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The regulatory effects of circulating normal immunoglobulins on autophagy and Th17 response

Mrinmoy Das

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**THESE DE DOCTORAT DE L'UNIVERSITE PARIS VI
PIERRE ET MARIE CURIE**

ECOLE DOCTORALE: Physiologie, Physiopathologie et Thérapeutic

Specialite: *IMMUNOLOGIE*

Présentée par Monsieur
Mrinmoy DAS

Pour l'obtention du grade de
Docteur de l'Université Paris VI

Sujet de la thèse

**The regulatory effects of circulating normal
immunoglobulins on autophagy and Th17 response**

Soutenu le 7 septembre 2017

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Résumé en français

Les immunoglobulines circulantes jouent un rôle critique dans l'homéostasie immunitaire en modulant les fonctions des cellules du système immunitaire. Au cours de ma thèse, j'ai exploré les effets régulateurs des immunoglobulines G thérapeutiques (IVIG) et des immunoglobulines A monomériques circulantes (mIgA) sur l'autophagie et les réponses Th17 respectivement.

Les IVIG sont une préparation thérapeutique d'IgG normales et provenant de milliers de donneurs sains. Elles ont été utilisées comme agent anti-inflammatoire dans le traitement de maladies auto-immunes et inflammatoires variées. Cependant, les mécanismes ne sont pas complètement élucidés et plusieurs mécanismes mutuels et non exclusifs ont été proposés. L'autophagie est un important processus biologique impliquant la dégradation lysosomale des composants cellulaires endommagés et des protéines mal repliées. Il y a plusieurs preuves montrant l'implication de l'autophagie dans les maladies auto-immunes et auto-inflammatoires incluant la découverte de polymorphismes dans des gènes liés à l'autophagie. J'ai donc émis l'hypothèse que les IVIG modulent l'autophagie dans les cellules immunes, ce qui explique en partie les mécanismes par lesquels les IVIG sont bénéfiques aux patients avec des maladies auto-immunes et inflammatoires. J'ai montré que les IVIG chez des patients avec une maladie auto-immune induisent l'autophagie dans les cellules mononuclées du sang (PBMCs). De plus, j'ai observé que l'autophagie médiée par les IVIG est dépendante de $F(ab')_2$ et requiert l'endocytose d'IgG. Structurellement, l'induction de l'autophagie médiée par les IVIG implique l'activation de l'AMPK, de beclin-1, de la PI3K de classe III et de la p38MAPK et l'inhibition de mTORC. Donc, l'induction de l'autophagie par les IVIG représente un nouveau mécanisme d'action permettant leur effet thérapeutique dans les maladies auto-immunes et inflammatoires.

Du fait de leur implication dans la physiopathologie de plusieurs maladies inflammatoires et auto-immunes, les Th17 représentent une cible attractive pour traiter ces conditions pathologiques. Malgré le fait qu'elles sont le deuxième anticorps le plus abondant dans la circulation, la fonction immunorégulatrice des IgA n'est relativement pas explorée. C'est pourquoi, j'ai exploré comment les IgA monomériques isolées à partir de plusieurs donneurs sains son en interface avec la réponse Th17 en étudiant l'effet des mIgA sur la différenciation, l'amplification et les fonctions des Th17. J'ai montré que les mIgA inhibent la différenciation et l'amplification des cellules Th17 humaines et la production de leur cytokine effectrice IL-17A. Les mIgA suppriment également les réponses $IFN-\gamma$ sous ces conditions expérimentales. Les mIgA augmentent significativement les cellules T régulatrices $Foxp3^+$ (Tregs) parmi les

cellules T CD4⁺ mémoires, indiquant donc la régulation réciproque des Tregs et des Th17 par les mIgA. Les effets inhibiteurs des mIgA sur les Th17 sont F(ab')₂ dépendants and indépendants de FcαRI (CD89) et DC-SIGN. L'effet des mIgA sur les Th17 impliquent la suppression de la phosphorylation de STAT3. De plus, j'ai trouvé que les mIgA reconnaissent les T CD4⁺ aussi bien que les récepteurs pour des cytokines (IL-6Rα and IL-1RI) qui médient les réponses Th17. Donc, mes résultats montrent que les mIgA naturelles ont des fonctions immunomodulatrices et l'utilité potentielle des mIgA dans les maladies auto-immunes et inflammatoires au cours desquelles les Th17 sont impliqués.

Résumé in Anglais

Circulating immunoglobulins play a critical role in the immune homeostasis by modulating the functions of immune cells. In my thesis, I investigated the regulatory effects of therapeutic immunoglobulin G (IVIg) and circulating monomeric immunoglobulin A (mIgA) on autophagy and human Th17 response respectively.

IVIg is a therapeutic preparation of pooled normal IgG. It is used as an anti-inflammatory agent in the treatment of a wide variety of autoimmune and inflammatory diseases. However, the mechanisms are not yet fully elucidated and several mutually non-exclusive mechanisms have been proposed. Autophagy is an important biological process involving lysosomal degradation of damaged cellular components and misfolded proteins. There are several evidences that support the involvement of autophagy in autoimmune and auto-inflammatory disorders including the discovery of polymorphisms in autophagy-related genes. Therefore, I hypothesized that IVIg modulates autophagy in immune cells that explains in part the mechanism(s) by which IVIg therapy benefits patients with autoimmune and inflammatory diseases. I show that IVIg therapy in autoimmune patients induces autophagy in peripheral blood mononuclear cells (PBMCs). Furthermore, I have observed that IVIg-mediated autophagy induction is F(ab')₂-dependent and requires endocytosis of IgG. Mechanistically, IVIg-mediated autophagy induction implicates activation of AMPK, beclin-1, class III PI3K and p38MAPK and inhibition of mTORC. Thus, induction of autophagy by IVIg represents a novel mechanism of action in achieving therapeutic effect in autoimmune and inflammatory diseases.

Because of their implication in the pathogenesis of several inflammatory and autoimmune diseases, Th17 cells represent an attractive target to treat these pