for 18 h with CpG-B. (D) EAE mean clinical score in control mice (n= 30) and mice injected at day 12 of the disease with 60,000 cells per mouse either of WT CpG-proB cells (n=30) or CpG-proB cells prepared from IFN- $\gamma$  deficient mice (n=20). Data are pooled from two experiments. \*\*\*P < 0.001 when comparing disease scores for control mice and mice injected with IFN- $\gamma$  KO CpG-proB cells with mice injected with WT CpG-proB cells by two-way repeated measures ANOVA test.



**Fig 3. Cytokine profile of autoimmune responses at the peak of the disease in control mice or mice injected with WT CpG-proBs or IFN- $\gamma$  KO CpG-proB cells.** Mice were sacrificed at day 21 of the disease and their lymph nodes (A, C) were analyzed for the intracytoplasmic production of the cytokines IL-17, GM-CSF, IFN- $\gamma$  and IL-10 after re-stimulation with the MOG peptide for 4 days and activation with PMA + ionomycin for 5h whereas cells recovered from the spinal cord (B, D) were analyzed the same day after activation with PMA + ionomycin for 3h. (A, B) : Intracytoplasmic production of cytokines IL-17, IFN- $\gamma$  and GM-CSF in gated CD4<sup>+</sup> T cells of control mice or mice injected either with WT CpG-proBs or with IFN- $\gamma$  KO CpG-proB cells, collected from (A) the lymph nodes and (B) the spinal cord. (C, D): Production of the anti-inflammatory cytokine IL-10 by CD4<sup>+</sup> T cells and B220<sup>+</sup> B cells within (C) the lymph nodes or (D) the spinal cord of control or CpG-proB-recipient mice. (E, F): Nuclear expression of the transcription factor Foxp3 analyzed by flow cytometry on the same day in CD4<sup>+</sup> T cells of (E) lymph nodes and (F) spinal cord and expressed as percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> cells within gated CD4<sup>+</sup> cells. (G, H): The levels of Foxp3 expression by CD4<sup>+</sup> T cells was compared between control mice and mice that have

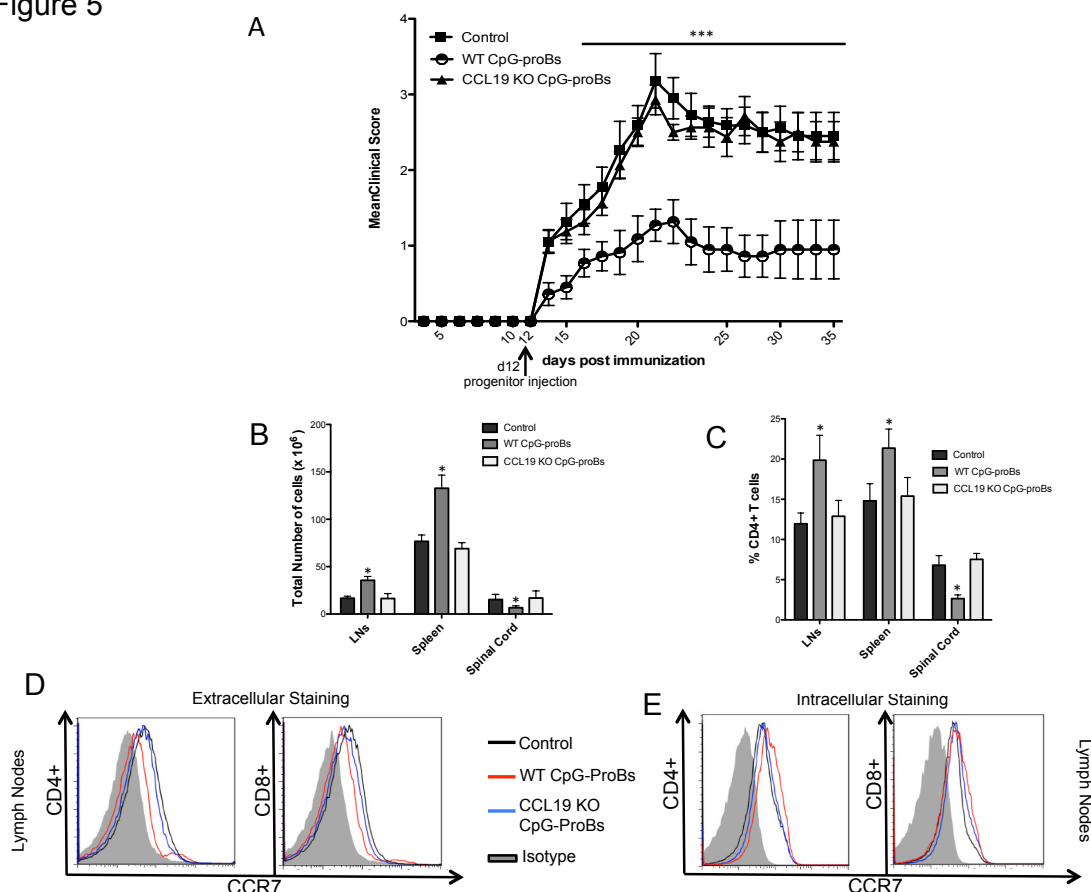
been injected with WT CpG-proBs and IFN $\gamma$  KO CpG-proB cells within either (G) the lymph nodes or (H) the spinal cord. Cells were stained for Foxp3 (open histograms) and for the control isotype antibody (filled histograms). Representative results from 4 independent experiments.

Figure 4

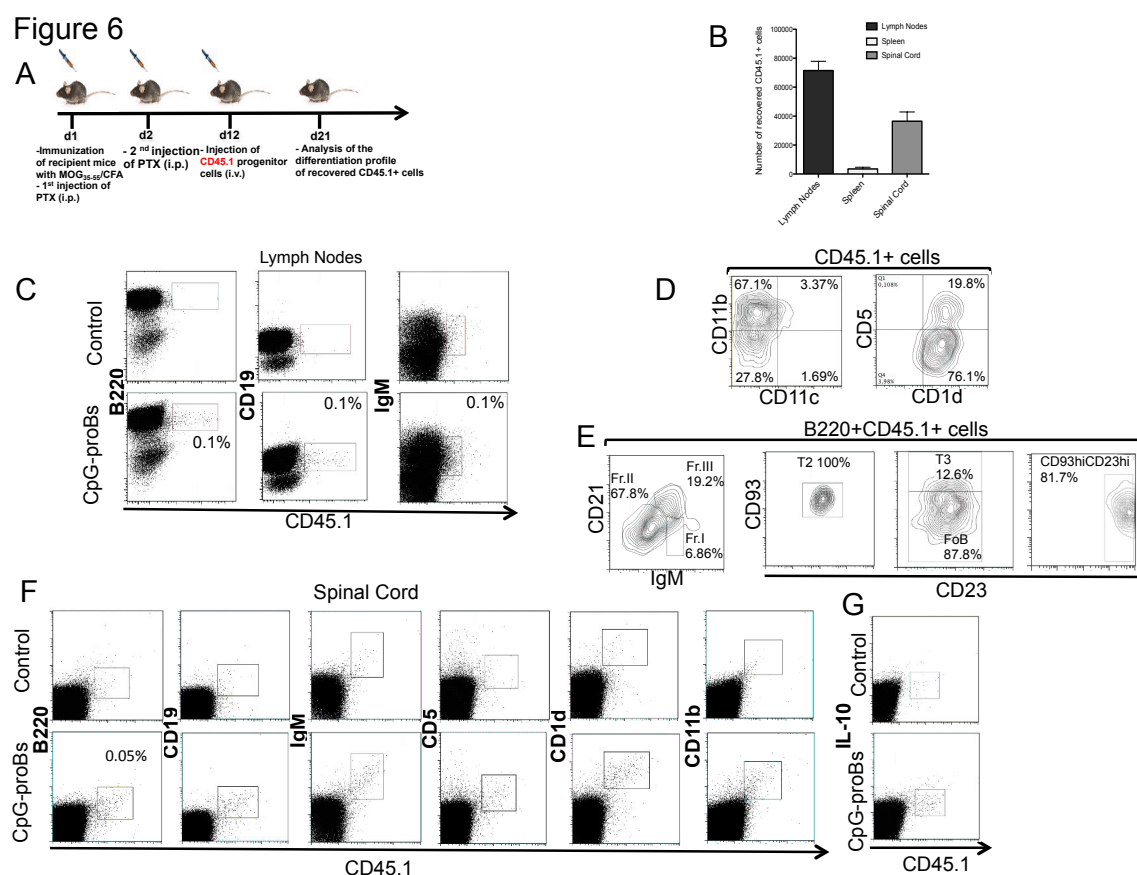


**Fig.4 WT CpG-proB cells restrain T-cells to the periphery by downregulating the expression of the chemokine receptor CCR7.** (A) Total number of cells within the lymph nodes, spleen and spinal cord of control or CpG-proB recipient mice at the peak of the disease. \*\*  $P < 0.005$ , \*  $P < 0.05$ . (B) Percentage of CD4<sup>+</sup> T cells found in all the above tissues \*  $P < 0.05$ . (C) The expression of the chemokine receptor CCR7 was analyzed by flow cytometry in CD4<sup>+</sup> and CD8<sup>+</sup> T cells present within the lymph nodes of mice of the control group and of mice injected with either WT or IFN- $\gamma$  KO CpG-proB cells. Cells were stained with the specific anti-CCR7 antibody (open histograms) or with the control isotype antibody (filled histograms). (D) qRT-PCR determination of CCL19 mRNA levels relative to 18S in WT or IFN- $\gamma$  KO CpG-proB cells after their FACS sorting and stimulation with PMA + ionomycin for 5h. Data are expressed as means  $\pm$  SD of three experiments. \*\* $P < 0.005$  by non parametric Mann-Whitney t test.

Figure 5

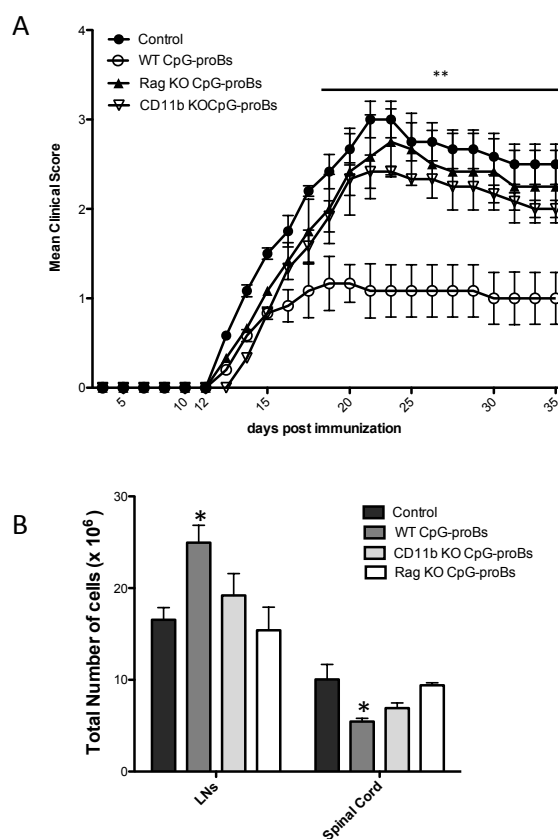


**Fig.5 Role of CCL19 in the protective effect of WT CpG-proB cells through the internalization of CCR7.** (A) The EAE mean clinical scores ( $\pm$  SD) were followed in control mice (n=10) or mice injected either with WT CpG-proBs (n=10) or CpG-proB cells prepared from the bone marrow of mice deficient for CCL19 (n=10). \*\*\*P < 0.001, when comparing control and/or CCL19 KO CpG-proB recipient mice with WT CpG-proB recipients. No significant difference was noted between control and CCL19 KO CpG-proB injected mice using the two-way repeated measures ANOVA test (B, C): Mice of all above groups were sacrificed at day 21 of the disease and their number of cells within the lymph nodes, spleen and spinal cord was counted. (B) Total number of cells recovered within all organs of each mouse groups and (C) Percentage of CD4<sup>+</sup> T cells found in each organ \*P < 0.05, \*\*P < 0.005 (D, E): Lymph node cells were analyzed for their extracellular or intracytoplasmic expression of the chemokine receptor CCR7 by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Cells were stained for extracellular (D) or intracellular (E) expression of CCR7 with a specific anti-CCR7 antibody (open histograms) or with a control isotype antibody (filled histogram).



**Fig. 6. Phenotypic characterization of CD45.1<sup>+</sup> recovered cells in the lymph nodes and spinal cord. (A):** Experimental protocol: EAE disease was induced in CD45.2<sup>+</sup> C57BL/6J mice and WT CpG-proB cells, prepared from the bone marrow of congenic CD45.1<sup>+</sup> C57BL6/J, after stimulation with CpG-B for 18h, were injected at day 12 of the disease. The recovery (B) and differentiation status (C – F) of CD45.1<sup>+</sup> injected cells was analyzed at day 21. (B) Number of CD45.1<sup>+</sup> cells recovered within the lymph nodes, spleen and spinal cord of injected mice. (C) CD45.1<sup>+</sup> cells in lymph nodes at day 21 were stained for cell-surface expression of the B-cell markers B220, CD19 and IgM and flow cytometry performed. (D) Flow cytometry analysis of the expression of CD11b, CD11c, CD1d and CD5 of CD45.1<sup>+</sup> cells within the lymph nodes. (E) Frequency of the B220<sup>+</sup>CD45.1<sup>+</sup> progeny in the lymph nodes of the WT CpG-induced proB cell recipients, in diverse B-cell subfractions analyzed by flow cytometry according to the expression of CD21, IgM, CD23 and CD93. (F) Flow cytometry analysis of the expression of B220, CD19, IgM, CD5, CD1d, CD11b at the cell-surface of recovered CD45.1<sup>+</sup> cells within the spinal cord of WT CpG-proB injected mice at day 21 of the disease. (G) CD45.1<sup>+</sup> cells recovered in the spinal cord were also analyzed by flow cytometry for the intracytoplasmic production of IL-10, after 5h of stimulation with PMA + ionomycin.

Figure 7



**Fig. 7 Role of the progeny of CpG-activated proB cells in the protective effect against EAE.** (A) EAE incidence was followed in control mice or mice that have been injected with WT CpG-proB cells or CpG-proB cells prepared from the bone marrow of Rag2 KO and CD11b KO mice, after simulation with CpG-B for 18h. The progenitor cells were injected at day 12 of the disease.  $n = 8$  mice per group.  $** P < 0.005$ , only between mice injected with WT CpG-proBs and other groups, N.S between all other groups by two-way repeated measures ANOVA test. (B) Total number of cells found per mouse of each group within the lymph nodes and spinal cord. Results are expressed as mean  $\pm$  SD of data obtained in 3 mice per group.  $*P < 0.05$ , when comparing number of cells WT CpG-proB-injected mice and other groups.



## Discussion

Here, we provided evidence for the emergence of a transient population of innate pro-B (CpG-proB) cells within the bone marrow of C57BL/6J mice after stimulation with CpG-B for 18h, that exhibits immunoregulatory properties, differentiating into more mature B regulatory cells which can display protective properties against the MOG<sub>35-55</sub>-induced EAE model of MS. We have already demonstrated that the same population of cells can also emerge in NOD mice under exactly the same conditions, whose adoptive transfer could prevent the development of T1D (Montandon et al., 2013). In C57BL/6 mice, this population was sorted as  $\text{ckit}^+\text{Sca-1}^+\text{B220}^+\text{PDCA-1}^-\text{IgM}^-$  cells and one single injection of 60,000 cells at the onset of clinical signs was sufficient for conferring protection against MOG<sub>35-55</sub>-induced EAE disease. Similar to NOD mice, in C57BL/6J mice not only CpG-B but also all ligands for TLRs dependent on MyD88 signaling could induce the emergence of these progenitor cells. In our experimental protocol, the onset of EAE disease after immunization with MOG<sub>35-55</sub>/CFA is between the days 10-14 post-immunization whereas the peak of the clinical symptoms normally occurs between days 18 and 21, resulting in a chronic and stable clinical score. The injection of CpG-proBs was performed at day 12 (onset of the disease) in order to investigate a potential therapeutic tool which could be applied also in MS patients, in whom no therapies can be applied before the diagnosis or the appearance of the first pathological symptoms. The activation of bone marrow cells with CpG is necessary for conferring protective properties to innate pro-B cells since the injection of control pro-B cells at d12, isolated from non-stimulated bone marrow cells was unable to prevent the development of EAE disease.

CpG-proB cells are producers of massive amounts of IFN- $\gamma$  immediately after their isolation, even without stimulation with PMA + ionomycin. The important role of IFN- $\gamma$  in the protective effect against EAE was confirmed with the use of CpG-proB cells derived from mice deficient for IFN- $\gamma$ , whose injection at day 12 of the disease could not confer protection to EAE mice. Only injection of WT CpG-proB cells could change the cytokine profile of recipient mice reducing the production of pathogenic cytokines such as GM-CSF in both periphery (lymph nodes) and spinal cord (CNS). The reduced production of the pro-inflammatory cytokines was associated also with an enhanced production of the anti-inflammatory cytokine IL-10 and expansion of  $\text{CD4}^+\text{Foxp3}^+$  Tregs.

Despite the fact that IFN- $\gamma$  is considered as a pro-inflammatory cytokine mainly produced by Th1 cells, which are highly involved in the pathogenesis of EAE, there is evidence also for a possible (indirect) regulatory role. In EAE, it has been reported that both IFN $\gamma$ <sup>-/-</sup> and IFN $\gamma$ R<sup>-/-</sup> mice display more severe disease in both chronic and RR-EAE models (Tran et al., 2000). It has been also shown that IFN- $\gamma$  can display a regulatory function through its signaling on astrocytes. When EAE was induced in mice bearing a deficiency for IFN- $\gamma$ R restricted to astrocytes, there was increased demyelination at the peak of the disease and acute disease correlated with sustained clinical symptoms. Interestingly, decreased expression of the anti-inflammatory cytokines IL-10 and IL-27 has been documented inside the CNS of these mice (Hindinger et al., 2012). WT CpG-proB cells changed the cytokine profile of CD4<sup>+</sup> T cells reducing the production of selectively GM-CSF in the lymph nodes and both GM-CSF and IL-17 in the spinal cord. GM-CSF has been shown to be the main pathogenic cytokine in the context of EAE which can induce EAE pathogenesis even in the absence of IL-17 and IFN- $\gamma$  (Codarri et al., 2011; El-Behi et al., 2011). IFN- $\gamma$  or other additional direct or indirect factors provided by the injected CpG-proB cells may be responsible for the reduced production of GM-CSF by CD4<sup>+</sup> T cells in both periphery and spinal cord since the cytokine profile of CD4<sup>+</sup> T cells in recipients of IFN- $\gamma$  KO CpG-proB cells is similar to control mice.

Downstream, the enhanced production of the anti-inflammatory cytokine IL-10 by CD4<sup>+</sup> T cells and host B220<sup>+</sup> B cells may be responsible for this effect. The ability of B cells to produce IL-10 is one of the most important mechanisms of action of several regulatory B cell subsets known to suppress autoimmune responses (Fillatreau et al., 2002; Mauri et al., 2003; Mizoguchi et al., 2002). Whether the activated CpG-proB cells can provide for additional signals which can induce B cells to acquire regulatory properties, possibly through the production of IFN- $\gamma$ , has to be investigated in more details, studying the direct effect of WT CpG-proB cells upon host B cells. B regulatory cells have been shown also to interact with T regulatory cells triggering their expansion (Amu et al., 2010; Blair et al., 2009; Carter et al., 2011; Mann et al., 2007; Mauri and Bosma, 2012). Only in WT CpG-proB-recipient mice there was an expansion of T regs suggesting that host B cells in addition to their production of IL-10, may also favor the in-vivo expansion of CD4<sup>+</sup>Foxp3<sup>+</sup> cells. Whether the presence of these IL-10-competent host B220<sup>+</sup> cells and CD4<sup>+</sup> T cells is necessary for the protective effect of CpG-proB cells remains to be investigated by injecting CpG-proB cells into IL-10 KO recipient mice.

Additionally, IFN- $\gamma$ -competent, CpG-proB cells could display their protective effect by blocking the migration of CD4<sup>+</sup> T cells to the spinal cord. Only in recipient mice of WT CpG-proB cells there was downregulation in the expression of CCR7, a chemokine receptor which is highly involved in the extravasation of CD4<sup>+</sup> T cells inside the inflamed CNS (Columba-Cabezas et al., 2003), resulting in an accumulation of CD4<sup>+</sup> T cells in the secondary lymphoid organs. Since no direct link has been as yet reported between IFN- $\gamma$  and CCR7 expression, we investigated whether IFN- $\gamma$  could indirectly affect CCR7 expression by T cells. Indeed, we show that only IFN- $\gamma$ -competent, WT CpG-proB cells can produce the chemokine ligand CCL19, one of the two specific ligands (together with CCL21) for CCR7. It has been shown that incubation of CCR7-transfected cells with the ligand CCL19 led to the internalisation of the CCR7 receptor whereas CCL21 had a much weaker effect (Bardi et al., 2001; Britschgi et al., 2008). The production of CCL19 by WT CpG-proB cells was necessary for their protective effect since the injection at day 12 of CpG-proB cells isolated from mice deficient for CCL19, was unable to restrain T cell homing into the spinal cord and to protect against EAE disease. CCL19 KO-CpG-proB-recipient mice did not display downregulated expression of CCR7 by CD4<sup>+</sup> T cells within the lymph nodes whereas WT CpG-proB-recipients were characterized by a downregulation of its extracellular expression associated with an upregulated intracellular expression, suggesting that the production of CCL19 by CpG-proBs is responsible for the internalization of CCR7 in T cells.

The ability of T cells to migrate inside the CNS passing through the BBB is a crucial step for the pathogenesis of MS and EAE. Many already existing treatments against MS focus on that process. Besides S1PR, several chemokine receptors have been shown to affect the homing process of activated T cells inside the spinal cord, including CCR6, CXCR4 and CCR7 (Fletcher et al., 2010). FTY720, an oral treatment of MS patients, has been shown to act through its active phosphorylated metabolite which resembles S1P, a ligand for the sphingosine-1 phosphate 1 (S1P1) receptor, occupying this receptor and resulting in a blocking of T cell migration into the CNS (Choi et al., 2011). Because of the fact that other immune cells express also this S1P1 receptor, a more specific S1P1-agonist (AUY954) has been designed and shown to target specifically effector T cells inhibiting their migration into the CNS. At the same time, the ability of pDCs to enter into the CNS was highly related with the beneficial effect of this specific agonist AUY954 (Galicia-Rosas et al., 2012). Interestingly, in the absence of S1P1R, CCR7<sup>+</sup> naïve and central memory T cells are unable to override CCR7 retention signals in the secondary lymphoid organs, resulting in their

entrapment in the lymph nodes (Brinkmann, 2009). The interaction CCL19/CCR7 has been already shown to be necessary for the development of EAE disease since CCR7<sup>-/-</sup> mice or plt/plt mice that are deficient for the expression of the CCR7 ligands CCL19 and CCL21, are resistant to EAE (Kuwabara et al., 2009). The production of CCL19 by IFN- $\gamma$ -competent CpG-proB cells was responsible for the internalization of CCR7 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Whether the production of CCL19 by the injected progenitor cells targets specifically the effector T cells or other immune cells which also express CCR7, among them regulatory populations, has to be investigated in more details, since the migration of regulatory populations to the site of inflammation is desirable. In our case, Tregs seem to migrate normally in the spinal cord of injected mice where they were found even in higher numbers than in control or EAE mice injected with CpG-proB cells from IFN- $\gamma$  KO mice. Interestingly, the targeting of leukaemic T-cells into the CNS has been reported to depend on the chemokine receptor CCR7 which represents their essential adhesion signal (Buonamici et al., 2009).

Whether the signal provided by CpG-proB cells which results in the enhanced production of IL-10, comes from IFN- $\gamma$  or CCL19 or another molecule remains to be investigated. It has been shown that IL-10 production by B-ALL CD23<sup>+</sup>CD5<sup>+</sup> B cells is elevated upon costimulation with CCL19 in case of B cell acute lymphocytic leukemia (Wang et al., 2007) whereas in ovalbumin-specific murine asthmatic model, plt mice display impairment regarding the production of IL-10 comparing to WT Balbc mice (Yamashita et al., 2006).

Here, we demonstrate that the injected CpG-proB cells can differentiate into several more mature B cells that can be recovered at the peak of the disease either within the lymph nodes or in the spinal cord. With the use of CpG-proB cells derived from mice deficient for Rag2 or CD11b, we provided evidence for the important role of their progeny in their protective effect. CpG-proB cells gave rise to follicular B cells, transitional 2 and 3 as well as CD11b<sup>+</sup> and CD5<sup>+/-</sup> B cells in the lymph nodes. The progeny seems to be necessary for maintaining the long-term protection against EAE and for restraining T cell homing to the spinal cord since the injection of Rag2 KO CpG-proBs could not lead to the differentiation of mature B lymphocytes. Whether these cells retain the ability to produce the chemokine ligand CCL19 which downregulates the expression of CCR7 or whether they display an additional mechanism of action has to be investigated by performing more detailed analysis of Rag2 KO CpG-proB recipient mice.

In the spinal cord, the recovered differentiated cells from the injection of WT CpG-proB cells exhibit an exclusive phenotype of B220<sup>+</sup>CD11b<sup>+</sup>CD1d<sup>+</sup>CD5<sup>+</sup>IgM<sup>+</sup> B cells, which can produce the anti-inflammatory cytokine IL-10. This phenotype of cells is very similar to already described B10 (CD5<sup>+</sup>CD1d<sup>hi</sup>) cells known to display their suppressive functions through the production of IL-10 with the difference that these cells were unable to migrate to the site of inflammation, the spinal cord (Yanaba et al., 2008b). The important role of the differentiated B cells in the spinal cord was confirmed by the use of CD11b<sup>-/-</sup> mice. CpG-proB cells derived from these knock-out mice were not able to protect against EAE, suggesting that the emergence of this phenotype exclusively inside the spinal cord is necessary for completing the protective effects. Interestingly, it has been shown that the expression of the integrin CD11b facilitates the entry of grafted bone marrow cells inside the CNS (Lebson et al., 2010). It is strongly possible that the CpG-proB cells derived from CD11b<sup>-/-</sup> donor mice lose their migratory capacity to the spinal cord where inflammatory signals can induce their production of IL-10 in order to suppress autoimmune responses by activated T cells. Moreover, whether the protective mechanism of the recovered activated B cells inside the spinal cord is dependent on their ability to produce IL-10 is still unknown. It will be interesting to investigate whether the injection of CpG-proB cells derived from IL-10<sup>-/-</sup> mice can provide protection against EAE. Similar to the migratory profile of CpG-proB cells in NOD mice where injected progenitor cells could be recovered as soon as day 5 in the pancreas, in the case of EAE, the site of inflammation, i.e the spinal cord, seems to attract the injected cells. It remains to investigate whether CpG-proB cells migrate directly to the spinal cord where inflammatory signals favor their differentiation into IL-10-producing B220<sup>+</sup>CD1d<sup>+</sup>CD5<sup>+</sup>IgM<sup>+</sup>CD11b<sup>+</sup> B cells or if they arrive in that tissue as already differentiated cells. In any case, their presence in the site of autoimmune responses seems to be necessary for inducing changes in the cytokine profile of the already existing activated T cells which may have migrated before the injection of CpG-proB cells.

Whether CpG-proB cells, adoptively transferred at low number at the onset of clinical signs, exert immunoregulatory properties at the progenitor stage is still unclear. However, their capacity to reach the site of inflammation and differentiate into more mature B regulatory cells undoubtedly prolongs their efficacy and provides long term protection against EAE. These interesting properties suggest that they may represent a promising tool for cell therapy of autoimmune diseases even after the onset of clinical signs.

## Material and Methods

**Mice** : Wild type CD45.2<sup>+</sup> C57BL/6, congenic CD45.1<sup>+</sup> C57BL/6, MyD88<sup>-/-</sup>, IFN- $\gamma$ <sup>-/-</sup>, CD11b<sup>-/-</sup>, Rag2<sup>-/-</sup> C57BL/6J were bred in our animal facility under specific pathogen-free conditions. S. Luther, from the department of Biochemistry at the University of Lausanne, generously provided us with CCL19<sup>-/-</sup> C57BL/6J mice. The Necker Animal Experimentation and Ethics Committee approved live animal experiments. Female C57BL/6 mice were used at the age of 12 weeks of age as a model for MOG<sub>35-55</sub>-induced Experimental Autoimmune Encephalomyelitis and received intravenous progenitor cell transfers at day 12 post-immunization.

**EAE induction:** Active EAE was induced in 12-wk-old female mice by s.c. immunization at two sites, upper back and lower back, with 1 mg myelin oligodendrocyte glycoprotein (MOG)<sub>35–55</sub> peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in CFA containing 2.5 mg heat-killed Mycobacterium tuberculosis H37Ra (Hooke Laboratories, Lawrence, MA, USA), on day 0. Additionally, mice received 200 ng pertussis toxin (Hooke Laboratories, Lawrence, MA, USA) i.p. in 0.1 ml/mouse on days 0 and 1. Clinical signs of EAE were assessed daily with a 0- to 5-point scoring system, as follows: 0, no obvious changes in motor function compared to non-immunized mice; 0.5, tip of tail is limp; 1, limp tail; 1.5, limp tail and hind leg inhibition; 2, limp tail and weakness of hind legs; 2.5, limp tail and dragging of hind legs; 3, limp tail and complete paralysis of hind legs or paralysis of one front and one hind leg; 3.5, limp tail and complete paralysis of hind legs, in addition to: mouse is moving around the cage, but when placed to its side, is unable to right itself; 4, limp tail complete hind leg and partial front leg paralysis; 4.5, complete hind leg and partial front leg paralysis, no movement around the cage, mouse is not alert; 5, mouse is spontaneously rolling in the cage. Mice with score  $\geq 4$  for two consecutive days and mice with score 5 were euthanized. Disease scores over the course of the 30-d experiments were totalized for each animal, and the mean clinical scores compared between experimental groups.

**Isolation of TLR-Activated Bone Marrow Progenitors:** Bone marrow cells isolated from tibiae and femurs of 8- to 12-wk-old C57BL/6J mice were incubated in RPMI-1640 medium (PAA) supplemented with 10% (vol/vol) FCS and 1% antibiotics (penicillin and streptomycin) for 18 h with 1  $\mu\text{g}/\text{mL}$  of the oligodeoxynucleotides CpG 1585 (CpG-A) (InvivoGen), CpG 1668 (CpG-B) (Eurogentec) or with the respective agonists of TLR1-9 supplied in a commercial kit (InvivoGen): Pam3CSK4 (0.5  $\mu\text{g}/\text{ml}$ ), FSLI (1  $\mu\text{g}/\text{ml}$ ), HKLM ( $2 \times 10^6$  cells/ml), Poly I:C (HMW) (5  $\mu\text{g}/\text{ml}$ ), Poly I:C (LMW) (5  $\mu\text{g}/\text{ml}$ ), LPS-EK standard (1  $\mu\text{g}/\text{ml}$ ), FLA- ST standard (1  $\mu\text{g}/\text{ml}$ ), and ssRNA40/LyoVec (2  $\mu\text{g}/\text{ml}$ ) as well as CpG 1826 (CpG-B) (1  $\mu\text{g}/\text{ml}$ ). c-kit<sup>+</sup> bone marrow cells were sorted by c-kit<sup>+</sup> immuno-magnetic separation using a RoboSep automaton (StemCell Technologies). Sorted cells were further stained with appropriate fluorochrome-conjugated mAbs against Sca-1, B220, PDCA-1, IgM and electronically sorted into small-size, c-kit<sup>low</sup>Sca-1<sup>low</sup>B220<sup>+</sup>PDCA-1<sup>-</sup>IgM<sup>-</sup> cell subset using a FACS-Aria II (BD Biosciences).

**Isolation of Immune cells from the Spinal cord:** Spinal cord isolated from control and CpG-ProBs-recipient mice were incubated for 30 min in digestion buffer of DNase and Liberase (27 WU/ml) in PBS 1X at 37°C, mixing every 5 minutes. 500 $\mu\text{l}$  of EDTA (100mM) was added for 1 min to end the digestion. The cells were passed through a cell strainer, using a syringe plunger (back side) to smash the tissue. A percoll separation was followed resuspending the cells in 3-5ml of 40% Percoll and underlayered with the same volume of 70% Percoll (in PBS) and the cells were centrifuged for 35 minutes at 1300g (2800 rpm) without brake in order to get an interface. The cells of the interface were collected with a Pasteur pipette and diluted 10 times with complete medium RPMI 10% SVF. The cells were centrifuged and resuspended in 2-3 ml of complete medium.

**Staining of Cells for Flow Cytometry Analysis:** To block non-specific Fc receptor binding, cells were preincubated for 10 min at room temperature with FcR blocker 2.4G2 mAb. Cells were then stained with appropriately labeled mAbs against CD4, B220, CD21, CD23, CD24, IgM, CD1d, CD5, CD43, CD93, PDCA-1, CD8 (eBiosciences) CD19, CD127, CD25, CD11b, CD11c, c-Kit (CD117), Sca-1 (anti-Ly6A/E), CCR7, CD25, CD45.1 (BD Biosciences) and PDCA-1 (Miltenyi Biotec). Nuclear Foxp3 expression was measured by FACS analysis as per the manufacturer's instructions (eBioscience). Intra-cytoplasmic expression of cytokines was assessed after a 5-h stimulation with PMA (10 ng/mL) plus ionomycin (500 ng/mL) in the presence of Brefeldin A (2 mg/mL), followed by fixation/permeabilization with PFA/saponin and subsequent staining with specific antibodies

including APC-labeled anti-IL-23p19 (BD Biosciences), APC-labeled anti-IL12p40 (BD Biosciences), APC-labeled anti-IL-10 (from BD Biosciences), PE-labeled anti-IFN- $\gamma$  (from BD Biosciences), PE-labeled anti-GM-CSF (from eBioscience) and APC-labeled anti-IL-17 (BD Biosciences) or isotype controls (BD Biosciences and eBiosciences). Intracellular expression of CCR7 was analyzed by FACS, as previously described for cytokines. Membrane and intracellular antigen expression was analyzed in a FACS Canto II cytometer (BD Bio- sciences) using FlowJo software (Treestar).

**Cytokine Measurements in cellular supernatants.** Cytokines were measured at day 4 of co-culture supernatants after a cell culture of lymph node cells or splenocytes isolated from control or CpG-ProB-recipient mice at the peak of the disease, re-stimulated with the MOG<sub>35-55</sub> peptide plus 5-h of stimulation with PMA + ionomycin. For determination of spinal cord cytokines, control and CpG-ProB-recipient mice were killed at the peak of the disease, 18-21 days after adoptive transfer of progenitors. Isolation of spinal cord cells was followed by an incubation at 37<sup>0</sup>C/ 5% CO<sub>2</sub> in the presence of Golgi-stop (+PMA/Ionomycin) for 3h. Th1/Th2/Th17 cytokines were measured using Flow Cytomix analyte detection reagent from eBioscience

**Isolation of mRNA and Real-Time RT-PCR:** Live CpG-proB cells were electronically sorted and their mRNA was extracted using RNAqueous-4PCR (Ambion). Reverse transcription was performed with high capacity cDNA reverse-transcription kits (Applied Biosystems) followed by quantitative PCR (qPCR) analysis. TaqMan primers and probes for murine CCL19 and CCL21 were purchased from Applied Biosystems and samples were analyzed after preamplification with the Taqman PreAmp Master Mix kit, using the StepOne Plus analyzer (Applied Biosystems) with 18S as endogenous control.



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## **Paper 3**

**Mobilized multipotent progenitor-derived IL-1 $\beta$  drives enhanced regulatory Foxp3<sup>+</sup> T cell expansion and protects against experimental autoimmune encephalomyelitis**

**Sarantis Korniotis\*, Maud D'Aveni\*, Julien Rossignol, Christophe Gras, Guillemette Fouquet, Ruddy Montandon, Nicolas Cagnard, Marie-Thérèse Rubio, Olivier Hermine and Flora Zavala \*contributed equally (*in preparation*)**

## **Mobilized multipotent hematopoietic progenitor-derived IL-1 $\beta$ drives enhanced regulatory Foxp3<sup>+</sup> T cell expansion and protection against experimental autoimmune encephalomyelitis**

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### **Abstract**

Mobilized multipotent hematopoietic progenitors at the MPP2 stage of differentiation have the capacity to enhance the proliferation of TCR-activated Foxp3<sup>+</sup> regulatory CD4<sup>+</sup> T-cells not only in vitro but also in vivo. We now show that their adoptive transfer at 25,000 cells per mouse effectively reduced the development of experimental autoimmune encephalomyelitis (EAE), a pre-clinical model for multiple sclerosis. Adoptively transferred MPPs suppressed the production of the pathogenic cytokines IL-17, GM-CSF and IFN- $\gamma$  by peripheral and spinal cord CD4<sup>+</sup> T cells in protected recipients. MPPs also enhanced the levels of expression of Foxp3 within Tregs and actively promoted their expansion, as assessed by Ki67 nuclear staining, both in the periphery and in the spinal cord. Depletion of Tregs after protection by MPPs was established, abolished the protection against disease, demonstrating the key role of Tregs in the protective effect of MPPs against EAE. Transcriptomic analysis revealed that MPPs enhanced the expression of genes related to the IL-1 $\beta$  pathway within Tregs. Accordingly, MPPs prepared from IL-1 $\beta$  deficient donors were unable to provide protection against EAE, to enhance Treg expansion and to suppress pathogenic cytokine production, demonstrating the key role played by MPP-derived IL-1 $\beta$  in the immunoregulatory properties of the progenitors. Mobilized MPPs may be worth considering for enrichment of the HSC graft in autologous bone marrow transplantation already implemented in patients with severe refractory multiple sclerosis.

**Key words:** Multipotent hematopoietic progenitors/Mobilization/Tregs/Proliferation/IL-1 $\beta$

### **Introduction**

Autologous transplantation of hematopoietic progenitors (HSCs) is being performed in patients with severe, refractory, autoimmune diseases and singularly, multiple sclerosis (Burt et al., 2003, 2009; Fassas et al., 1997; Shevchenko et al., 2012). Although beneficial effects of the procedure have been ascribed to the resetting of a naïve, non-activated immune system, the hypothesis that selective HSC subsets might exert an active immunoregulatory role should not be neglected.

Taking into consideration that HSCs are increasingly isolated from the peripheral blood after mobilization, the possibility that mobilization might confer immunoregulatory properties to HSCs was explored. Using a cocktail of hematopoietic growth factors composed of G-CSF and Flt3-ligand (Flt3-L) for a synergistic HSC mobilization from the bone marrow to the periphery, we could show that hematopoietic progenitors at the multipotent MPP2 stage of differentiation were enriched by mobilization and acquired the capacity to enhance the proliferation of TCR-activated Foxp3<sup>+</sup> Tregs. The direct expansion of Tregs also took place in vivo, and accounted for the protection against spontaneous type 1 diabetes (T1D) in NOD mice induced by adoptive transfer of as few as 10,000 mobilized MPPs per mouse (Kared et al., 2006). Both contact- and soluble factor-dependent mechanisms (Kared et al., 2006) (Kared et al., 2008) were implicated in this acquired property of mobilized MPPs.

Tregs play an important role in the control of tolerance in multiple sclerosis as well as in its animal model of experimental autoimmune encephalomyelitis (EAE). Considerable efforts are being produced aiming at promoting expansion of Tregs in vitro for subsequent administration to patients with autoimmune diseases. Promoting the expansion of Tregs directly in vivo might represent a therapeutic strategy worth of interest. We therefore investigated in a murine model whether adoptive transfer of mobilized MPPs could be used to protect against EAE by promoting direct in vivo Treg expansion. We herein report that mobilized MPPs effectively reduced the disease score. They enhanced Foxp3 levels of expression within Tregs and promoted their proliferation both in vitro and in vivo, by an IL-1 $\beta$ -dependent mechanism. The key role of Tregs in the protection against EAE conferred by MPPs was demonstrated by disease relapse occurring upon selective depletion of Tregs performed once protection was established. Therefore, in vivo expansion of functional Tregs can be efficiently induced by MPPs, and could represent a therapeutic strategy against autoimmune diseases such as multiple sclerosis.



## **Results and discussion**

### **Adoptive transfer of mobilized MPPs protects against EAE and reduces pathogenic cytokine autoimmune production**

Adoptive transfer of as few as 25,000 mobilized MPPs per mouse, cell-sorted as shown in Fig.1A, at day 0 of immunization with the myelin oligodendrocyte glycoprotein 35-55 peptide (pMOG) according to the administration protocol outlined in Fig.1B, significantly reduced the clinical scores of EAE, relative to mice injected with PBS (Fig.1C). Accordingly, both in the periphery and in the spinal cord of MPP recipients, the CD4<sup>+</sup> T-cell production of the major pathogenic cytokines, IL-17, GM-CSF and IFN- $\gamma$  measured at the peak of the disease (day 21) was reduced (Fig.2A,B).

### **Peripheral and CNS Tregs proliferate at higher rates in MPP recipients**

The levels of Foxp3 were enhanced in gated CD4<sup>+</sup> cells in the spleen, lymph nodes and spinal cord of MPP recipient mice compared to control mice (Fig. 3A). Additionally, Treg proliferation, measured by nuclear staining of Ki67 within gated Foxp3<sup>+</sup> CD4<sup>+</sup> cells in control and MPP recipient mice at the peak of the disease was significantly enhanced in the periphery as well as in the spinal cord of MPP recipients relative to control mice with EAE (Fig.3B).

### **The protection against EAE conferred by adoptively transferred MPPs, once established, can be abrogated by Treg depletion**

To assess whether Tregs that accumulated in MPP recipients played a role in the observed protection against disease, MPP recipient mice with established protection were divided into two separate groups (n = 6 mice per group) that received one single injection at day 17 either of the anti-CD25 PC61 mAb (200  $\mu$ g/mouse, i.p.), which depletes Tregs (Sakaguchi et al., 2006) or of a control isotype mAb (Fig. 4A). Three days after mAb injection, the PC61-injected group started to develop more severe clinical signs of EAE and by day 25, had reached the clinical score of the control group that had not received MPPs. Conversely, the MPP-recipient group injected with the same amount of an isotype mAb did not significantly change its mean clinical score, and remained protected against EAE till the end of the experiment (day 30) (Fig.4 B).

Therefore, Tregs play a key role in the protection conferred by the adoptive transfer of MPPs since their removal, after protection is established, triggers disease relapse.

### **Role of IL-1 $\beta$ in the enhanced proliferation of Tregs conferred by mobilized MPPs**

Tregs sorted from Foxp3-GFP-KI mice and co-cultured in vitro at 1:1 ratio with MPPs showed enhanced proliferation, assessed by CFSE dilution over 3 days in response to stimulation by anti-CD3 + anti-CD28, compared to Tregs cultured alone. Transcriptomic analysis performed with Ingenuity Pathway Analysis on sorted Tregs either alone or co-cultured with MPPs over 3 days revealed enhanced expression of genes dependent of the IL-1 $\beta$  and possibly TNF $\alpha$ -pathways in Tregs whose proliferation was enhanced by MPPs (Fig.5A, B), such as IFN- $\gamma$ , *tbx-21*, *Prdm1* and *CCL5* genes.

The role of IL-1 $\beta$  was confirmed by the observation that MPPs from IL-1 $\beta$  KO mice (Fig.6A) were less efficient than WT MPPs at enhancing Treg proliferation in vitro (Fig. 6B left). Moreover addition of an anti-TNF- $\alpha$  neutralizing antibody abrogated differences between WT and IL-1 $\beta$  KO MPPs (Fig. 6B right) and also reduced basal proliferation rate of Tregs suggesting that TNF- $\alpha$  production is partly induced by IL-1 $\beta$  and may play a complementary role with IL-1 $\beta$  in MPP-enhanced proliferation rate.

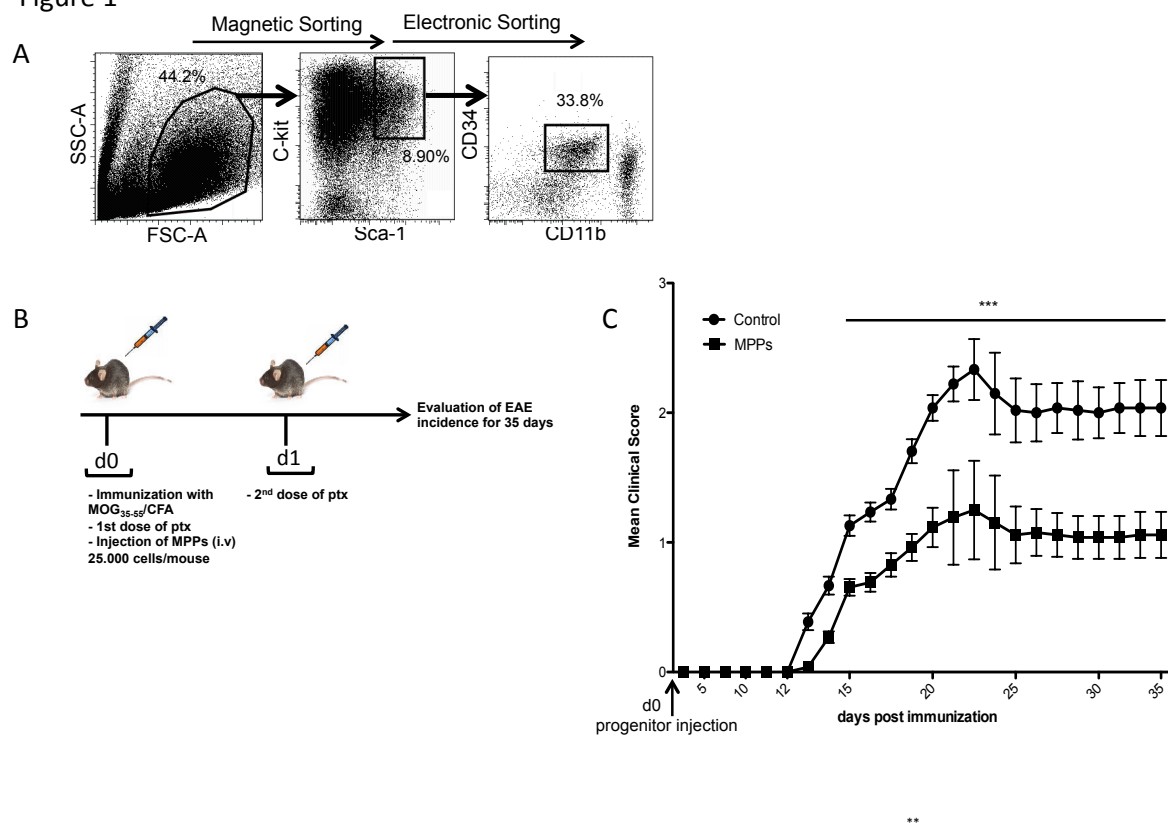
### **IL- $\beta$ -dependency of the protection against EAE, the cytokine switches and the enhanced Treg numbers induced by MPPs**

Unlike WT MPPs, MPPs prepared from IL-1 $\beta$  KO-donor mice (Fig. 6A) and adoptively transferred at day 0 of immunization (Fig.6C) were unable to confer protection in vivo against EAE (Fig.6D), confirming the key role of this cytokine in the in vivo effect of MPPs against the disease.

Moreover, in recipient mice adoptively transferred with IL-1 $\beta$  KO MPPs, the encephalitogenic cytokine production remained at levels comparable to those in control mice with EAE in both peripheral lymph nodes and spinal cord (Fig.7). In addition, recipients of IL-1 $\beta$  KO MPPs did not display the strongly enhanced production of TGF- $\beta$  observed particularly in the spinal cord in WT-MPP recipients (Fig.7D).

Finally, conversely to recipients of WT MPPs, recipients of IL-1 $\beta$  KO MPPs showed no increase in the percentages of Treg in their spinal cord, compared to non-injected EAE controls (Fig.7E, F).

Figure 1



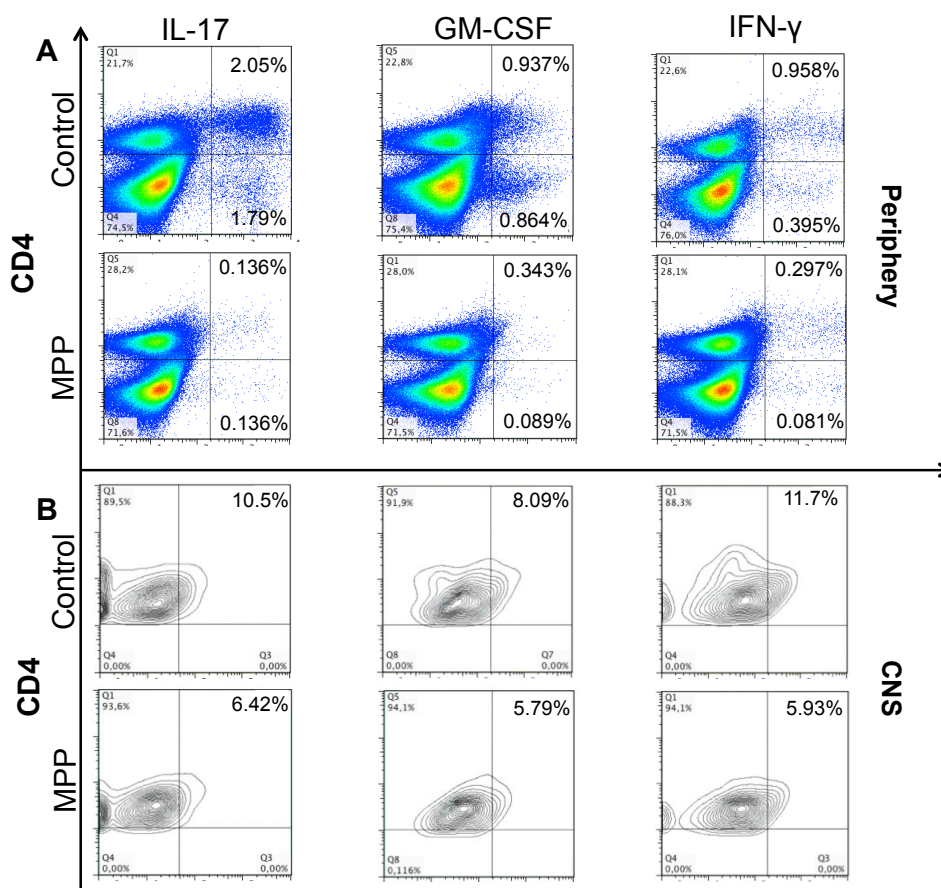
**Fig 1. Adoptive transfer of mobilized multipotent progenitor cells at day 0 confers protection against MOG<sub>35-55</sub>-induced EAE mice** (A) Mobilized multipotent progenitor cells (MPPs) from the spleen of C57BL/6J mice were magnetically selected for c-kit<sup>+</sup> cells, further stained for Sca-1, CD34 and CD11b and electronically sorted into c-kit<sup>high</sup>Sca-1<sup>high</sup>CD34<sup>+</sup>CD11b<sup>-</sup> cells. (B) Experimental protocol for MOG<sub>35-55</sub> EAE disease induction in C57BL/6J mice after immunization with MOG<sub>35-55</sub>/CFA at day 0, and injections of pertussis toxin at day 0 and 1. Mice received intravenous progenitor cell transfers at day 0 and the incidence of the disease was followed for 35 days. (C) EAE incidence in C57BL/6J mice after an adoptive transfer at the time of immunization. Adoptive transfer of 25,000 MPPs per mouse at day 0 conferred protection to EAE in vivo. n=26 mice per group, \*\*\*P < 0.001 when comparing cumulative incidence curves by two-way repeated measurement ANOVA test.

Table 1 Clinical Parameters

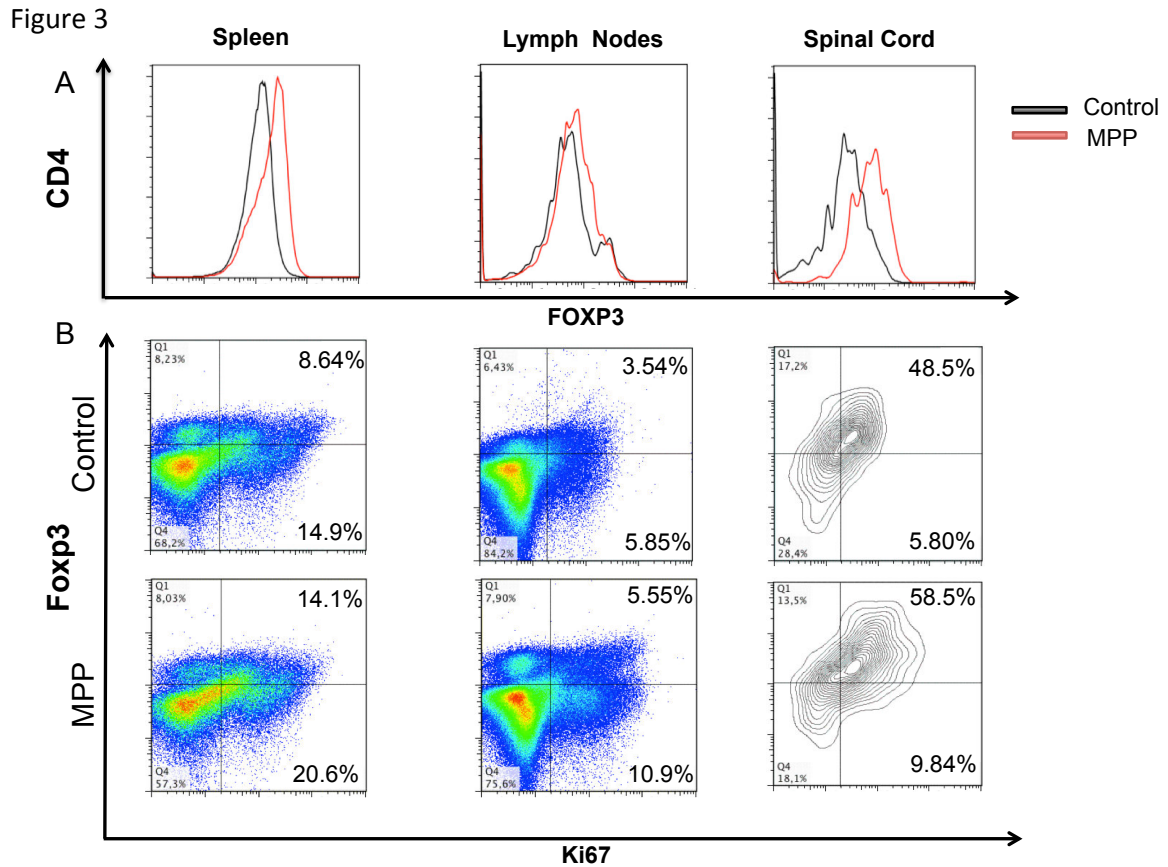
Group of Mice	Mice/group	Age at immunization	Mean Maximum Score $\pm$ SD	Day of onset $\pm$ SD	Disease Incidence
Control	26	12 weeks of age	2.6 $\pm$ 1.3	13 $\pm$ 1.06	90%
MPP injected	26	12 weeks of age	1.3 $\pm$ 1.2 *	14.9 $\pm$ 2.3	61.5% **

EAE disease was induced in C57BL/6J mice at the age of 12 weeks. Mice received 25,000 of MPPs intravenously at day 0 or injected with PBS (control). It is shown the number of mice immunized per group, the maximum clinical score (mean  $\pm$  SD), the day of onset among mice with EAE and the total incidence of the disease in each group. \*P < 0.05 \*\*P < 0.005 using the non-parametric Whitney t test.

Figure 2

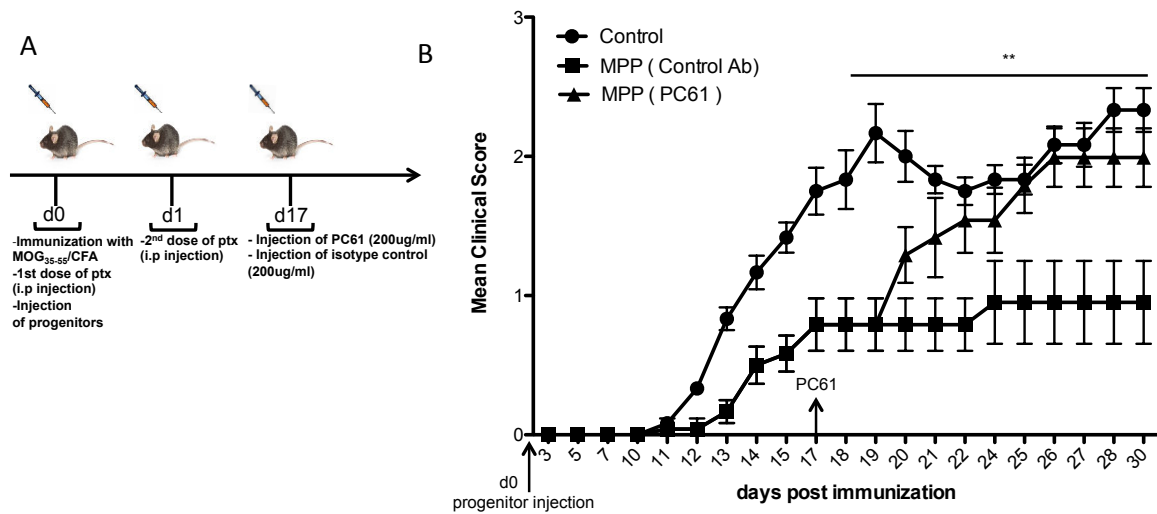


**Fig 2. Cytokine profile of autoimmune responses in control mice or mice injected with MPPs at the peak of the disease.** Mice were sacrificed at day 21 of the disease and intracytoplasmic production of the cytokines IL-17, GM-CSF and IFN- $\gamma$  analyzed by FACS in gated CD4<sup>+</sup> T cells. (A): Lymph nodes (Periphery) were analyzed for the production of the cytokines after re-stimulation with the MOG peptide for 4 days and activation with PMA + ionomycin for 5h. (B): Cells recovered from the spinal cord (CNS) were analyzed the same day after activation with PMA + ionomycin for 3h.



**Fig.3** The adoptive transfer of MPPs enhances both Foxp3 expression and the *in vivo* proliferation of T regulatory cells (A) Intranuclear expression of Foxp3 in CD4<sup>+</sup> T cells purified from the lymph nodes, spleen and spinal cord either of control mice or mice injected with MPPs was analyzed by FACS. (B) The *in vivo* proliferation of Foxp3<sup>+</sup> cells was analyzed by measuring the intranuclear expression of Ki67 by FACS.

Figure 4

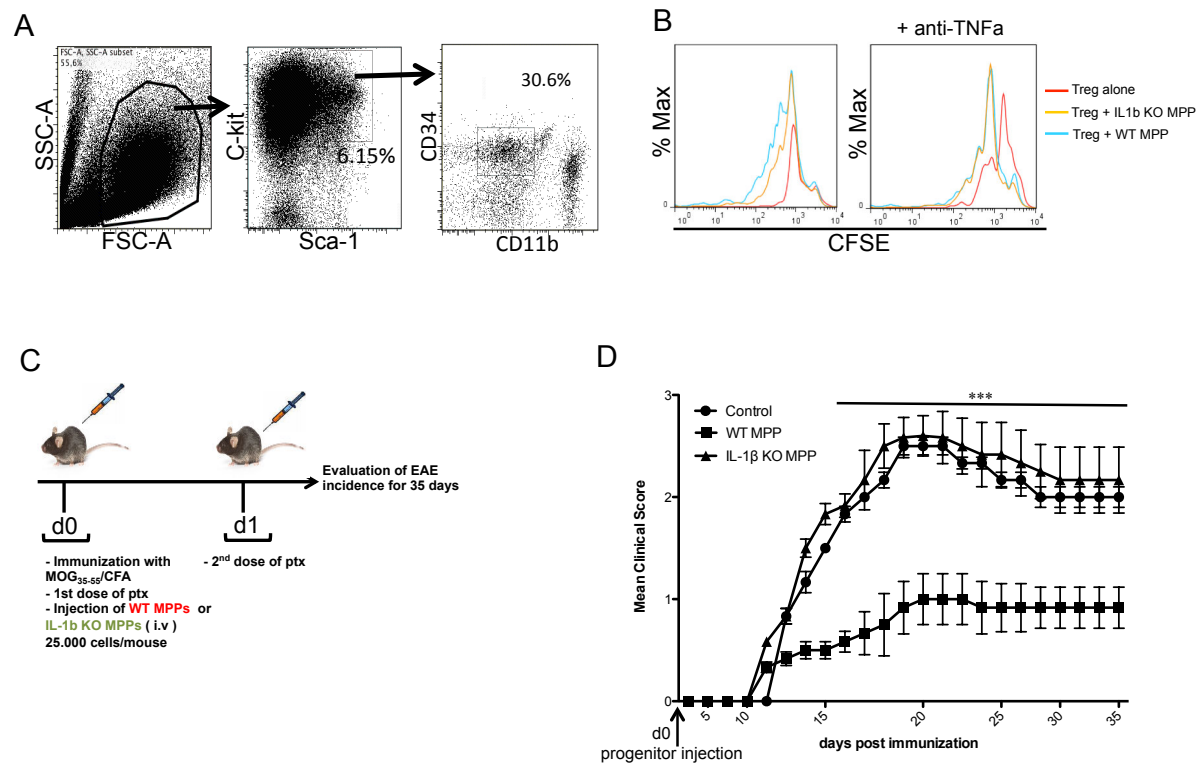


**Fig.4 Role of Tregs in the protection against EAE in MPP recipients.** (A) EAE disease was induced upon immunization with MOG<sub>35-55</sub>/CFA and mobilized progenitor cells were adoptively transferred at day 0. EAE development (mean clinical score  $\pm$  SD) was followed in control mice (n=10) or mice injected with MPPs (n=12) and at day 17, once the protection due to the injection of MPPs established, 6 of the recipient mice of MPPs were injected intravenously with the anti-CD25 mAb (PC61) and 6 mice with an isotype control antibody. (B) EAE mean clinical score after the adoptive transfer of MPPs at day 0 and injection of the PC61 or its isotype control antibody at day 17 of disease. \*\*P < 0.005 by two-way repeated measurements ANOVA test.



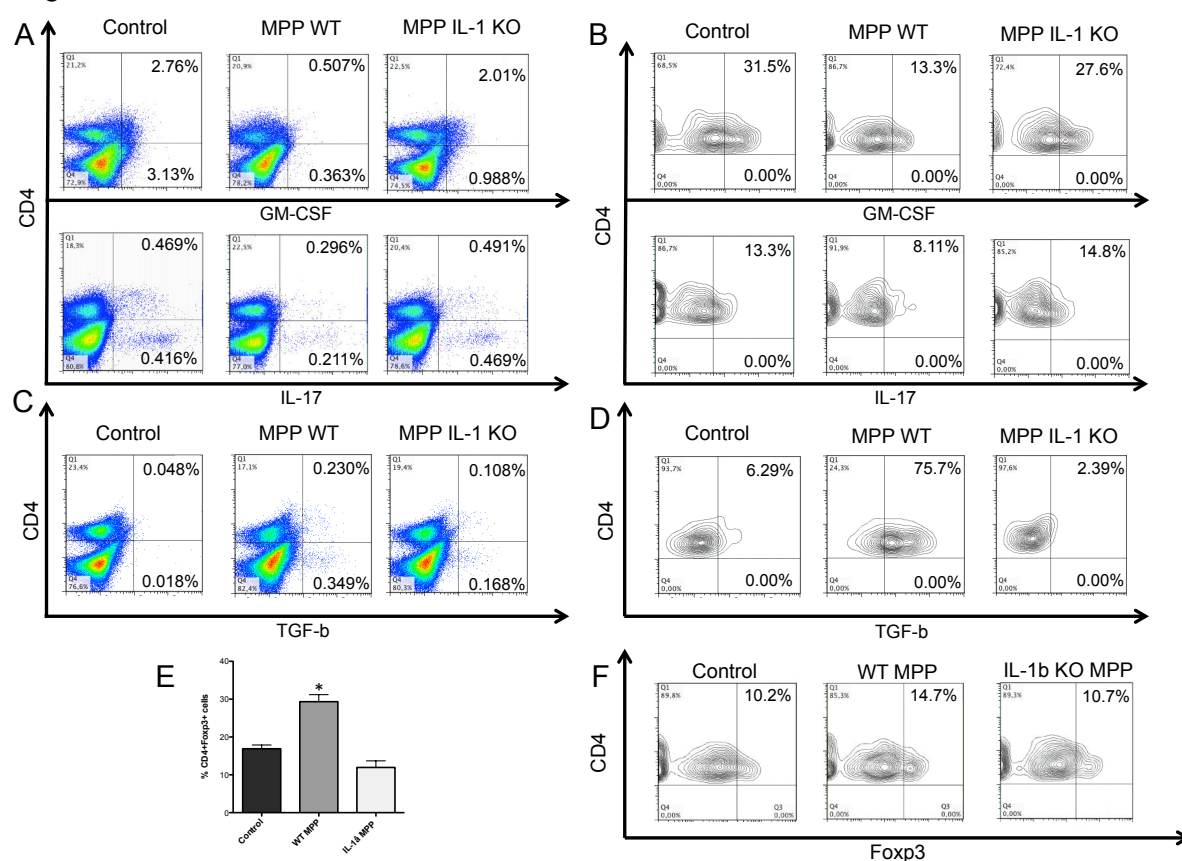


Figure 6



**Fig. 6 Role of IL-1 $\beta$  produced by MPPs in the expansion of Tregs and protection against EAE.** (A) Mobilized multipotent progenitor cells were magnetically sorted for c-kit<sup>+</sup> cells from the spleen of C57BL/6J mice deficient for IL-1 $\beta$  and further stained for Sca-1, CD34 and CD11b and electronically sorted into c-kit<sup>high</sup>Sca-1<sup>high</sup>CD34<sup>+</sup>CD11b<sup>-</sup> cells. (B) Proliferation of CFSE-loaded CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T regulatory cells purified from the secondary lymphoid organs of Foxp3-GFP-KI C57BL/6J mice, 3d after activation with anti-CD3 + anti-CD28 either cultured alone or co-cultured with MPPs from WT or IL-1 $\beta$  KO B6 mice. (C) Experimental protocol for MOG<sub>35-55</sub> EAE disease induction in C57BL/6J mice after immunization with MOG<sub>35-55</sub>/CFA at day 0, injections of pertussis toxin at day 0 and 1 and adoptive transfer of progenitor cells at day 0. (D) EAE disease was followed in control mice (n=10) or mice that have been injected either with WT (n=10) or IL-1 $\beta$  KO MPPs (n=10) at day 0. The development of the disease was followed for 35 days. \*\*\* P < 0.001, by two-way repeated measurement ANOVA test.

Figure 7



**Fig.7 WT but not IL-1 $\beta$  KO MPPs reduce the pathogenic cytokine profile of CD4<sup>+</sup> T cells and trigger the production of TGF- $\beta$  and the expansion of Treg.** Mice were sacrificed at the peak of the disease (d21) and autoimmune responses were analyzed by FACS within lymph nodes after 4d of re-stimulation in vitro with MOG peptide plus activation with PMA + ionomycin for 4h whereas cells recovered from the spinal cord were analyzed the same day after 3h of stimulation with PMA + ionomycin + golgi stop. (A) Intracytoplasmic production of the cytokines IL-17 and GM-CSF by CD4<sup>+</sup> T cells within the lymph nodes and (B) spinal cord of control mice or mice that have been injected either with WT or IL-1 $\beta$  KO MPPs. (C, D): Intracytoplasmic production of the cytokine TGF- $\beta$  by CD4<sup>+</sup> T cells in the (C) lymph nodes or (D) spinal cord. (E) Percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T regulatory cells found in the spinal cord of EAE control mice or mice injected either with WT or IL-1 $\beta$  KO MPPs, (n=3 mice per group, results are expressed as mean  $\pm$  SD) \* P< 0.05. (F) Intranuclear expression of Foxp3 in gated CD4<sup>+</sup> T cells of the spinal cord cells from control mice or mice injected with either WT or IL-1 $\beta$  KO MPPs

## Discussion

Mobilized hematopoietic progenitor subsets at the developmental stage of MPP2, displayed the remarkable property to promote the expansion of TCR-activated Tregs. The functional outcomes of this property were highly significant, since as few as 25,000 mobilized MPP cells adoptively transferred per recipient conferred protection against EAE, and this protection was highly dependent on Tregs as demonstrated by disease relapse upon Treg depletion by the PC61 mAb treatment in MPP-recipient mice.

The molecular mechanism by which C57Bl/6 mice derived MPPs afforded protection against autoimmune disease is different from that previously described in spontaneous diabetes in NOD mice in which mobilized MPPs triggered both Notch- and GM-CSF-dependent signaling (Kared et al., 2006). Genetic variations between strains may account for this inconsistency as it has been shown that GM-CSF effectively enhanced Treg proliferation both in the NOD (Kared et al., 2008) and the BALB/c strains but not in the CBA nor in the C57Bl/6 strains (Zou et al., 2010). We herein show that in C57Bl/6 mice immunized with MOG<sub>35-55</sub>, IL-1 $\beta$  was the key molecular mediator produced by mobilized MPPs promoting the expansion of TCR-activated Tregs in vitro as well as in vivo.

This may appear paradoxical since IL-1 $\beta$  is mostly associated with inflammation in MS and EAE. Schiffenbauer et al have shown an absolute requirement of IL-1R for the development of EAE (Schiffenbauer et al., 2000). IL-1 $\beta$  is present in MS lesions (Cannella and Raine, 1995) and IL-1R $\alpha$  moderates the induction of EAE (Badovinac et al., 1998). Both IL-1 $\beta$  and IL-1RA appear in white and grey matter lesions in a model of relapsing EAE at sites of active demyelination (Prins et al., 2013). An elevated IL-1 $\beta$  over IL-1RA ratio corresponds to higher risks of developing MS (de Jong et al., 2002). In addition, IL-1 $\beta$  plays a role in glutamate-mediated synaptic excitability and neurotoxicity taking place in EAE (Mandolesi et al., 2013; Rossi et al., 2012).

However, IL-1 $\beta$  priming reduced EAE severity in rats (Huitinga et al., 2000). Additionally, the capacity of IL-1 $\beta$  to enhance TCR-activated Treg proliferation but not to trigger resting Treg proliferation was recently reported in several in vitro studies. IL-1 $\beta$  has been shown to promote TGF- $\beta$  and IL-2 dependent Foxp3 expression in Tregs and to display co-stimulatory effects on their expansion and differentiation (Brinster and Shevach, 2008; Ganesh et al., 2011). It has been proposed that Foxp3<sup>-</sup> T cells may be the primary target of IL-1 $\beta$  and actually support Foxp3<sup>+</sup> Treg expansion. Here, IL-1 $\beta$  produced by mobilized MPPs was able to enhance the proliferation of Foxp3<sup>+</sup> Tregs isolated from Foxp3-GFP-KI mice, thus not contaminated by non Tregs.

TNF- $\alpha$  signaling was also enhanced in Treg co-cultured with MPPs. The possibility that IL-1 $\beta$  induces TNF- $\alpha$  production by Tregs, that in turn promotes their autocrine proliferation, is suggested by the effect of anti-TNF- $\alpha$  neutralization in co-cultures in vitro which abolished the differences in Treg proliferation induced in the presence of WT versus IL-1 $\beta$  KO MPPs. TNF- $\alpha$  interacts on TNF-R2 expressed on Tregs and is important for the stabilization, function and expansion of Tregs (Chen et al., 2013). Its implication in MPP-driven expansion of Tregs might be investigated using mice that are TNF-R2 KO selectively within Tregs.

TNF- $\alpha$  can exert both enhancing and regulatory activities in the pathogenesis of MS and EAE. Treatment with anti-TNF- $\alpha$  at the time of immunization reduced the incidence and delayed the onset of EAE but had no effect on disease severity once EAE was established (Suvannavejh et al., 2000). Conversely, selective targeting of TNFR1 could ameliorate the symptoms of the most common models of MS disease (Eugster et al., 1999).

In vitro, mobilized MPPs had no effect on the proliferation of isolated CD4<sup>+</sup>CD25<sup>-</sup> effector T-cells and did not induce their conversion into Foxp3<sup>+</sup> cells (not shown). Notably, we observed in recipients of WT but not IL-1 $\beta$  KO mobilized MPPs a quite significant increase in TGF- $\beta$  production, but no change in IL-10 levels (not shown), that could account for the reduction of the pathogenic cytokine production observed in MPP recipients. TGF- $\beta$  production was induced in a large proportion of CD4<sup>+</sup> T-cells, surpassing the proportion of Tregs, suggesting that non-Tregs cells are also switched towards the production of this immunoregulatory cytokine.

Foxp3<sup>+</sup> Tregs have been shown to specialize to selectively regulate effector T cell responses (Chaudhry et al., 2009; Cipolletta et al., 2012; Koch et al., 2009; Zheng et al., 2009). Interestingly, several of the genes with enhanced expression in Tregs co-cultured with MPPs including IFN- $\gamma$ , *tbx21*, *Prdm1*, have been reported to confer to Tregs the capacity to suppress Th1 and Th17 inflammatory cells (Joller et al., 2014), most appropriate to control EAE disease. Indeed, such cytokine reduction was induced in CD4<sup>+</sup> T cells of MPP recipients. Whether these Treg-induced changes in Teff affect their capacity to transfer disease remains to be investigated. Moreover, whether MPPs may only target Teff through Tregs should be tested by verifying that MPPs display no effect in vivo on EAE induced by passive transfer in Rag-KO C57BL/6 recipients of Teff strictly deprived of Tregs.

Non mobilized bone-marrow progenitors at the MPP stage are devoid of the capacity to promote Treg expansion in NOD mice (Kared et al., 2006, 2008) and in C57BL/6 mice (data not shown). However, they share the same multilineage differentiation potential as mobilized

MPPs, ruling out the possibility that their progeny plays a major role in their protective properties against autoimmune diseases.

The herein described protective effects of MPPs occurred when the progenitors were injected at day 0, at the time of immunization. The therapeutic potential of MPPs when injected later in disease, after the onset of clinical signs, would be interesting to investigate. MPPs would then represent a promising tool for cell therapy of autoimmune diseases either injected on their own or as complement of autologous HSC transplantation, already performed in MS patients with severe, non-responsive disease.

## Material and Methods

**Mice:** Wild type C57BL/6, C57BL/6 Foxp3-GFP-KI and C57BL/6 IL-1 $\beta$ <sup>-/-</sup> mice were bred in our animal facility under specific pathogen-free conditions. Live animal experiments were approved by the Necker Animal Experimentation and Ethics Committee. Female C57BL/6 mice were used at the age of 10-12 weeks of age as a model for MOG<sub>35-55</sub>-induced Experimental Autoimmune Encephalomyelitis and received intravenous progenitor cell transfers at day 0.

**EAE induction:** Active EAE was induced in 10-12-wk-old female mice by s.c. immunization at two sites, upper back and lower back, with 1 mg myelin oligodendrocyte glycoprotein (MOG)<sub>35–55</sub> peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in CFA containing 2.5 mg heat-killed Mycobacterium tuberculosis H37Ra (Hooke Laboratories, Lawrence, MA, USA), on day 0. Additionally, mice received 200 ng pertussis toxin (Hooke Laboratories, Lawrence, MA, USA) i.p. in 0.1 ml/mouse on days 0 and 1. Clinical signs of EAE were assessed daily with a 0- to 5-point scoring system, as follows: 0, no obvious changes in motor function compared to non-immunized mice; 0.5, tip of tail is limp; 1, limp tail; 1.5, limp tail and hind leg inhibition; 2, limp tail and weakness of hind legs; 2.5, limp tail and dragging of hind legs; 3, limp tail and complete paralysis of hind legs or paralysis of one front and one hind leg; 3.5, limp tail and complete paralysis of hind legs, in addition to: mouse is moving around the cage, but when placed to its side, is unable to right itself; 4, limp tail complete hind leg and partial front leg paralysis; 4.5, complete hind leg and partial front leg paralysis, no movement around the cage, mouse is not alert; 5, mouse does not move any more in the cage. Mice with score  $\geq 4$  for two consecutive days and mice with score 5 were euthanized. Disease scores over the course of the 35-d experiments were totalized for each animal, and the mean for the experimental group was expressed as a cumulative EAE score.

**In vivo treatments and isolation of mobilized multipotent progenitors (MPPs):** Mobilized progenitor cells (MPPs) were prepared as follows : Wild type C57BL/6J or IL-1 $\beta$ <sup>-/-</sup> mice (8- to 12-week-old) were injected s.c for 4 consecutive days with human recombinant G-CSF (200  $\mu$ g/kg/day) (Zarzio 48MU/0.5ml, Sandoz) + recombinant murine Flt3-L (20  $\mu$ g/kg/day) (Immunotools, Friesoythe, Germany). Total splenocytes were magnetically sorted for c-kit<sup>+</sup> cells with an automated magnetic cell sorter (Robosep, StemCell Technologies, Vancouver, BC, Canada), further stained with the mAbs directed against Sca-1, CD34 and CD11b and electronically sorted into c-kit<sup>high</sup>Sca-1<sup>high</sup>CD34<sup>+</sup>CD11b<sup>-</sup> cells with the FACS Aria II cell sorter (BD Biosciences). Each mouse received intravenously 25,000 cells of this above subset

at the same day of immunization with MOG<sub>35-55</sub>/CFA. Intravenous treatment with anti-CD25 mAb (PC61) (200 µg/ml) or the control isotype antibody was performed at day 17 of the disease, once protection by MPP as established.

**Isolation of Immune cells from the Spinal cord.** Spinal cord isolated from control and MPP-recipient mice were incubated for 30 min in digestion buffer of DNase and Liberase (27 WU/ml) in PBS 1X at 37°C, mixing every 5 minutes. EDTA (500µl, 100mM) was added for 1 min to end the digestion. The cells were passed through a 100 µm cell strainer, using a syringe plunger (back side) to smash the tissue. A percoll separation was followed resuspending the cells first in 3-5ml of 40% Percoll and underlayered with the same volume of 70% Percoll (in PBS) and the cells were centrifuged for 35 minutes at 1300g (2800 rpm) without brake. The cells of the interface were collected with a pipette Pasteur and diluted 10 times with complete medium RPMI 10% fetal cell serum (FCS). The cells were centrifuged and resuspended in 2-3 ml of complete medium.

**Staining of Cells for Flow Cytometry Analysis.** To block nonspecific Fc receptor binding, cells were preincubated for 10 min at room temperature with FcR blocker 2.4G2 mAb. Cells were then stained with appropriately labeled mAbs against CD4, c-kit (CD117), CD11b, CD34, Sca-1 (anti-Ly6A/E), (eBioscience). Nuclear Foxp3 and Ki67 expression was measured by FACS analysis as per manufacturer's instructions (eBioscience). Cytokines were measured at day 4 after a cell culture of lymph node cells or splenocytes isolated from control or MPP-recipient mice, re-stimulated with the MOG<sub>35-55</sub> peptide and in spinal cord cells the same day. Intracytoplasmic expression of cytokines was assessed after a 5-h stimulation with PMA (10 ng/mL) plus ionomycin (500 ng/mL) in the presence of Brefeldin A (2 mg/mL) for lymph nodes and spleen, and 3h for spinal cord, followed by fixation/permeabilization with PFA/saponin and subsequent staining with specific antibodies including APC-labeled anti-IFN-γ (from eBiosciences), PE-labeled anti-GM-CSF (from BD Biosciences), PE-labeled anti-TGF-β (R & D Systems), APC-labeled anti-IL-17 (BD Biosciences) or isotype controls. Membrane and intracellular antigen expression was analyzed in a FACS Canto II cytometer (BD Biosciences) using FlowJo software (Treestar).

**Proliferation assays:** Proliferation Assays. CD4<sup>+</sup>CD25<sup>high</sup> (all Foxp3<sup>+</sup>) cells isolated from the secondary lymphoid organs were magnetically sorted from the spleen of Foxp3-GFP-KI C57BL/6J mice. They were loaded with CFSE (Life Technologies) and cultured (5 × 10<sup>4</sup> cells per well) in RPMI medium 1640 supplemented with 5% (vol/vol) FCS (Life Technologies),

1% antibiotics, and  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol. Cells were plated in 96-well round-bottomed culture plates, either alone or with sorted MPPs at 1:1 T:MPP cell ratios, and stimulated with 2.5  $\mu\text{g}/\text{mL}$  of anti-CD3 mAb (clone 145–2C11) and 5  $\mu\text{g}/\text{mL}$  of anti-CD28 mAb (clone 37.51, eBioscience) for 3d.

**Transcriptomic Assay:** To analyze the possible mechanisms involved in mouse  $\text{CD4}^+\text{CD25}^{\text{high}}$  regulatory T-cells (Treg cells) expansion promoted by mobilized MPPs (MPPs), we sequenced the transcriptome of activated Treg extracted from simple culture or from co-culture with MPPs. Tregs and MPPs were sorted as previously described. Tregs were cultivated for 3 days with anti-CD3 and anti-CD28 alone or together with freshly purified MPPs. After 3 days,  $\text{CD4}^+$  cells (Treg) were sorted for extraction of RNA using RNeasy Micro kit (QIAGEN). Its quality was verified in an Agilent Bioanalyzer. Total RNA was amplified and converted to biotinylated cRNA according to the manufacturer's protocol (Illumina TotalPrep RNA Amplification Kit; Ambion). Two biological duplicates were hybridized to the Sentrix BeadChips Array mouse WG-6 v2 (Illumina) and read at the Imagine Genomic platform, Paris, France. RetroMicroarray experiments were performed on Affymetrix Mouse Genome 430\_2 GeneChips. Gene expression levels were normalized using the GC-RMA algorithm and flags were computed using MAS5. Quality assessment of the chips has been performed with affyQCReport R package (R project for Statistical Computing : [<http://www.r-project.org/>]). The group comparisons were done using Student's *t* test. We filtered the results using a 1.5-fold variation and *p* values  $< 0.05$ . Cluster analysis was performed by hierarchical clustering using the Spearman correlation similarity measure and average linkage algorithm. Data were submitted to Ingenuity Pathway Analysis (IPA) to model relationships among genes and proteins, to construct pathways and unveil relevant biological processes (Ingenuity Pathways : [<http://www.ingenuity.com/>]).



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**Contribution to other scientific papers**

**Paper 4**

**CCL22-producing CD8 $\alpha$  myeloid dendritic cells mediate regulatory T cell recruitment in response to G- CSF treatment**

**Layseca-Espinosa E, Korniotis S, Montandon R, Gras C, Bouillie M, Gonzalez-Amaro R, Dy M, Zavala F**

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## CCL22-Producing CD8 $\alpha$ <sup>-</sup> Myeloid Dendritic Cells Mediate Regulatory T Cell Recruitment in Response to G-CSF Treatment

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G-CSF prevents type 1 diabetes in the NOD mouse by promoting the local recruitment of T regulatory cells (Tregs). This is an indirect effect because adoptive transfer of G-CSF-induced tolerogenic dendritic cells (DCs) promotes Treg accumulation. However, the identity of the particular DC subset and the molecule(s) mediating this effect remain unknown. We demonstrate in this study that the adoptive transfer of CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs isolated from pegylated G-CSF (pegG-CSF) recipients, but not that of other DC subtypes, enhanced the pancreatic recruitment of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, which generated increased amounts of TGF- $\beta$ . Likewise, only CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs from pegG-CSF recipients secreted the chemokine CCL22 at levels that effectively attracted Tregs. PegG-CSF was more efficient at enhancing the synthesis of CCL22 by CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs from the pancreatic lymph nodes compared with those from the spleen. Accordingly, CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs from the pancreatic lymph nodes of pegG-CSF recipients were more efficient than their splenic counterparts in the recruitment of Tregs upon adoptive transfer. Predictably, CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs failed to recruit these Tregs both in vivo and in vitro following intracellular neutralization of CCL22. These data assign a key role to CD8 $\alpha$ <sup>-</sup> DCs and CCL22 in Treg recruitment in the protection of NOD mice against type 1 diabetes following the treatment with G-CSF. *The Journal of Immunology*, 2013, 191: 2266–2272.

**O**ne major immunotherapeutic trend in autoimmune diseases is to readjust the balance between regulatory T cells (Tregs) and autoreactive effector T cells, both by increasing the number of Tregs and by enhancing their suppressive function.

Interestingly, some biological agents already used in the clinics for other applications display such properties. Thus, treatment with G-CSF provides protection against various experimental autoimmune diseases, including lupus nephritis, experimental autoimmune encephalomyelitis, colitis, and type 1 diabetes (T1D) (1–5). T1D is an autoimmune disease characterized by the infiltration in

the pancreatic islets of autoreactive T cells that target insulin-producing  $\beta$  cells. This process, named insulinitis, leads ultimately to  $\beta$  cell death and insulin deficiency. NOD mice develop a spontaneous form of T1D, gradually evolving from peripheral insulinitis at 4–8 wk of age to invasive insulinitis at 8–12 wk of age, followed by overt hyperglycemia from 15 wk of age onward. Despite inherent limitations, NOD mice represent a relevant animal model of T1D widely used for investigating novel therapeutic strategies (6).

The hematopoietic growth factor G-CSF prevented T1D in NOD mice if administered during consecutive 5 d every 4 wk at 4, 8, 12, and 16 wk of age (5). This treatment promoted the recruitment to the pancreatic lymph nodes (PLN) of Tregs, which play a key role in the protection against T1D conferred by G-CSF. Indeed, the G-CSF–recruited Tregs were more suppressive than control Tregs by generating enhanced levels of TGF- $\beta$  and preventing the onset of T1D induced by adoptively cotransferred diabetogenic splenocytes in NOD-SCID recipients (5). Subsequent adoptive cell transfer experiments revealed the role of dendritic cells (DCs) in the Treg accumulation in G-CSF recipients (5). However, whole DC populations were transferred in those experiments. Although plasmacytoid DCs (pDCs) accumulated preferentially over other DC subsets in G-CSF-treated NOD mice that were protected against T1D (5), the DC subset primarily responsible for Treg recruitment and the underlying molecular mechanisms remained to be identified.

G-CSF rescues NOD mice with recent diabetes onset (7), when administered in conjunction with antithymocyte globulin, and enhances pancreatic islet graft survival (8) in combination with rapamycin. These beneficial effects have prompted the evaluation of stabilized pegylated G-CSF (pegG-CSF) in various ongoing clinical trials, to investigate its capacity to induce Tregs in patients with T1D, and to reverse recent onset T1D in association with antithymocyte globulin.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; mDC, myeloid DC; pDC, plasmacytoid DC; pegG-CSF, pegylated G-CSF; PLN, pancreatic lymph node; T1D, type 1 diabetes; Treg, T regulatory cell.

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In this study, we determined the therapeutic window of efficacy for pegG-CSF in NOD mice and evaluated its ability to modulate the distribution of various DC subsets as well as the DC subtype involved in Treg recruitment. We established that CD11c<sup>high</sup> CD8 $\alpha$ <sup>-</sup> DCs rather than other DC subsets mediated the recruitment of Tregs in response to pegG-CSF, through their production of the chemokine CCL22. PegG-CSF induced higher production levels of CCL22 by DCs located in the PLNs compared with those localized in the spleen. Accordingly, PLN-derived DCs show an enhanced ability to attract Tregs, which synthesized increased amounts of TGF- $\beta$ . As expected, these cells were no longer recruited when CCL22 was neutralized.

## Materials and Methods

### Animal experiments

Congenic CD45.1<sup>+</sup> and CD45.2<sup>+</sup> NOD mice were bred in our animal facilities under specific pathogen-free conditions. Based on the demonstration that a single accumulated injection of pegG-CSF (Neulasta) was equivalent to five daily consecutive injections of nonpegylated G-CSF (Neupogen) (5, 9), NOD mice were treated with a single s.c. injection of 1 mg/kg recombinant human pegG-CSF at 4, 8, or 11 wk of age. The excipient consisted of 5% dextrose in sterile H<sub>2</sub>O. Colorimetric strips were used to monitor glycosuria twice per week (Glukotest; Boehringer-Mannheim, Mannheim, Germany) and glycemia (Hemoglukotest and Reflolux F; Boehringer-Mannheim). Mice with blood glucose levels >250 mg/dl on two consecutive readings were considered diabetic. Animal experiments were approved by the local Animal Ethical Committee.

### Abs and flow cytometry

CD4, CD25, and Foxp3 Abs were purchased from eBioscience (San Diego, CA), and intranuclear staining for Foxp3 was performed following the manufacturer's instructions. DC subtypes were stained with Abs to CD11c purchased from BD Biosciences (Le Pont de Claix, France), to PDCA-1 from Miltenyi Biotec (Bergisch Gladbach, Germany), and to CD11b, CD8 $\alpha$ , and CD103 from eBioscience. Membrane and intracellular Ag expression was analyzed on a FACSCanto II cytometer (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR).

### FACS analysis of cytokine expression

Pancreata were collected from DC recipients 2 wk after adoptive transfer, cut into pieces, and lysed in 1 mg/ml Liberase TL and 10 U/ml DNase (Roche Diagnostics, Meylan, France) for 30 min at 37°C. Cells recovered from the lysate were stimulated for 5 h with PMA (10 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (2 mg/ml) and surface expression of CD4 and CD25, as well as intracytoplasmic expression of cytokines was assessed using the Fix/Perm kit (BD Biosciences), according to the manufacturer's instructions, with specific Abs, including allophycocyanin-labeled anti-IL-10 (BD Biosciences) and PE-labeled anti-TGF- $\beta$  (R&D Systems, Abingdon, U.K.).

### Isolation of spleen DCs from excipient- and pegG-CSF-treated NOD mice and adoptive transfer into secondary NOD recipients

Six days after the injection into 6- to 8-wk-old mice of excipient or pegG-CSF (1 mg/kg/mouse), myeloid DCs (mDCs) were immunomagnetically sorted from the spleen of NOD mice with the PanDC kit of Miltenyi Biotec, according to manufacturer's instructions, and stained with anti-PDCA-1, anti-CD11c, and anti-CD8 $\alpha$  Abs. CD11c<sup>high</sup> PDCA-1<sup>-</sup> mDCs were further separated into >98% pure CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> cells by electronic sorting on a BD FACS Aria. pDCs were immunomagnetically isolated (90% pure) from the spleen with the pDC isolation kit II (Miltenyi Biotec). DCs from the PLNs of Exc- or pegG-CSF-treated NOD mice were electronically sorted (FACSARIA II; BD Biosciences) after staining with CD11c, CD8 $\alpha$ , and PDCA-1. Adoptive transfer into naive 6-wk-old female NOD recipients was performed with i.v. injection of either  $2.5 \times 10^5$  cells/mouse for pDCs,  $2.5 \times 10^5$  or  $7 \times 10^4$  cells/mouse for CD8 $\alpha$ <sup>-</sup> mDCs, and  $1 \times 10^7$  cells/mouse for CD8 $\alpha$ <sup>+</sup> mDCs.

### ELISA

CCL22 protein levels were measured by ELISA (R&D Systems) in supernatants of DCs ( $10^6$  cells/ml) after 48-h incubation in RPMI 1640 cell

culture medium with 10% FCS. Cytokine production was measured with a Multiplex ELISA kit (Life Technologies).

### Quantitative RT-PCR

Total RNA was isolated from the sorted DC subsets by using the Ambion (Life Technologies) PureLink RNA extraction kit. TaqMan primers and probes for murine CCL22 were purchased from Applied Biosystems (Life Technologies), and samples were analyzed using the StepOne Plus analyzer (Applied Biosystems) with 18S as endogenous control.

### Vector and Ab transfection of DCs

CD8 $\alpha$ <sup>-</sup> mDCs, isolated by electronic cell sorting, were incubated for 15 min with the Chariot vector (Active Motif, La Hulpe, Belgium) previously coupled with a neutralizing anti-CCL22 Ab (R&D Systems) or a control rat Ab, according to the manufacturer's instructions. Transfected DCs (>95% as assessed by FACS with a fluorescent control Ab) were washed twice and thereafter cultured for 48 h in complete medium at a density of  $1 \times 10^6$  per ml, and their supernatant was collected and assessed for their capacity to trigger Treg migration in vitro. Freshly transfected and twice washed DCs were otherwise adoptively transferred by i.v. injection ( $2.5 \times 10^5$  or  $7 \times 10^4$  cells/mouse) into naive 6-wk-old female NOD recipients.

### Migration of Tregs in response to CCL22

CD4<sup>+</sup>CD25<sup>high</sup> (>99% Foxp3<sup>+</sup>) cells were electronically sorted from the spleen of NOD mice. A total of  $10^5$  cells was suspended in 100  $\mu$ l complete medium and placed in the upper compartment of a 24-well sterile Transwell device (Greiner Bio One, Monroe, NC) separated from the bottom well by an 8- $\mu$ m filter. Migration was allowed to proceed for 1 h, and the migrated Tregs were counted by flow cytometry analysis within the bottom compartment and calculated as the number of Tregs having migrated either in response to the recombinant mouse chemokine CCL22 (ImmunoTools, Friesoythe, Germany) or to DC supernatants, minus the number of Tregs having migrated to medium only.

### Statistical analysis

Data were analyzed using the nonparametric Mann-Whitney *U* test when two groups were compared. Kaplan-Meier estimates and log rank analysis were performed for diabetes incidence curves, with Prism software (GraphPad). The *p* values <0.05 were considered statistically significant.

## Results

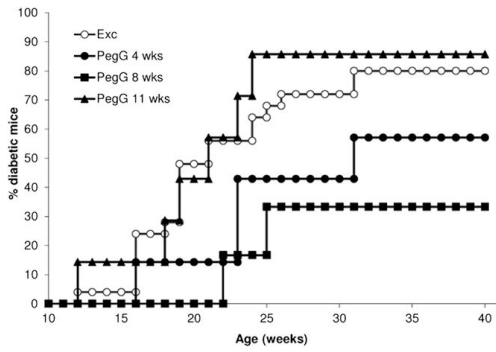
### PegG-CSF delays diabetes onset in NOD mice if administrated before the development of insulinitis

We have previously reported that nonpegylated G-CSF prevents spontaneous (4) and cyclophosphamide-accelerated (4) T1D onset if administrated during consecutive 5 d to mice aged 4, 8, 12, and 16 wk, at a dose of 200  $\mu$ g/kg/day. We found that a single s.c. injection of pegG-CSF at an equivalent cumulative dose of 1.0 mg/kg to 4-wk-old mice delayed the onset of T1D of ~12 wk compared with excipient-treated mice. Likewise, when this treatment was administered at the age of 8 wk, we also observed a significant reduction in T1D incidence (Fig. 1). However, treatment of 11-wk-old mice with pegG-CSF had no effect. These results suggest that, like G-CSF (7), the pegylated factor provided long-term protection only if administrated at the onset of invasive insulinitis.

### PegG-CSF modifies the distribution of DC subsets in NOD mice

DCs were extracted from the spleen of 8-wk-old NOD mice 6 d after injection of pegG-CSF and analyzed by flow cytometry. We found that CD11c<sup>low</sup>B220<sup>+</sup>PDCA-1<sup>+</sup> pDCs and CD11c<sup>high</sup> CD8 $\alpha$ <sup>+</sup> DCs accumulated in pegG-CSF recipients, but not in control mice, whereas the proportion of CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs remained unchanged (Fig. 2A). Total DC counts per spleen were further increased at day 6 in pegG-CSF-treated mice, as a result of total splenocyte count increment of  $1.59 \pm 0.10$ . Similar changes in the proportions of the DC subsets were observed in mesenteric lymph nodes and PLNs in excipient- versus pegG-CSF-treated mice (Fig. 2B). In addition, PegG-CSF reduced the expression of

2268



**FIGURE 1.** Therapeutic window for pegG-CSF treatment in NOD mice. PegG-CSF was injected s.c. once at a cumulative dose of 1 mg/kg/mouse at 4, 8, or 11 wk of age, and diabetes incidence was measured thereafter. A significantly reduced T1D incidence is observed only in mice treated at 8 wk of age ( $p = 0.0024$  versus excipient-treated mice). Excipient (Exc),  $n = 27$  mice; pegG-CSF at 4 wk,  $n = 7$  mice; pegG-CSF at 8 wk,  $n = 12$  mice; pegG-CSF at 11 wk,  $n = 7$  mice per group. Shown is one representative experiment of two.

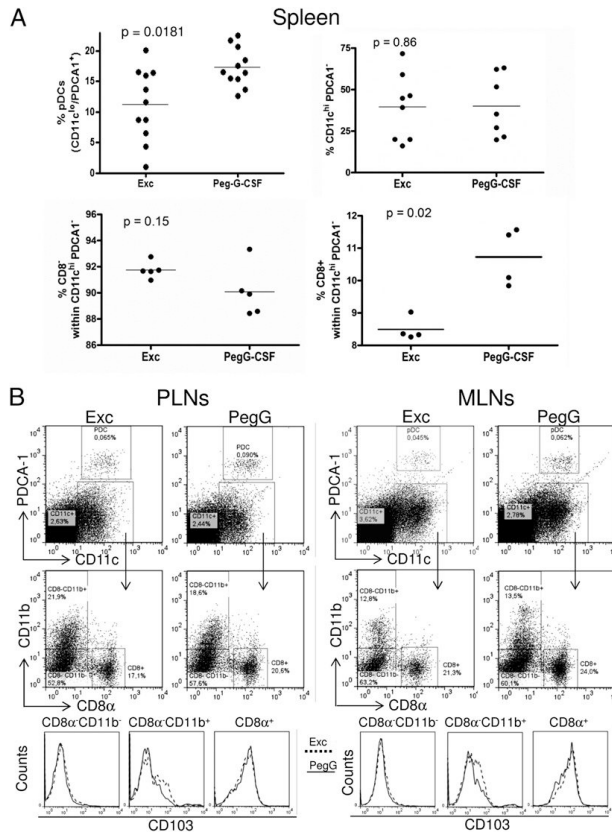
CD103 by the CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup> DC subset, with no effect on CD11c<sup>high</sup>CD8 $\alpha$ <sup>+</sup> DCs (Fig. 2B).

G-CSF-INDUCED CD8 $\alpha$ <sup>-</sup> mDCs RECRUIT Tregs VIA CCL22

CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs from pegG-CSF recipients are able to recruit Tregs after adoptive transfer

We observed that when splenic CD45.2<sup>+</sup> NOD DCs were transferred into CD45.1<sup>+</sup> NOD recipients, they migrated not only to the spleen, but also to the PLNs and pancreas (Supplemental Fig. 1A). To determine whether a particular DC subset was responsible for Treg recruitment, we isolated the three major DC subsets from the spleen of NOD mice, 6 d after injection of pegG-CSF or excipient, and performed adoptive cell transfer experiments. We found that Treg recruitment was enhanced in the spleen and the PLNs 1 wk after adoptive transfer of whole splenic DCs (4) or CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> splenic DCs isolated from pegG-CSF-treated mice (Fig. 3B). In addition, 2 wk after DC transfer, a significant higher recruitment of Tregs was observed in PLNs (Fig. 3A, left) and pancreas (Fig. 3C), but not the spleen of pegG-CSF CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DC recipients (Fig. 3A, right), compared with recipients of the same DC subset derived from excipient-treated mice. Furthermore, Tregs showed an increased expression of TGF- $\beta$ , whereas IL-10 synthesis was not affected (Fig. 3D). Paradoxically, no significant accumulation of Tregs was observed in recipients of pDCs or CD8 $\alpha$ <sup>+</sup> mDCs, generally believed to exhibit tolerogenic potential (10–12). Spleen of mice transferred with pDCs isolated from pegG-CSF-treated mice displayed highly variable, but not significantly enhanced Treg counts.

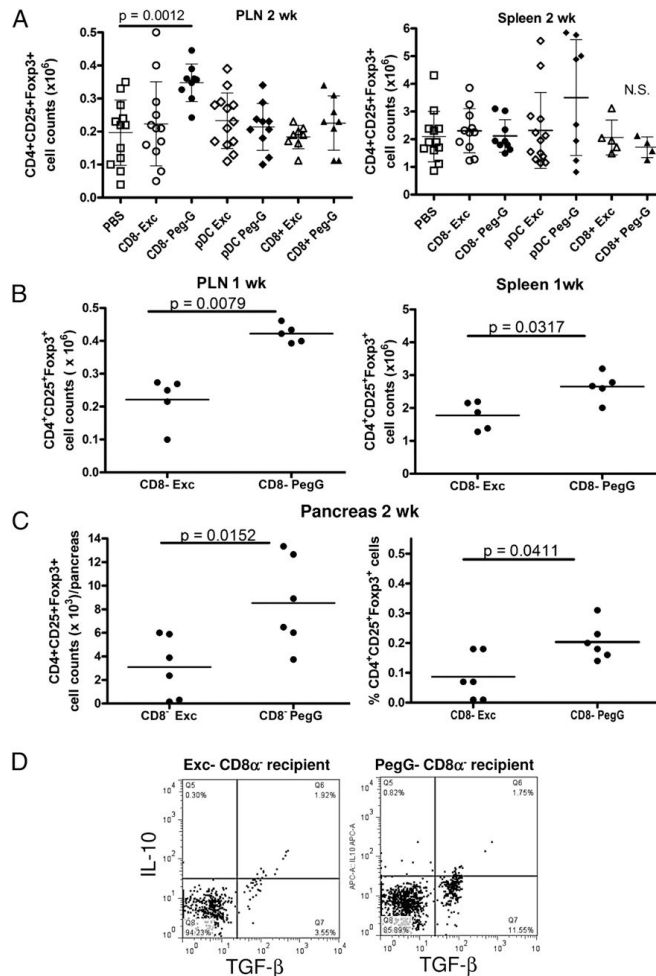
**FIGURE 2.** Relative proportions of splenic, pancreatic, and mesenteric lymph node DC subsets after pegG-CSF treatment. (A) PegG-CSF (1 mg/kg/mouse) or excipient (Exc) were injected s.c., and 6 d later DCs were isolated from the spleen. Flow cytometry analysis was performed to evaluate the relative proportions within DCs of CD11c<sup>low</sup>PDCA-1<sup>+</sup> pDCs and CD11c<sup>high</sup>PDCA-1<sup>-</sup> mDCs, including CD11c<sup>high</sup>PDCA-1<sup>-</sup>CD8 $\alpha$ <sup>+</sup> and CD11c<sup>high</sup>PDCA-1<sup>-</sup>CD8 $\alpha$ <sup>-</sup> subsets.  $n = 4$ –11 mice analyzed per group. pDCs ( $p = 0.0181$ ) and CD11c<sup>high</sup>PDCA-1<sup>-</sup>CD8 $\alpha$ <sup>+</sup> ( $p = 0.02$ ) were significantly different. (B) Flow cytometry analysis of DC subsets in PLNs (left) and mesenteric lymph nodes (right) depicting the relative proportions of CD11c<sup>+</sup>PDCA-1<sup>+</sup> or CD11c<sup>+</sup>PDCA-1<sup>-</sup> DCs, as well as their relative expression of CD11b, CD8 $\alpha$ , and CD103 in Exc- and pegG-CSF-injected mice. Flow cytometry patterns of one representative experiment of three are shown (B).



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**FIGURE 3.** The CD8 $\alpha$ <sup>-</sup> mDC subset recruits TGF- $\beta$ -producing Tregs to the PLNs and pancreata. The 8-wk-old NOD mice were injected either with excipient or with pegG-CSF, and 6 d later, splenic DCs were sorted, as detailed in *Materials and Methods*. Each subset was thereafter adoptively transferred into naive 6-wk-old NOD recipients by i.v. injection of  $2.5 \times 10^5$  pDCs or CD8 $\alpha$ <sup>-</sup> mDCs and  $1 \times 10^5$  CD8 $\alpha$ <sup>+</sup> mDCs per mouse. **(A)** Two weeks after adoptive transfer of the different DC subsets, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells were quantified by flow cytometry within PLNs (*left*) and spleen (*right*);  $n = 4$ –12 mice per group.  $p = 0.0012$ , in PLN of mice injected with CD8 $\alpha$ <sup>-</sup> mDCs from pegG-CSF (Peg-G)–treated donors compared with PBS-injected controls, and  $p = 0.0142$ , when compared with mice injected with CD8 $\alpha$ <sup>-</sup> mDCs from excipient-treated (Exc) donors; no statistically significant differences were found among Treg counts measured in the spleen of noninjected or any recipient groups; **(B)** Treg counts were quantified in PLNs and spleen of DC recipients 1 wk after transfer of CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs from excipient- or pegG-CSF-treated donors;  $n = 5$  mice per group;  $p = 0.0079$  in PLNs and  $p = 0.0317$  in spleen when comparing Treg counts in recipients of CD8 $\alpha$ <sup>-</sup> mDCs from either excipient- or pegG-CSF-treated donors; **(C)** Tregs were quantified in cell counts (*left*) and percentages (*right*) within mononuclear cells recovered from pancreata of the recipients ( $n = 6$  mice per group) of CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs isolated from either excipient- or pegG-CSF-treated donors.  $p = 0.0152$  when comparing Treg counts, and  $p = 0.0411$  when comparing Treg percentages. Results shown in (A–C) are from one representative experiment of two. **(D)** The intracytoplasmic production of TGF- $\beta$  and IL-10 was measured by flow cytometry, after PMA + ionomycin activation, in gated CD4<sup>+</sup>CD25<sup>+</sup> pancreatic cells, isolated from recipients of CD8 $\alpha$ <sup>-</sup> mDCs from excipient (*left*)– or pegG-CSF (*right*)–treated mice. One representative experiment of three is shown.



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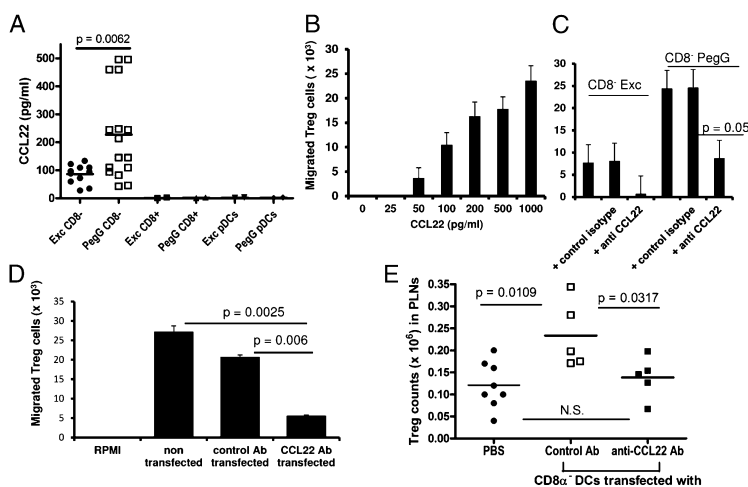
*CCL22 mediates the tolerogenic effect of CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs*

To identify the molecular mechanisms accounting for the tolerogenic effect of CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs, we examined their phenotypic and functional properties. We first assessed the expression of OX40L, which has been reported for mediating in vitro expansion of Tregs by the DCs derived from GM-CSF-treated C57BL/6 mice (13). We observed that OX40L expression gradually decreased from the surface of DCs, and disappeared at 11 wk of age from all DC subsets. Although OX40L expression by CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs isolated from 9-wk-old, pegG-CSF NOD recipients tended to be higher compared with cells from their excipient-treated counterpart, no significant differences were observed in the expression of this molecule in the three DC subsets (Supplemental Fig. 2). By contrast, only CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs produced the chemokine CCL22 (Fig. 4A), which has been shown to attract Tregs expressing CCR4 (14, 15). As depicted in the dose-response curve evaluating Treg migration upon exposure to CCL22 (Fig. 4B), culture supernatants of CD8 $\alpha$ <sup>-</sup>CD11c<sup>high</sup> DCs

from pegG-CSF-treated animals contained enough CCL22 to induce an effective Treg migration in vitro (Fig. 4C). By contrast, CD8 $\alpha$ <sup>-</sup>CD11c<sup>high</sup> DCs from control NOD mice released CCL22 at levels that scarcely induced Treg chemotaxis (Fig. 4C).

To further assess the role of CCL22 in the tolerogenic effect of CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs recovered from pegG-CSF-treated mice, we performed chemokine neutralization experiments by two different methods. As shown in Fig. 4C, a specific anti-CCL22 neutralizing mAb (5  $\mu$ g/ml), but not its isotype-matched control, reduced by two-thirds the in vitro chemotactic effect induced by culture supernatants from CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs collected from pegG-CSF-treated mice on Tregs. Additional experiments testing the effect of supernatants from pegG-CSF-derived CD8 $\alpha$ <sup>-</sup> mDCs transfected with the same anti-CCL22 mAb coupled to the Chariot delivery reagent showed that intracellular neutralization of this chemokine efficiently blocked the chemotactic activity of these cells (Fig. 4D). A similar effect was observed in vivo, when CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs from pegG-CSF recipient mice, having received anti-CCL22 mAb intracellularly, were transferred to





**FIGURE 4.** Enhanced secretion of CCL22 enables CD8 $\alpha$ <sup>-</sup> mDCs from pegG-CSF-treated recipients to attract and recruit Tregs in vitro and in vivo: loss of activity after intracellular neutralization of the chemokine. **(A)** Only CD8 $\alpha$ <sup>-</sup> mDCs upregulate their CCL22 secretion upon treatment with pegG-CSF. CD8 $\alpha$ <sup>+</sup> and CD8 $\alpha$ <sup>-</sup> mDCs as well as pDCs isolated from spleen of excipient- and pegG-CSF-treated individual NOD mice were incubated ( $1 \times 10^6$  cells/ml) for 48 h in RPMI 1640/10% FCS when their supernatant was collected and measured for CCL22 content by ELISA.  $p = 0.0062$  comparing CCL22 levels in supernatants of CD8 $\alpha$ <sup>-</sup> mDCs isolated from excipient (Exc)- versus pegG-CSF (PegG)-treated mice. **(B)** Dose response of Treg migration to CCL22. Sorted Tregs ( $1 \times 10^5$  cells) were placed in the upper well of a Transwell device, over increasing concentrations of recombinant mouse CCL22 in the bottom well. Migration was allowed to proceed for 1 h, and then cells were recovered from the bottom well and counted by flow cytometry. Migrated CD4<sup>+</sup>CD25<sup>+</sup> cell counts were determined as cell counts at a given CCL22 concentration minus cell counts in the bottom well containing RPMI 1640 alone. **(C)** Supernatants of CD8 $\alpha$ <sup>-</sup> mDCs isolated from excipient or pegG-CSF recipients were cocultured for 15 min with a neutralizing anti-CCL22 Ab ( $5 \mu\text{g/ml}$ ) or with isotype control and thereafter assessed for their capacity to trigger Treg migration in vitro.  $p = 0.05$  for Treg migrated toward supernatants of CD8 $\alpha$ <sup>-</sup> mDCs isolated from pegG-CSF recipients preincubated with either a neutralizing anti-CCL22 versus a control Ab. Results are expressed as means  $\pm$  SEM of three experiments for (A–C). **(D)** Supernatants of CD8 $\alpha$ <sup>-</sup> mDCs isolated from pegG-CSF recipients, either nontransfected or transfected with the Chariot vector associated with a control Ab or a neutralizing anti-CCL22 Ab, were tested for their capacity to trigger Treg migration in vitro, assessed as in (B). Results are expressed as means  $\pm$  SEM of three experiments.  $p = 0.0025$  when comparing nontransfected DCs with anti-CCL22 Ab-transfected DCs, and  $p = 0.006$  when comparing Treg migration induced by DCs transfected with a control Ab with that induced by DCs transfected with an anti-CCL22 Ab. **(E)** Intracytoplasmic neutralization of CCL22, but not transfection with the isotype control, selectively abrogates the capacity of adoptively transferred CD8 $\alpha$ <sup>-</sup> mDCs from pegG-CSF recipients to recruit Tregs to the PLNs. Two weeks after adoptive transfer of transfected DCs, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cell counts were measured by flow cytometry in the PLNs of 8-wk-old NOD recipients ( $n = 5$  mice per group) of either isotype- or anti-CCL22 Ab-transfected CD8 $\alpha$ <sup>-</sup> DCs as well as in control, PBS-injected mice ( $n = 8$  mice);  $p = 0.0109$  when comparing Treg counts in PBS-injected controls and in mice injected with CD8 $\alpha$ <sup>-</sup> mDCs from pegG-CSF recipients transfected with a control Ab;  $p = 0.0317$  when comparing Treg counts in recipients of CD8 $\alpha$ <sup>-</sup> mDCs transfected with either a control Ab or the neutralizing anti-CCL22 Ab. Treg counts from noninjected mice and mice injected with CD8 $\alpha$ <sup>-</sup> mDCs from pegG-CSF recipients transfected with the neutralizing anti-CCL22 Ab are not significantly different (N.S.).

naive NOD mice. As shown in Fig. 4E, these cells had lost their capacity to attract Tregs to PLNs, whereas the same DCs having received a control Ab with the same method still displayed this property. We verified that DCs transfected with the Chariot reagent coupled to a PE-labeled Ab were still able to migrate efficiently to the PLNs after their adoptive transfer (Supplemental Fig. 1B).

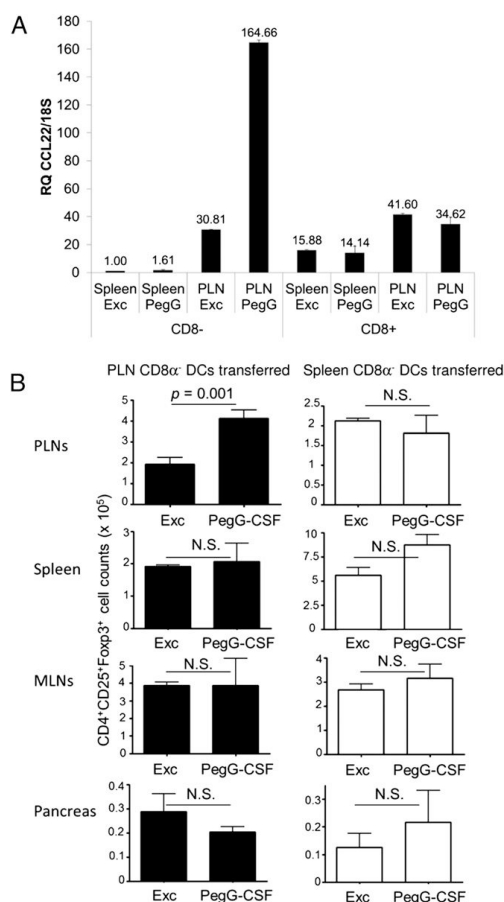
*PLN CD8 $\alpha$ <sup>-</sup> DCs from the pegG-CSF-treated mice show an enhanced synthesis of CCL22, and an increased ability to recruit Tregs*

Finally, we investigated the possibility that DCs found within the PLNs could be even more efficient than splenic DCs at producing CCL22 and at recruiting Tregs. We observed that splenic CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs from pegG-CSF animals showed a 1.8-fold increased ability to synthesize CCL22 mRNA compared with excipient-treated mice (Fig. 5A). In contrast, pegG-CSF treatment enhanced >5-fold the mRNA levels of CCL22 in CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs derived from PLNs. Notably, CCL22 mRNA levels expressed by CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs were 30- and 100-fold higher in PLNs than in spleen of excipient- and pegG-CSF-treated mice, respectively (Fig. 5A). In contrast, no significant differences in the

synthesis of CCL22 mRNA were observed in the case of CD11c<sup>high</sup>CD8 $\alpha$ <sup>+</sup> DCs. Finally, CD11c<sup>high</sup>CD8 $\alpha$ <sup>-</sup> DCs isolated either from PLNs or spleen were further compared for their ability to recruit Tregs. As shown in Fig. 5B,  $7 \times 10^4$  PLN-derived CD8 $\alpha$ <sup>-</sup> DCs significantly recruited Tregs and selectively to the PLNs. In contrast, spleen-derived CD8 $\alpha$ <sup>-</sup> DCs, which efficiently attracted Tregs in the PLNs when injected at  $2.5 \times 10^5$  cells/mouse (Fig. 3A), lost this capacity when injected at such low numbers. Therefore, higher CCL22 production levels by PLN CD8 $\alpha$ <sup>-</sup> DCs correlate with their higher Treg recruiting capacity, relative to their splenic counterparts.

## Discussion

Tregs play a key role in the long-lasting protection against T1D conferred by G-CSF, as established by their secondary transfer from G-CSF recipients to naive NOD-SCID mice, which blocked disease onset induced by diabetogenic splenocytes (4). This is an indirect effect of G-CSF on Tregs, which was mediated by DCs that promoted Treg accumulation in naive NOD recipients after adoptive transfer from G-CSF recipients, as compared with splenic DCs from excipient-treated control mice.



**FIGURE 5.** Comparison of spleen and PLN-derived CD8 $\alpha^{-}$  and CD8 $\alpha^{+}$  DC subsets for their CCL22 mRNA levels and their capacity to recruit Tregs in vivo upon adoptive transfer. **(A)** Quantitative RT-PCR analysis was performed to measure relative quantification ratio of CCL22 mRNA levels relative to 18S in cell-sorted CD8 $\alpha^{+}$  and CD8 $\alpha^{-}$  CD11c<sup>high</sup> DC subsets from spleen and PLNs from NOD mice treated with excipient (Exc) or pegG-CSF (PegG). Results shown are expressed as the mean  $\pm$  SEM of three experiments performed in duplicates. **(B)** Cell counts of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs recruited into PLNs, spleen, mesenteric lymph nodes, and pancreas were obtained by flow cytometry analysis 2 wk after adoptive transfer of only 70,000 CD8 $\alpha^{-}$  DCs isolated from PLNs or spleen of excipient- or pegG-CSF-treated NOD mice.  $n = 3$  mice per group.  $p = 0.001$  when comparing Treg counts in PLNs of recipients of CD8 $\alpha^{-}$  DCs isolated from the PLNs of excipient- or pegG-CSF-treated donors. N.S., Nonsignificant differences in all other cases.

In this study, we have used the stabilized pegylated form of G-CSF, pegG-CSF, and shown that a single injection at a cumulative dose of 1.0 mg/kg provides long-lasting protection against diabetes onset if administrated not later than at 9 wk of age, that is, at the onset of invasive insulinitis.

We have now investigated by a series of adoptive transfer experiments which particular DC subset from G-CSF-treated mice caused Treg recruitment. Surprisingly, pDCs and CD11c<sup>high</sup> CD8 $\alpha^{+}$  DCs, which increased proportionally after pegG-CSF

treatment and can allegedly become tolerogenic in certain circumstances (10–12), failed to trigger Treg accumulation in naive recipients. Despite their unchanged proportions in the spleen after pegG-CSF treatment, only CD11c<sup>high</sup>CD8 $\alpha^{-}$  DCs became able to attract Tregs to the pancreas and its draining lymph nodes upon pegG-CSF treatment in adoptively transferred, naive NOD recipients. The ability of adoptively transferred splenic DCs from pegG-CSF recipients to trigger Treg recruitment at different compartments corresponded with their capacity to migrate to these tissues, as shown when DCs from CD45.2<sup>+</sup> congenic NOD mice treated with pegG-CSF were transferred into CD45.1<sup>+</sup> NOD recipients (Supplemental Fig. 1).

Conversely to previous results implicating interactions between OX40 and OX40L in the capacity of GM-CSF-elicited CD11c<sup>high</sup> CD8 $\alpha^{-}$  DCs to promote the in vitro expansion of Tregs (13), the in vivo tolerogenic effect of pegG-CSF-derived CD11c<sup>high</sup>CD8 $\alpha^{-}$  DCs was not explained by this mechanism of action. Indeed, all DC subsets expressed the OX40L molecule, albeit at different levels in aged NOD mice, which is inconsistent with the selective effect of CD11c<sup>high</sup>CD8 $\alpha^{-}$  DCs on Treg accumulation in vivo.

Instead, and although CD11c<sup>high</sup>CD8 $\alpha^{+}$  DCs expressed higher levels of CCL22 mRNA than CD11c<sup>high</sup>CD8 $\alpha^{-}$  DCs, only CD11c<sup>high</sup>CD8 $\alpha^{-}$  DCs were actually producing the chemokine CCL22 at the protein level, as detectable by ELISA. CCL22 can attract Tregs via the highly expressed corresponding CCR4. PegG-CSF treatment enhanced this production to levels that could effectively attract CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, both in vitro and in vivo. This was demonstrated by neutralization of CCL22 activity, which caused the loss of migration of Tregs toward CD11c<sup>high</sup>CD8 $\alpha^{-}$  DCs or their supernatant in vitro. To optimize CCL22 neutralization, we coupled the Ab to the Chariot reagent that improved its intracytoplasmic delivery. In this regard, it is worth mentioning that the treatment of DCs with the Chariot delivery reagent alone did not significantly affect cell viability nor cell migration in vivo (Supplemental Fig. 1B), whereas it achieved a >95% efficiency in introducing the anti-CCL22 mAb into these cells, as assessed by flow cytometry analyses. Interestingly, the blocking effect lasted at least 48 h, as assessed by in vitro migration studies, which is consistent with the in vivo half lifespan of mDCs (16).

CCL22 production levels were higher and their enhancement by pegG-CSF treatment more pronounced in CD8 $\alpha^{-}$  DCs within the PLNs than within the spleen. Notably, pegG-CSF did not induce significant changes in CCL22 mRNA levels on CD8 $\alpha^{+}$  DCs. Accordingly, as few as  $7 \times 10^4$  CD11c<sup>high</sup>CD8 $\alpha^{-}$  DCs from the PLNs were still effective upon adoptive transfer in attracting Tregs to the PLNs, but no more to the pancreas, whereas their splenic counterparts were unable to do so at such numbers. Therefore, DCs presenting higher levels of CCL22 production displayed higher capacity to recruit Tregs.

The observation that synthesis of TGF- $\beta$  was enhanced in pancreatic Tregs recruited by CCL22-producing splenic CD8 $\alpha^{-}$  mDCs, although IL-10 was not detected (Fig. 3D), is in accordance with the report that human CD8 $\alpha^{-}$  mDCs are able to synthesize CCL22 upon activation with thymic stromal lymphopietin, promoting the accumulation of TGF- $\beta^{+}$  rather than IL-10<sup>+</sup> Tregs (17).

CCL22 attracts CCR4-expressing T cells that include activated memory T cells and Tregs, the latter expressing high levels of the chemokine receptor CCR4 (15). It is worth mentioning that we detected variable but similar levels of CCR4 expression, whether Tregs were recovered from mice having received CD11c<sup>high</sup> CD8 $\alpha^{-}$  DCs from excipient- or pegG-CSF-treated mice (Supplemental Fig. 3), indicating that enhanced recruitment in the latter is not due to an increased expression of CCR4.

The role of CCL22 in the pathogenesis of T1D has been previously explored, but led to apparently controversial results. Indeed, enhanced CCL22 transcripts have been detected by microarray analysis in spleen, but not in PLN cells from NOD mice having received G-CSF, as compared with excipient controls (7). CCL22 transcripts have also been evidenced both in pancreata of patients suffering from T1D and of NOD mice (18), with increased levels of transcription induced by inflammatory signals. In addition, earlier reports demonstrated that transgenic pancreatic expression of CCL22 accelerated insulinitis and T1D transfer in NOD/SCID recipients of diabetogenic spleen cells (19). In the same model, *in vivo* neutralization of CCL22 retarded T1D transfer. In contrast, more recent observations suggest that the ectopic production of this chemokine by  $\beta$  cells promotes the recruitment of Tregs, preventing spontaneous T1D in NOD mice (20). Likewise, it has been reported that, in a mouse model of cardiac allograft, donor-specific tolerance is dependent on the synthesis of CCL22 and the expression of its receptor CCR4 by Foxp3<sup>+</sup> Tregs (21).

Although CCL22 can be produced by cells other than DCs, like B and NK lymphocytes, our adoptive transfer experiments indicate that DCs, mainly the PLN CD8 $\alpha$ <sup>-</sup> subset, is the major effector of G-CSF-induced recruitment of Tregs (4). However, it is very likely that other cells may contribute to the effect of pegG-CSF on Tregs. In this regard, we have previously reported that multipotent hematopoietic progenitors mobilized by G-CSF also promote the expansion of Tregs (22, 23). Additionally, we observed that DCs from pegG-CSF-treated mice produced more inflammatory cytokines and chemokines in response to LPS or R848 stimulation than DCs from excipient-treated mice, suggesting that their tolerogenic potential may eventually get lost in infectious or inflammatory conditions (Supplemental Fig. 4). Despite this general increase in cytokine and chemokine production by DCs from G-CSF-treated mice, the fact that CCL22 neutralization nearly abrogated both *in vitro* migration and *in vivo* recruitment of Tregs suggested that other cytokines and/or chemokines are unlikely to play such a key role as CCL22 in the Treg accumulation.

In conclusion, our data demonstrate that CD8 $\alpha$ <sup>-</sup> mDCs participate in the protection of G-CSF-treated NOD mice against T1D, by their capacity to increase their production of CCL22 to levels that induce chemotaxis of Tregs.

Whether pegG-CSF will be able to upregulate CCL22 expression in DCs from patients with T1D, thus promoting the recruitment of Tregs (8, 24), warrants further investigation.

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### Disclosures

The authors have no financial conflicts of interest.

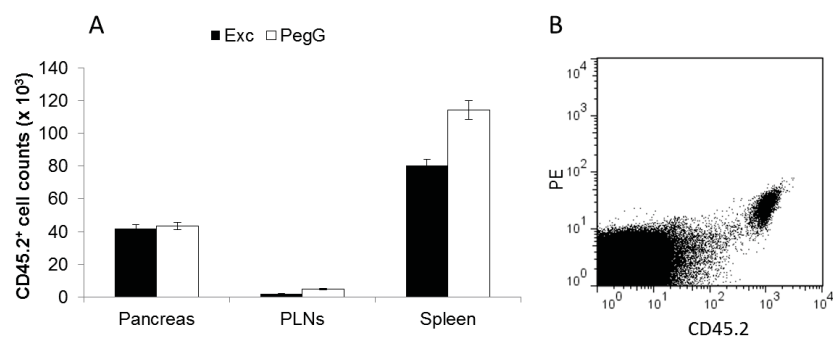
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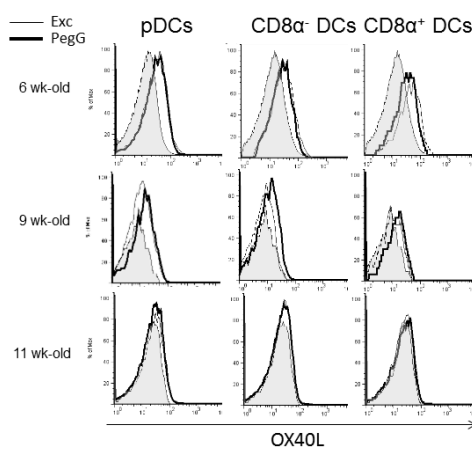
### G-CSF-INDUCED CD8 $\alpha$ <sup>-</sup> mDCs RECRUIT Tregs VIA CCL22

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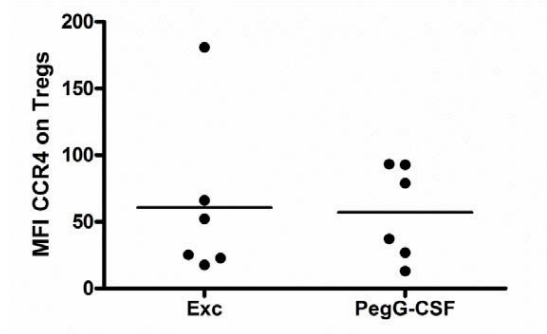
**Figure S1: Migration of adoptively transferred DCs, transfected or not with a fluorescent antibody coupled to the Chariot® reagent.** A: DCs isolated from the spleen of excipient- or pegG-CSF-treated CD45.2<sup>+</sup> NOD mice were transferred intravenously ( $2.5 \times 10^5$  cells/mouse) to CD45.1<sup>+</sup> NOD recipients and their presence as CD11c<sup>+</sup> CD45.2<sup>+</sup> cells analyzed 7 days later in the spleen, PLNs and pancreas of recipients. Values of CD45.2<sup>+</sup> cell counts are the mean  $\pm$  SEM of 3 experiments. B: DCs isolated from the spleen of CD45.2<sup>+</sup> NOD mice were transfected with the Chariot reagent coupled to a PE-labeled antibody and adoptively transferred by intravenous injection ( $5 \times 10^5$  cells/mouse) into CD45.1<sup>+</sup> recipients. The presence of PE-positive CD45.2<sup>+</sup> CD11c<sup>+</sup> cells was detected by flow cytometry in the PLNs of recipient mice 3 days later. One representative experiment out of 2 is shown.



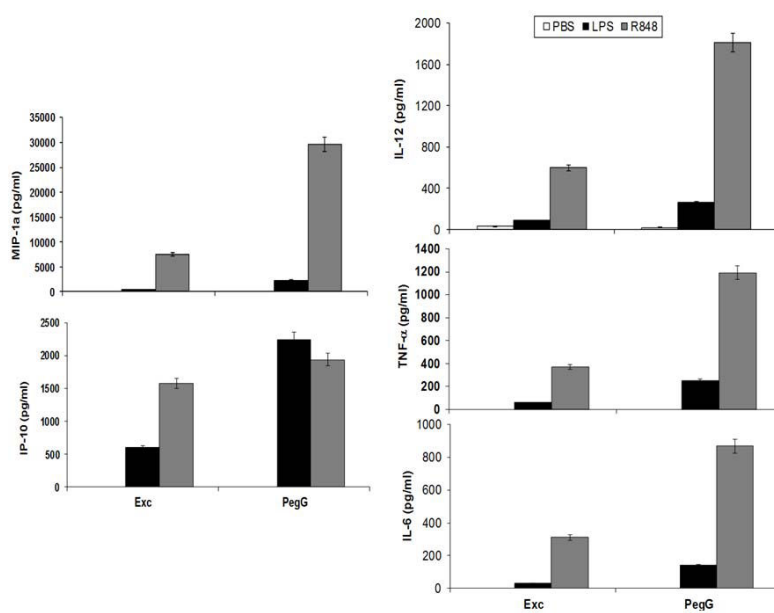
**Figure S2: Decline of OX40L expression over age on splenic DC subsets from excipient- and PegG-CSF-treated NOD mice.** DCs were isolated from the spleen of excipient- (Exc-) or pegG-CSF-treated NOD mice, at day 6 after treatment from 6, 9 and 11 week-old NOD mice and their expression of OX40L measured by flow cytometry on CD11c<sup>low</sup>PDCA-1<sup>+</sup> pDCs, CD11c<sup>hi</sup>PDCA-1<sup>-</sup>CD8 $\alpha$ <sup>-</sup> mDCs (CD8 $\alpha$ <sup>-</sup> DCs) and CD11c<sup>hi</sup>PDCA-1<sup>-</sup>CD8 $\alpha$ <sup>+</sup> mDCs (CD8 $\alpha$ <sup>+</sup> DCs). OX40L expression was detected at similar levels on all DC subsets from Exc- and PegG-CSF-treated mice at 6 weeks of age and declined afterwards. At 9 weeks of age, OX40L expression was less reduced on CD8 $\alpha$ <sup>-</sup> DCs from pegG-CSF-treated mice than on CD8 $\alpha$ <sup>-</sup> DCs from Exc-treated mice. At 11 weeks of age, OX40L was no more expressed on the various DC subsets. Shown is one representative experiment out of 3.



**Figure S3: CCR4 expression on pancreatic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs is not affected by pegG-CSF treatment.** Pancreatic mononuclear cells were recovered 7 days after injection of  $2.5 \times 10^5$  CD8 $\alpha$ <sup>-</sup> DCs isolated from the spleen of Exc- or PegG-CSF-treated NOD mice and the level of expression of CCR4 was measured on CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells. N = 6 mice per group. N.S.



**Figure S4: DCs from PegG-CSF recipients produce enhanced chemokine and cytokine levels upon activation.** DCs isolated from the spleen of excipient (Exc)- or PegG-CSF (PegG)- treated mice were incubated ( $2 \times 10^5$  cells/ml) in the presence of PBS, LPS or the TLR-7 agonist R848 at 1  $\mu$ g/ml during 48h. Collected supernatants were measured for chemokine (MIP-1 $\alpha$ , IP-10) and cytokine (IL-6, TNF- $\alpha$  and IL-12 p40/p70) content using multiplex ELISA (Life Technologies). Values are expressed as mean  $\pm$  SEM of triplicate measurements.



## Discussion and Perspectives

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It is interesting that until today, the largest part of studies focusing on the immune regulation has mainly provided evidence for mature cells with regulatory properties. Besides MDSC progenitors, known for almost 20 years, other populations of progenitor cells have been described in the literature<sup>635</sup>. In our laboratory, it has been reported previously that mobilization of bone marrow cells with hematopoietic growth factors conferred tolerogenic properties to hematopoietic progenitors at the multipotent progenitor (MPP2) stage of differentiation that enabled them to promote the expansion of regulatory cells, preventing spontaneous autoimmune T1D in NOD mice<sup>109</sup>.

Direct interactions between pathogens and hematopoietic stem/progenitor cells can occur through Toll-like receptor (TLR) activation, driving their differentiation along myeloid pathways to enforce anti-infectious defenses<sup>97,98</sup>. TLR agonists are known to promote hematopoiesis by enhancing the production of the hematopoietic growth factor G-CSF, with whom they synergize to mobilize hematopoietic stem cells from the bone marrow to the periphery<sup>100</sup>. TLR-mediated innate-type stimulation by infectious<sup>370</sup> and parasitic<sup>636</sup> agents has been shown to be involved in the emergence of regulatory B cells (Bregs), along with acquired-type stimulation, such as B-cell receptor (BCR) engagement concomitant or not with CD40 activation<sup>359,361</sup>. Many subsets of B cells have been described today to be able to display regulatory properties, among them Marginal Zone B cells, Follicular B cells, peritoneal CD5<sup>+</sup> B-1a cells and the most immature T2-MZP B cells. It is still unknown whether Bregs derive from one or several progenitors or solely from conventional B-cell subsets. Moreover, an immature B-cell progenitor population endowed with suppressive properties per se or after differentiation into more mature B regulatory cells had not been described as yet.

### 1) CpG-proBs in T1D and EAE

In the first part of my PhD, we showed that the stimulation of bone marrow cells of NOD mice with TLRs through the MyD88 signaling can trigger the emergence of a population of hematopoietic progenitor cells at the stage of pro-B cells, with tolerogenic properties.

During our experiments, we mainly used a TLR-9 agonist ODN-CpG as a stimulator for these progenitor cells and this why we named them as “CpG-proBs”.



Performing a cell culture of bone marrow cells from NOD mice in the presence of the CpG-B, a population of progenitor cells at the stage of pro-B cells emerged. Firstly, we asked about the exact conditions, which favor the emergence of CpG-proB cells. In comparison with the emergence of mature regulatory B cells which require the activation of TLR pathways<sup>388,631</sup>, together with parallel stimulation of CD40/CD40L<sup>361</sup>, whether the CpG-proB cells depend on any other cell populations inside the bone marrow in order to emerge, it remains to be investigated. Both in vitro and in vivo, CpG-B could trigger the emergence of a bone marrow population characterized by the expression  $c\text{-kit}^{\text{low}}\text{Sca-1}^{\text{low}}\text{B220}^+\text{CD43}^+\text{CD19}^+\text{IgM}^-$ , similar enough to the phenotype of control pro-B cells characterized by the expression  $c\text{-kit}^{\text{low}}\text{B220}^+\text{CD43}^{\text{hi}}\text{CD24}^{\text{hi}}\text{CD19}^+\text{IgM}^-$ .

An adoptive transfer of 60,000 CpG-proBs could restore self-tolerance and protects against T1D in NOD mice, either if injected (intravenously) at the age of 6 or 16 weeks, in comparison with control pro-B cells, which did not confer similar protection.

Progenitor populations with similar phenotype of CpG-proBs emerge also upon activation of TLR-2, -4, -5, -6, and -7 but not of TLR-1 and -3 whereas control GpC had no such effect. The suppressive properties of these other TLR-induced progenitor populations remain to be established. The fact that CpG-A, a poor direct activator of B cells, triggers the emergence of a similar progenitor population, may suggest that other non-B cells may be the primary target of CpG in the bone marrow, leading to the emergence of the CpG-proB cells. Concerning the immunosuppressive functions of CpG-proBs, we tried to provide evidence for their mechanism of action both in vitro and in vivo.

In vitro, CpG-proBs seem to require a cross-talk with activated T cells, which produce IFN- $\gamma$  in order to upregulate the FasL expression on CpG-proBs. As a result, CpG-proBs become capable of inducing apoptosis of target T cells, including diabetogenic Teffs. CpG-proBs produce large quantities of IFN- $\gamma$  even without an additional stimulation of PMA-ionomycin but it has been shown that they need Teffs to provide for more IFN- $\gamma$  in order to induce the expression of FasL at their surface. When CpG-proBs were cultured together with Teff isolated from mice deficient for IFN- $\gamma$ , CpG-proBs displayed reduced expression of FasL.

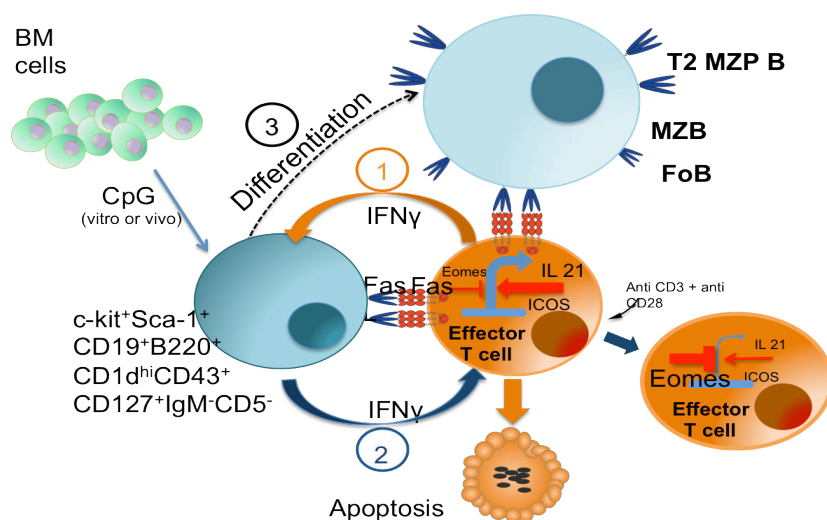
In addition to its role to favor the upregulation of FasL at the surface of CpG-proBs, IFN- $\gamma$  derived from the Teffs enhanced the production of IFN- $\gamma$  from CpG-proBs, inducing a shift of Teff functions and a cytokine profile dominated by a clear-cut enhancement of IFN- $\gamma$  target genes such as IL-12R, granzyme B, and the t-Box transcription factors t-Bet and eomesodermin. Upregulation of eomesodermin<sup>637</sup>, a negative regulator of IL-21 production, together with down regulation of ICOS, a positive regulator of IL-21<sup>638</sup> in target Teffs, led to

dramatically reduced IL-21 production. CpG-proBs from IFN- $\gamma$ <sup>-/-</sup> NOD mice were far less potent in shifting the cytokine profile of Tregs in vitro and similarly unable to provide protection against T1D when transferred to NOD recipients. Concerning the important role of IFN- $\gamma$  in vivo, it remains to be demonstrated in more details since CpG-proBs that emerge from cultures set up with bone marrow cells from IFN- $\gamma$ <sup>-/-</sup> mice might also be modulated by the absence of various IFN- $\gamma$  dependent factors. A definitive proof of the key role of T-cell-derived IFN- $\gamma$  in the differentiation of pro-B cells with suppressive functions would require experiments with CpG-proB cells generated from mice in which only B cells would lack IFN- $\gamma$ R. It would be also interesting to investigate if the CpG-proB cells can still display their protective effects when injected in IFN- $\gamma$ <sup>-/-</sup> recipients in order to identify if the inflammatory environment, triggered by the activated T effector cells through the production of IFN- $\gamma$  is indeed necessary for CpG-proB cells to become activated and suppressive.

The role of IFN- $\gamma$  in T1D developed by NOD mice is not clear since genetic absence of IFN- $\gamma$  delays but does not prevent diabetes in NOD mice<sup>639</sup>. Although IFN- $\gamma$  is cytotoxic for pancreatic  $\beta$  cells, its production in the spleen leads to a paradoxical protective effect against T1D in bacillus Calmette–Guérin-treated mice<sup>640,641</sup>. In addition to the important role of IFN- $\gamma$  in T1D, IL-21 has been shown to be a pathogenic cytokine while it is encoded by *idd3*, a gene locus of predisposition to diabetes in the NOD mouse<sup>642</sup> whereas polymorphisms for IL-21 and its receptor are linked to prevalence of T1D in humans<sup>643</sup>. Several studies have shown that IL-21 is produced at high levels in NOD mice. IL-21R deficiency protects against T1D<sup>253,587</sup> and neutralization of IL-21 with either IL-21R-Fc or anti-IL-21 prevents disease development at early and late prediabetes stages<sup>642</sup>. Most mature B cells that have been shown to suppress autoimmune responses, display these regulatory functions through the production of the anti-inflammatory cytokine IL-10<sup>362</sup>. Interestingly, CpG-proBs were not able to produce IL-10 after their recovery from bone marrow cultures, a capacity generated only in response to PMA + ionomycin. Additionally, even the progeny of CpG-proB cells did not produce IL-10, even after PMA + ionomycin stimulation alone or with LPS or anti-CD40 or CpG. The suppressive function of CpG-proBs can be added to other already known IL-10-independent mechanisms of suppression by B cells. Some of the regulatory pathways that do not require production of IL-10, include cell-to-cell contact interactions involving MHC<sup>644</sup> or B7 molecules<sup>323</sup> and blockade of Treg expansion via FasL-induced apoptosis<sup>405,645,646</sup>.

The inflammatory signals and cytokines that they encounter in their microenvironment engender diverse immunosuppressive mechanisms. For example, LPS-activated mature B cells prevented T1D in NOD mice<sup>601</sup> by FasL-dependent apoptosis of diabetogenic Tregs, similarly to the mechanism of action reported here. In addition, these LPS-activated B cells down-regulated Th1 cytokines, possibly through TGF- $\beta$  production, but Th2 cytokines were not increased. This mechanism could not account for the action of CpG-proBs that was not associated with detectable TGF- $\beta$  production. However, other reports have shown that TLR activation of B cells could instead foster Th1 differentiation, through MyD88-dependent B-cell activation driving IFN- $\gamma$  producing T-cell differentiation and leading to an IgG2c primary antibody response<sup>647</sup>. MyD88-dependent activation can therefore induce similar activities in mature B cells and pro-B-cell progenitors.

Here we provide evidence for the rapid recruitment of the early progeny of CpG-proB-cell progenitors to the pancreas as FasL<sup>hi</sup>c-kit<sup>+</sup>B220<sup>+</sup>IgM<sup>low</sup> cells, leading to a reduction in the numbers of active IL-21-producing Tregs as early as 15–20 days after injection. In addition, the suppressive properties and high FasL expression are maintained throughout the differentiation process into several mature B-cell subsets in the spleen and the PLNs, thereby providing long-term protection against T1D. Indeed, CpG-proBs differentiated into various B-cell subsets, but not into CD1d<sup>hi</sup>CD5<sup>+</sup> B10 cells, suggesting that they are not the common precursors of this regulatory cell lineage in the NOD mice. Instead, follicular B cells, MZ B cells, and T2-MZP-Bs largely prevailed among the suppressive CpG-proB progeny in NOD recipients. Notably, tolerogenic properties of T2-MZP-Bs have been abundantly reported in several experimental models of autoimmune diseases<sup>373,374,620,648,649</sup> (**Figure 21**).



**Figure 21. Mechanism of action of CpG-proB cells in NOD mice** 1) CpG-proB cells need to be together with activated T effector cells in order to display their suppressive functions. The production of IFN- $\gamma$  by T effectors is essential for the upregulation of FasL expression on the surface of CpG-proBs. Due to the interaction of CpG-proBs with Teffs through Fas/FasL, CpG-proB cells can induce the apoptosis of T effector cells. 2) Additionally, the production of IFN- $\gamma$  by T cells is responsible for enhancing the production of IFN- $\gamma$  by CpG-proBs which switch T cells to upregulate the expression of the transcription factor Eomesodermin, a negative regulator of ICOS, resulting in the reduction of IL-21 production. 3) CpG-proB cells can differentiate in vivo into more mature B cells of several phenotypes such as T2-MZP, FoB and MZ B cells which can be recovered in the spleen up to 3 months post-injection. These differentiated B cells express still high levels of FasL, contributing to a more long-term control of T effector cells by apoptosis.

My main project was to investigate if the same population can also emerge in another mouse strain such as the C57BL/6 which can be used for the induction of a chronic type of EAE disease upon immunization with MOG<sub>35-55</sub>/CFA. Interestingly, it has been shown that the CpG-proB cells can emerge under exactly the same time conditions as in NOD mice both in vitro and in vivo.

Similar to NOD mice, in C57BL/6J mice not only CpG-B but also all ligands for TLRs dependent on MyD88 signaling could induce the emergence of these progenitor cells whereas incubation of bone marrow cells of mice deficient for MyD88 with CpG did not trigger the appearance of the CpG-proBs. In accordance with the data of Welner et al who used C57BL/6 mice to establish that incubation with CpG-A for 48h directed common lymphocyte progenitors toward differentiation into PDCA-1<sup>+</sup> plasmacytoid dendritic cells (PDCs) at the expense of the B-cell lineage<sup>99</sup>, the CpG-proB cells, induced in the C57BL/6 mice after 18h of incubation of bone marrow cells with CpG-B were heterogenous in terms of the expression

of PDCA-1. We sorted out the  $c\text{-kit}^+\text{Sca-1}^+\text{B220}^+\text{IgM}^-\text{PDCA-1}^-$  subset in order to compare with the protective CpG-proBs injected in the NOD mice that did not express the marker PDCA-1. These differences suggest that the genetical background of the mouse strain can affect the differentiation of progenitor cells in a more or less orientated phenotype of dendritic cells. CpG-proBs cells in both C57BL/6J and NOD mice did not express the dendritic cell marker CD11c.

In our experimental protocole, the onset of EAE disease after immunization with MOG<sub>35-55</sub> is between the days 10-14 post-immunization whereas the peak of the clinical symptoms normally occurs between days 18 and 21, resulting in a chronic and stable clinical score. The injection of CpG-proBs was performed at day 12 (onset of the disease) for mainly two reasons. Firstly, it is much more interesting to investigate a potential therapeutic tool which could be applied also in MS patients, in whom no therapies can be considered efficient before the diagnosis or the appearance of the first pathological symptoms. Secondly, according to our knowledge from the NOD mice, CpG-proB cells in order to become activated and to perform their suppressive properties, are dependent on the existence of an already inflammatory environment triggered by the presence of auto-reactive T effector cells, through the production of IFN- $\gamma$  for example in the case of T1D.

Only one single injection (intravenously) of 55,000 CpG-proBs cells was enough for conferring protection against EAE whereas mice that have been injected with control pro-B cells, isolated from non-stimulated bone marrow cells as  $c\text{-kit}^+\text{B220}^+\text{Sca-1}^-\text{CD43}^{\text{hi}}\text{CD24}^{\text{hi}}$  developed EAE disease similar to control (only immunized) mice.

Analyzing the functional properties of CpG-proB cells just immediately after their isolation, we found out that they are very similar to CpG-proBs from the NOD mice since they were good producers of IL-12p40 and IFN- $\gamma$  but they produced neither IL-23p19 nor IL-10. Interestingly, in contrast with the NOD mice, CpG-proB cells from B6 mice could not acquire the capacity to produce IL-10 even after stimulation with PMA-ionomycin.

Like in T1D, the production of IFN- $\gamma$  by CpG-proB cells was also important for their protective effect in the context of EAE, since CpG-proB cells isolated from mice deficient for IFN- $\gamma$  lost their ability to protect against EAE. Ex-vivo analysis at the peak of the disease, when the clinical symptoms are well established, revealed that CpG-proBs in EAE used a

distinct mechanism of action than the one found in NOD mice, since the injected and protected EAE mice were characterized by a strong accumulation of CD4<sup>+</sup> T cells in both spleen and lymph nodes and very few CD4<sup>+</sup> T cells infiltrating the CNS tissue (spinal cord). Indeed, the total number of cells as well the percentage of CD4<sup>+</sup> T cells in the lymphoid organs was two or three times more than of those in control mice or mice injected with CpG-proBs from IFN- $\gamma$  KO mice.

One of the important steps for the induction of EAE disease is the migration of activated T effector cells from the periphery to the CNS, where they are responsible for triggering inflammation and demyelination, either by activating other immune or CNS-resident cells with effector functions or via the secretion of pro-inflammatory cytokines such as IL-17, IFN- $\gamma$ , GM-CSF and IL-21. This process has attracted the interest of many scientists and many already existing treatments for MS patients aim at blocking the homing of T effector cells to the CNS. In contrast with MS where the inflammation is observed in the brain, in the B6-MOG model of EAE, the main organ where the inflammation occurs is the spinal cord. The observation that the accumulation of CD4<sup>+</sup> T cells in the periphery is related with a reduced number in the spinal cord led us to the hypothesis that CpG-proB cells may affect this homing process. As mentioned already, many factors, either chemokines or integrins have been shown to be involved in the homing of T effector cells into the CNS. Among them, the expression of CCR7 by T-cells, in addition to its role to direct the migration of T cells in the lymphoid organs, has been shown to affect the homing process inside the CNS: interaction with its ligands CCL19 and CCL21, expressed by epithelial cells of the BBB is necessary for the extravasation of activated T cells inside the CNS<sup>283,650,651</sup>. CCL19 has been shown to be more involved in that process since in both human MS patients and EAE mice, the expression of CCL19 mRNA is constitutively expressed in CNS and is upregulated in cases of neuroinflammation, favoring the entry of CCR7<sup>+</sup> T cells into the CNS where they display their inflammatory properties<sup>284,285</sup>.

In our study, we show that the accumulation of the CD4<sup>+</sup> T cells in the periphery of CpG-proB-injected mice was related with a downregulation in the expression of CCR7 at the surface of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the lymph nodes. The levels of the CCR7 expression were not modulated in mice that had been injected with CpG-proBs from IFN- $\gamma$  KO mice suggesting that IFN- $\gamma$  plays a role in this modification. According to our knowledge, there are no published studies demonstrating a direct relationship between IFN- $\gamma$  and CCR7.

Other factors such as the cytokine TNF or the miR21 have been shown to affect CCR7 expression in a direct way. However, it has been demonstrated recently that stable inhibition of miR21 led to significant induction of CCR7 protein expression whereas a direct interaction was confirmed in a dual luciferase report assay. Additionally, in patients with psoriasis, an early critical event for the clinical remission in response to TNF-inhibitors is the inhibition of CCL19/CCR7 axis supporting its role in psoriasis pathogenesis<sup>652,653</sup>.

We addressed the question whether IFN- $\gamma$  can affect the expression of CCR7 in an indirect way. Interestingly, we found that WT CpG-proBs (IFN $\gamma$ -competent) are also characterized by the expression of high levels of CCL19, one of the chemokine ligands for CCR7, but not of CCL21, the second specific ligand for CCR7. It has been shown that incubation of CCR7-transfected cells with the ligand CCL19 led to the internalisation of the CCR7 receptor whereas CCL21 had a much weaker effect<sup>654,655</sup>. Our hypothesis is that the production of CCL19 by WT CpG-proBs could occupy the CCR7 receptor at T cells leading to the internalisation of the receptor and downregulating its expression at the surface of T cells. Indeed, we found that in mice injected with WT CpG-proB cells, the downregulation of the expression of CCR7 at the surface of T cells was related with an increase in its intracellular expression. IFN- $\gamma$  seems to be necessary for making CpG-proB cells able to produce CCL19 since CpG-proBs with deficiency in IFN- $\gamma$  production were unable to produce CCL19.

The production of IFN- $\gamma$  by CpG-proB cells, as its cell source, is required for the induction of CCL19 production by CpG-proB cells. When we inject CpG-proB cells from IFN- $\gamma$ <sup>-/-</sup> mice, they cannot confer protection to EAE mice and do not restrain the number of CD4<sup>+</sup> (and CD8<sup>+</sup>) T cells in the periphery despite the fact that IFN- $\gamma$  is normally available from other activated cells such as Th1 or NK cells that are known to be present in EAE disease. IFN- $\gamma$  may trigger locally the production of CCL19 by CpG-proB cells, which act directly on the effector T cells. In vivo, the important role of CCL19 produced by CpG-proBs for their protective effect against EAE was confirmed by the use of CpG-proB cells deficient for CCL19 (derived from CCL19<sup>-/-</sup> mice), which were unable to protect mice against EAE and to restrain T cells in the periphery. Additionally, in these mice, there was no modulation in terms of the surface expression of CCR7 on T cells. Many groups have studied the role of CCL19/CCR7 in EAE. Interestingly, the expression of CCR7 seems to be necessary for the development of EAE since CCR7<sup>-/-</sup> mice or plt/plt mice that are deficient for the expression of the CCR7 ligands CCL19 and CCL21, are resistant to EAE<sup>444</sup>. In most cases where the

blocking of T-cell homing had been used for therapeutic reasons, different agonists of the targeted receptors have been tried to occupy these receptors in order to inhibit their interaction with their specific ligands and the migration of activated T cells into the CNS. FTY720 is an already existing oral treatment for MS patients and it has been shown that this active phosphorylated metabolite resembles S1P, a ligand for the sphingosine-1 phosphate 1 (S1P1) receptor, occupying this receptor and resulting in a blocking of T cell migration into the CNS<sup>656</sup>. Interestingly, in the absence of S1P1R, CCR7<sup>+</sup> naïve and central memory T cells are unable to override CCR7 retention signals in the secondary lymphoid organs, resulting in their entrapment in the lymph nodes<sup>657,658</sup>. Because of the fact that other immune cells express also this S1P1 receptor and of the lack of specificity of FTY720 for S1P only, the use of a more specific S1P1-agonist (AUY954) has been shown to target specifically effector T cells inhibiting their migration into the CNS whereas at the same time the ability of pDCs to enter into the CNS was highly related with the beneficial effect of this specific agonist AUY954<sup>292</sup>.

Interestingly, the observation regarding the inhibition of T cells to the site of autoimmune responses is not unique for the case of EAE mice injected with WT CpG-proB cells. In NOD mice, it has been shown that the injection of CpG-proBs was associated with a strong down regulation of the expression of s1p (no evidence for CCR7), limiting the migration of effector T cells to the pancreas where, indeed, the number of effector cells recovered in CpG-proB-recipient mice was less comparing with control mice. It is unknown whether in T1D, this effect was also IFN- $\gamma$ -dependent, so it would be interesting to investigate that in more details in order to provide more evidence about a possible regulatory role of IFN- $\gamma$  related with the down regulation of specific chemokine receptors.

A potential regulatory role of IFN- $\gamma$  in EAE has been reported by observations in both IFN- $\gamma$ <sup>-/-</sup> and IFN- $\gamma$ R<sup>-/-</sup> mice which displayed more severe disease in both chronic and RR-EAE models<sup>659</sup>. It has been shown also that IFN- $\gamma$  can display a regulatory function through its signaling from astrocytes. When EAE was induced in mice deficient for IFN- $\gamma$ R restricted on astrocytes, there was increased demyelination at the peak of the disease and acute disease correlated with sustained clinical symptoms. Interestingly, decreased expression of the cytokines IL-10 and IL-27 was also observed inside the CNS<sup>660</sup>.



Whether the production of CCL19 by the injected progenitor cells targets specifically the effector T cells or other immune cells which also express CCR7, among them regulatory populations, has to be investigated in more details, since the migration of regulatory populations to the site of inflammation is desirable. In our case, Tregs seem to migrate normally in the spinal cord of injected mice, even at higher rates than in control or EAE mice injected with CpG-proB cells from IFN- $\gamma$  KO mice.

The important role of the CCL19/CCR7 signaling has been shown also in a case of T-cell leukaemia. It has been reported that the chemokine receptor CCR7 is the essential adhesion signal required for the targeting of leukaemic T-cells into the CNS. *Ccr7* gene expression is controlled by the activity of the T-ALL oncogene *Notch1* and is expressed in human tumors carrying *Notch1*-activating mutations. Silencing of either CCR7 or its chemokine ligand CCL19 in an animal model of T-ALL specifically inhibits CNS infiltration<sup>661</sup>.

In comparison with the NOD mice where the performance of in-vitro experiments provided helpful observations regarding the suppressive mechanism of action of CpG-proB cells, in EAE, the adaptation of an in vitro model was more complicated given that the migration process of T lymphocytes was a major point in the protective mechanism of these progenitor cells in EAE. It requires immunization of mice and 10-12 days later, isolation of total splenocytes or lymph node cells that should be re-activated for about 3 days with the peptide. Some preliminary results of in vitro experiments were not in accordance with our in vivo observations so the studies focused exclusively on in vivo analysis.

A switch to an anti-inflammatory environment in both lymph nodes and CNS further characterized the protection induced by the injection of WT CpG-proB cells. Surprisingly, in the periphery, T cells produced less GM-CSF but there was no change in terms of the expression of other pro-inflammatory cytokines such as IL-17 and IFN- $\gamma$ . T cells enhanced their production of the anti-inflammatory cytokine IL-10 and there was also an expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells. Host B220<sup>+</sup> B cells also enhanced their production of IL-10. The preferential suppression by CpG-proB cells of only the production of the cytokine GM-CSF may be explained by the enhanced expression of IL-27 by B cells in the lymph nodes of mice injected with WT CpG-proB cells (data not shown). In support of this hypothesis, it has been shown that IL-27 can suppress EAE, in part via Th17 cell inhibition and IL-10 upregulation whereas IL-27R<sup>-/-</sup> mice exhibit enhanced CNS inflammation<sup>662-664</sup>.

Additionally, more recently, Young A et al showed that IL-27 can suppress the production of GM-CSF in synergy with IL-10<sup>665</sup>.

Both in NOD and B6 mice, WT CpG-proB cells immediately after their isolation, they can produce IL-27. Compared to NOD mice where the mechanism of action was not at all related to the production of IL-10 either by the injected or host cells, in B6-EAE mice, the enhanced production of IL-10 by both T and B cells may involve a stronger participation of IL-27.

It is possible that the injection of WT CpG-proBs provide with additional signals which favor host B cells to become IL-10-competent. The secretion of IL-10 is one of the most important suppressive functions of the already described B regulatory cell types. Whether these signals are related directly to IFN- $\gamma$  or CCL19, since (IFN- $\gamma$  KO CpG-proB-recipient mice do not display enhanced IL-10 production by host B220<sup>+</sup> cells) remains to be investigated in more details by studying the direct role of these factors in host B cells.

The expansion of T regulatory cells in CpG-proB-injected mice is an interesting point since many studies investigating the suppressive mechanisms of more mature B regulatory populations have suggested a relation between B regulatory cells and the expansion of T regs and their migration to the site of inflammation<sup>321,407,644,666</sup>. The production of IL-10 does not seem to be sufficient for the direct induction of T regulatory cells but requires a cognate interaction with CD80 and CD86<sup>323,377</sup>. B regulatory cells can also trigger the differentiation of Tr1 cells, suggesting that the presence of B regs may be responsible for maintaining the balance between Th1/Th17 and Tregs. The exact mechanism by which Bregs can induce the expansion of Tregs is still unclear and it should be investigated if the involvement of other cytokines such as IL-6, which is known to induce the differentiation of iTregs into Th17 cells, is also affected by the presence of Bregs<sup>667</sup>. In our case, it remains to be shown if the expansion of T regulatory cells results from an interaction with the progenitor cells or rather with their more mature progeny. The differentiated cells may be able to interact with effector T cells, inducing the production of cytokines, which favor the expansion of Tregs.

In the CNS, the few activated T cells, which had already migrated into the spinal cord, displayed reduced production of both GM-CSF and IL-17 together with enhanced IL-10 in WT CpG-proB-recipient mice.

If the only mechanism of action of CpG-proB cells was to restrain T cells to the periphery, it would not be easy to explain why T cells inside the CNS lost their capacity to produce the pathogenic cytokines GM-CSF and IL-17.

A powerful point of these progenitor cells is their ability to differentiate into more mature cells that can also contribute to a more long-term protection. As we have already described in NOD mice, the differentiated mature B cells that could be recovered in the spleen even 3 months post-injection, expressed high levels of FasL leading targeted T-cells to apoptosis and contributing to the long protective effect observed after CpG-proB cell adoptive transfer.

In EAE, the injected WT CpG-proB cells could be recovered at the peak of the disease, only in the lymph nodes and in the spinal cord. Interestingly, the differentiation profile of these more mature recovered cells was not the same in these two organs. In lymph nodes, CpG-proB cells could give rise to several mature subsets of B cells such as transitional, marginal zone, follicular and CD5<sup>+</sup> or CD5<sup>-</sup> CD11b<sup>+</sup> cells. Among the CD5<sup>+</sup> cells, 16% of these cells were characterized also by the expression of CD1d<sup>hi</sup>, suggesting that the injected CpG-proB cells could also give rise to a phenotype of B10 cells whereas in the NOD mice, no such similar phenotype could be recovered. These recovered B cells in the periphery (lymph nodes) did not produce IL-10 (**Figure 22**).

It remains to be shown if these differentiated more mature B cells can also produce CCL19, contributing to the restraint of T cells in the periphery. This is a strong hypothesis since when we prepared CpG-proB cells from Rag2<sup>-/-</sup> mice, which lost their capacity to give rise to mature lymphocytes (either B or T cells), these injected progenitor cells neither conferred protection to EAE nor blocked T cell entry into the spinal cord. The first conclusion is that the progeny is also needed for the long-term protection and secondly that these differentiated cells keep the capacity to restrain T cell homing to the CNS. The fact that the injection of IFN $\gamma$  KO CpG-proB cells is unable also to induce the production of the anti-inflammatory cytokine IL-10 may suggest that the IFN- $\gamma$ -dependent production of CCL19 by the WT progenitor cells is responsible not only for restraining T cells to the periphery, but also enhance the production of IL-10. IL-10 has been shown already to be affected by the expression of some chemokines, such as the macrophage proinflammatory MIP-3 $\beta$ <sup>668</sup>.

The important role of the progeny in the protective effect of the injected CpG-proB cells has been shown also in the case of T1D not only through the ability of more mature differentiated B cells to express high levels of FasL in the spleen, but also via the finding that the adoptive transfer of CpG-proB cells derived from Rag KO NOD mice lost their capacity to confer protection to NOD mice against T1D (data not shown).

On the other hand, inside the spinal cord, CpG-proB cells could differentiate exclusively to a phenotype characterized by the expression of markers  $CD19^+B220^+CD1d^+CD5^+IgM^+CD11b^+$ . This phenotype is very similar to the B10 cells that have described by the group of Tedder <sup>366</sup>. B10 cells can display their suppressive properties through the production of large quantities of the anti-inflammatory cytokine IL-10, after exposure to TLR ligands (TLR-4 and -9) and additional stimulation with PMA-ionomycin. Similar to B10 cells, the recovered differentiated cells in the spinal cord were also able to produce IL-10 and we consider this production of IL-10 as the main factor which can suppress autoimmune responses by T effector cells reducing the production of pro-inflammatory cytokines IL-17 and GM-CSF.

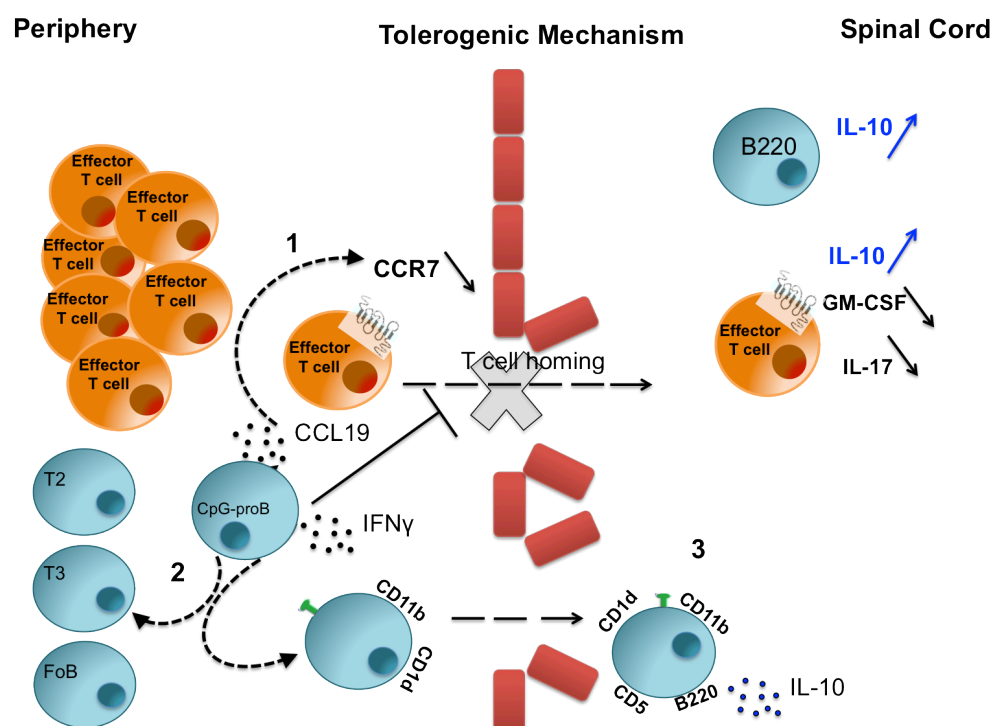
Recently, it has been demonstrated that the T cell Ig domain and mucin domain 1 (TIM-1) expression is associated with the competence of B cells to produce IL-10, proposing TIM-1 as a common marker of all IL-10-producing B regulatory cells<sup>367</sup>. Interestingly, the recovered progeny inside the CNS express TIM-1 whereas the differentiated B cells recovered in the lymph nodes were negative for the expression of TIM-1 (data not shown). This could additionally explain why IL-10 is secreted only by the B cells found in the spinal cord where some inflammatory signals could enhance the expression of TIM-1 on the activated B cells inducing the production of IL-10.

The important role of the differentiated B cells in the spinal cord was confirmed by the use of  $CD11b^{-/-}$  mice. CpG-proB cells derived from these knock-out mice were not able to protect against EAE, suggesting that the emergence of this phenotype exclusively inside the spinal cord is necessary for completing the protective effects. Interestingly, it has been shown that the expression of the integrin CD11b facilitates the entry of grafted bone marrow cells inside the CNS<sup>669</sup>. It is strongly possible that the CpG-proB cells derived from  $CD11b^{-/-}$  lose their migratory capacity to the spinal cord where inflammatory signals can induce their production of IL-10 in order to suppress autoimmune responses by activated T cells. Moreover, if the protective mechanism of the recovered activated B cells inside the spinal cord is dependent on their ability to produce IL-10 is still unknown. The use of CpG-proB cells from  $IL-10^{-/-}$  mice

could answer this question since these progenitor cells could normally differentiate in this exclusive phenotype of  $CD19^+B220^+CD1d^+CD5^+IgM^+CD11b^+$  with no capacity to produce IL-10. Whether the injection of CpG-proB cells derived from IL-10<sup>-/-</sup> mice can provide protection against EAE will be interesting to investigate.

Comparing with the differentiation profile of the injected cells in the NOD mice, we see that the same cells display different mechanisms of protective action and additionally, they give rise to different more mature B cells (**Table 4**). The fact that any B cell at any stage of differentiation has been shown to be able to acquire regulatory properties is not in support with the idea of the existence of a common progenitor for all the regulatory B cells subsets that have been described so far. Herein, we provide evidence for the emergence of a population of progenitor cells at the stage of pro-B, which can differentiate in several mature regulatory B cell subsets depending on the mouse strain, the inflammatory environment and the site where these cells migrate. Their emergence after stimulation with TLR ligands allow them to produce cytokines acting as regulatory cells against effector T cells. Since CpG-proBs do not express a BCR and therefore do not receive stimulation direct from the antigen, the inflammatory environment and mainly the production of inflammatory cytokines by activated T cells should be important to confer regulatory properties onto CpG-proB cells. In NOD mice, the production of IFN- $\gamma$  by Tregs is essential for inducing suppressive capacities onto CpG-proB cells whereas in EAE mice, the injection of CpG-proB cells at the day of immunization (day 0) was much less efficient than if they were injected at the onset of the clinical signs (not shown) when the inflammatory signals by activated T cells have been already established. However, in both animal models, we see that the injected cells are attracted by the inflammatory signals, mostly in the target tissues. Thus, in NOD mice, the injected cells migrate directly to the pancreas whereas in EAE mice, differentiated cells can be recovered in the spinal cord (**Table 4**).

The fact that the EAE model is a much faster model does not permit us to follow easily the differentiation profile of the injected cells so it is possible that even in EAE mice, some of the injected progenitor cells can migrate directly to the site of inflammation, the spinal cord where they remain at a progenitor cell stage before their differentiation in the exclusively recovered phenotype of activated  $CD19^+B220^+CD1d^+CD5^+IgM^+CD11b^+$  B cells (**Figure 22**).



**Figure 22. Tolerogenic mechanism of CpG-proBs in EAE** 1) CpG-proBs are characterized by the production of large quantities of IFN- $\gamma$  that can induce the autocrine production of the chemokine ligand CCL19. CCL19 occupy its specific receptor CCR7 which is expressed on the surface of T effector cells, leading to the down regulation of CCR7 expression. Activated T cells can't migrate inside the CNS since they can't interact with the chemokines CCL19 and CCL21, expressed on the BBB. This block of T cell homing results in an accumulation of CD4<sup>+</sup> T cells in the lymph nodes. 2) CpG-proB cells can differentiate into more mature B cells, either in the lymph nodes or in the spinal cord. The differentiated cells that could be recovered in the lymph nodes display several phenotypes of mature B cells such as Transitional and Follicular B cells. 3) In the spinal cord, it could be recovered an exclusive phenotype of differentiated B cells characterized by the expression of B220<sup>+</sup>CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>+</sup>CD11b<sup>+</sup>IgM<sup>+</sup>. These activated B cells were also able to produce the anti-inflammatory cytokine IL-10, which is considered to be the suppressive factor for the reduction of the production of inflammatory cytokines, IL-17 and GM-CSF by activated – already migrated T effector cells inside the CNS.

Herein, we provide evidence for the existence of a new population of immature hematopoietic progenitor cells, which can display regulatory properties against two different models of autoimmune diseases. It would be interesting to investigate if an equivalent population can also emerge in humans, in order to know if we can use this cell population as a potential therapeutic tool, either injected per se into recipient or as a complement of autologous HSC transplantation.

In the following table, we are trying to report the common and different points that have been found regarding the phenotype and functional properties of CpG-proB cells in the models of T1D and EAE.

	Phenotype	Role of IFN- $\gamma$ by Effector T cells	Role of IFN- $\gamma$ by CpG-proB cells	Mechanism of action (by CpG-proB cells)	Differentiation profile	Mechanism of action by their progeny
<b>T1D</b>	c-kit <sup>hi</sup> Sca-1 <sup>lo</sup> B220 <sup>+</sup> IgM <sup>-</sup>	Upregulation of FasL on CpG-proB cells. Enhance the production of IFN- $\gamma$ by CpG-proB cells.	Switch of effector T cells in a cytotoxic profile upregulating the expression of Eomes, T-bet and downregulating the expression of ICOS. Reduction of IL-21 production.	Apoptosis of targeted T cells through FasL. Reduction of IL-21 production through enhanced production of IFN- $\gamma$ .	Recovered cells in the pancreas until the day 15 post-injection displaying a phenotype of progenitor cells. Recovered mature B cells of T2, FoB and MZ B cells in the spleen even 3 months post-injection – no CD5 <sup>+</sup> CD1d <sup>hi</sup> phenotype	Expression of high levels of FasL, induction of apoptosis. No involvement of IL-10.
<b>EAE</b>	c-kit <sup>hi</sup> Sca-1 <sup>lo</sup> B220 <sup>+</sup> PDCA-1 <sup>-</sup> IgM <sup>-</sup>	No evidence	Enhance the production of CCL19 by CpG-proB cells.	Restrain of T cell homing to the CNS through the down regulation of CCR7. Reduction of pathogenic cytokines IL-17 and GM-CSF inside the spinal cord	More mature B cells recovered in the lymph nodes at the stage of T2, FoB, CD5 <sup>+</sup> CD1d <sup>hi</sup> , CD11b <sup>-</sup> and CD11b <sup>+</sup> An exclusive phenotype of B220 <sup>+</sup> CD19 <sup>+</sup> CD11b <sup>+</sup> CD1d <sup>+</sup> CD5 <sup>+</sup> IgM <sup>+</sup> in the spinal cord	Production of IL-10 by recovered cells in the spinal cord. Expression of CCL19 by recovered mature B cells in the lymph.

**Table 4. Comparison of the mechanism of action of CpG-proBs cells between T1D and EAE**

## 2) Mobilized MPPs protect against EAE

As it has been shown already by our laboratory, mobilized progenitor cells at the stage of MPP2 have the capacity to selectively enhance the proliferation of TCR-activated Foxp3<sup>+</sup> T regulatory cells both in vitro and in vivo, conferring protection to NOD mice against T1D<sup>110</sup>.

We addressed the question if the same mobilized progenitor cells can be also protective against a chronic model of MS, the MOG<sub>35-55</sub>/CFA-induced EAE. EAE disease was induced in C57BL/6J mice after immunization with the MOG<sub>35-55</sub> peptide/CFA together with two injections of pertussis toxin, at day 0 and 1 post-immunization. The mobilized progenitors were sorted as c-kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>+</sup>CD11b<sup>-</sup> cells. An adoptive transfer of only 25,000 mobilized progenitor cells per mouse at the time of immunization (d0) reduced the development of the experimental autoimmune encephalomyelitis.

In the case of T1D, the mobilized progenitor cells (MPPs) can trigger the expansion of regulatory T cells through a cell-to-cell contact mechanism. The expression of Jagged2 at the

surface of MPPs make them able to interact with its receptor Notch3 expressed by T regs and additionally, the ability of MPPs to secrete the soluble factor GM-CSF can also contribute to this cell-to-cell contact mechanism by binding to its receptor CD116 expressed at the surface of Tregs. Tregs play an important role in the control of tolerance in multiple sclerosis as well as in its animal model of experimental autoimmune encephalomyelitis (EAE). Promoting the expansion of Tregs directly *in vivo* might represent a therapeutic strategy worth of interest. We therefore investigated whether adoptive transfer of mobilized MPPs could be used to protect against EAE via Treg expansion.

We herein report that mobilized MPPs effectively reduced the disease score. The protection of the injected mobilized progenitor cells was also confirmed by looking at the production of pro-inflammatory cytokines by the pathogenic CD4<sup>+</sup> T cells at the peak of the diseases. Indeed, in the spinal cord (site of inflammation of autoimmune responses) of recipient mice there was reduced production of the pro-inflammatory cytokines IL-17, GM-CSF and IFN- $\gamma$ . To investigate whether the role of Tregs is also involved in the protective effect from MPPs for EAE, we studied the proliferation level of Foxp3<sup>+</sup> Tregs in the spleen, lymph nodes and spinal cord of MPP-recipients. In all organs, the levels of Foxp3 expression by CD4<sup>+</sup> T cells was higher in recipient mice together with higher levels of the expression of Ki67, a marker of proliferation, suggesting that in the injected mice, the MPPs enhanced the level of proliferation of regulatory T cells. The essential role of Tregs in the protection from MPPs was confirmed *in vivo* by depleting the Tregs with the use of anti-CD25 (PC61) mAb once the protection has been established. Interestingly, the depletion of Tregs was enough in order to abrogate protection against disease, as the clinical symptoms, reached the level seen in control mice. Comparing with the mechanism of action of MPPs in NOD mice in which the expansion of Tregs is induced through a cell-to-cell contact of the ligand Jagged2 and Notch3 receptor and the secretion of the soluble factor GM-CSF as well, in B6 mice it has been shown that the GM-CSF is not able to trigger the expansion of T regulatory cells. So the mechanism of actions in the case of EAE may be different from NOD mice.

The ability of these mobilized progenitor cells to induce the expansion of Foxp3<sup>+</sup> T regulatory cells has been confirmed also by *in vitro* studies. In cell cultures of activated (antiCD3/antiCD28) CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T regulatory cells together with MPPs, we found that MPPs could induce the proliferation of the Tregs. Performing a microarray transcriptome gene analysis of Tregs purified after 3 days of cell culture together with MPPs at 1:1 ration, it



has been documented that there was an upregulation in terms of the expression of molecules related to IL-1 $\beta$  and TNF- $\alpha$  pathways. Many studies have already reported the role of IL-1 $\beta$  and TNF- $\alpha$  in the expansion of Tregs. IL-1 $\beta$  has been shown to promote TGF- $\beta$ - and IL-2-dependent Foxp3 expression in T regulatory cells and to display also co-stimulatory effects on their expansion and differentiation<sup>670,671</sup>. Moreover, it is known that IL-1 $\beta$  is highly expressed in mobilized CD34<sup>+</sup> compared to CD34<sup>+</sup> fraction from steady state bone marrow, so IL-1 $\beta$  could be a strong candidate factor through which MPPs display their protective effect in EAE<sup>672</sup>. Taking advantage of the mice deficient for IL-1 $\beta$ , we showed that the mobilized progenitor cells derived from these mice were not as much efficient as the wild type ones to induce the proliferation of T regulatory cells in vitro. Interestingly, they lost their capacity to confer protection to EAE mice when injected at day 0. The clinical score of mice that have been injected with MPPs from IL-1 $\beta$  KO mice was very similar to control mice and comparing their ability to induce the expansion of Tregulatory cells, we found out that at the peak of the disease the number of Treg was impaired in mice injected with IL-1 $\beta$  KO MPPs. The lack of protection was also confirmed by the fact that there was no reduced production of the inflammatory cytokines IL-17 and GM-CSF. It is already known that Treg can display their suppressive effects through the secretion of anti-inflammatory cytokines such as IL-10 and TGF- $\beta$ . Comparing the cytokine profile of CD4<sup>+</sup> T cells in mice injected with WT MPPs and those injected with IL-1 $\beta$  KO MPPs, only CD4<sup>+</sup> T cells from the first group were characterized by a massive production of the TGF- $\beta$  in both periphery and CNS. Regarding the IL-10 production, there was no difference between the two groups. IL-1 $\beta$  seems to be necessary for the expansion of T regulatory cells, which enhance the production of the TGF- $\beta$ , reducing the production of inflammatory cytokines and resulting in a less pathogenic environment. The massive production of TGF- $\beta$  mainly by CD4<sup>+</sup> T cells within the spinal cord may suggest that not only CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs are responsible for the production of this anti-inflammatory cytokine, but IL-1 $\beta$  by MPPs may induce other conventional T cells to enhance the production of TGF- $\beta$ .

In the microarray gene analysis, it has been shown that the pathway of TNF- $\alpha$  may be also involved in the protective mechanism of action of mobilized progenitor cells. Many studies have shown the role of TNF- $\alpha$  and its receptors in several cases of autoimmune diseases. It has been demonstrated a direct relation between TNF- $\alpha$  and T regulatory cells showing that TNF- $\alpha$  is important for the stabilization, function and expansion of Treg<sup>316,673–675</sup>.

On the other hand, regarding the direct role of this cytokine in models of autoimmune diseases, several controversial observations make increasingly difficult to conclude if this cytokine display a regulatory effect or not. In case of EAE, it has been shown that treatment with anti-TNF $\alpha$  (at the time of immunization) reduced the incidence and delayed the onset of EAE but had no effect on disease severity once EAE had been established whereas more recently, it has been reported that selective targeting of TNFR1 could ameliorate the symptoms of the most common models of MS disease<sup>319,676</sup>.

In order to investigate the parallel role of TNF- $\alpha$  in the protective effect of these mobilized progenitor cells, we used an neutralizing antibody against TNF- $\alpha$  in the cell cultures of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells together with MPPs either from WT or IL-1 $\beta$  KO mice. WT MPPs lost their previous capacity to expand Tregs whose level of proliferation reached the level of proliferation that had been induced by IL-1 $\beta$  MPPs. The possibility that IL-1 $\beta$  induces TNF- $\alpha$  production by Tregs, that in turn promotes their autocrine proliferation, is suggested by the effect of anti-TNF- $\alpha$  neutralization in co-cultures in vitro which abolished the differences in Treg proliferation induced in the presence of WT versus IL-1 $\beta$  KO. It would be interesting to confirm this possible necessary role of TNF- $\alpha$  in vivo by injecting MPP cells from TNF- $\alpha$ <sup>-/-</sup> mice in order to investigate whether both cytokines need to get in synergy for triggering the expansion of T regulatory cells.

It should be mentioned that all our in vivo experiments arise from in vivo studies of MPPs injection at day 0. For clarifying a mechanism of action of the mobilized progenitor cells that could be applied in humans, we have to check whether these cells can confer protection to EAE mice when they are injected either at the onset of clinical signs (day 11 – 14) or at the peak of the disease. The finding that in mice that have been injected with WT MPPs, there was a high number of Tregs inside the spinal cord can make possible also the hypothesis that these regulatory cells upregulate the expression of a chemokine receptor which facilitates their entry to the site of inflammation and autoimmune responses. The number of Tregs as well as the level of Foxp3 expression by CD4<sup>+</sup> T cells was the same in the periphery of mice that have been injected either with WT or IL-1 $\beta$  KO MPP cells. So, it has to be investigated in more details if the only mechanism of action of these mobilized progenitor cells is just to trigger the expansion of Trgs through TNF- $\alpha$  and IL-1 $\beta$  or they acquire also a selective privilege of entry to the CNS where the inflammation occurs. It remains also to investigate whether this protective effect is exculsive in mobilized progenitor cells comparing with the

in-vivo capacity of non-mobilized MPPs cells from bone marrow cells to control or not the EAE development.

The injection of progenitor cells keep open also the possibility that their more mature differentiated cells can display a role either by contributing or not to their protective effects. It would be interesting to follow the differentiation profile, migration and functional properties of the injected cells in order to conclude if the protection is exclusively due to immunoregulatory properties of the mobilized progenitor cells.

It is interesting that hematopoietic progenitor cells are not only responsible for giving rise to more mature cells of several cell lineages but they can also acquire immunoregulatory properties under specific conditions.

We provide evidence for the existence of a population of mobilized progenitor cells at the stage of MPP2 whose adoptive transfer in two different animal models can confer protection against T1D and EAE through the expansion of T regulatory cells whereas, the intravenous injection of innate CpG-activated pro-B cells seems to control the incidence of these above models targeting the functions of effector T cells.

A combination protocole using both the mobilized progenitor MPPs which act through the expansion of T regulatory cells and the activated CpG-proB cells targeting the effector T cells, would be a promising potential therapeutic tool for cell therapies of autoimmune diseases.

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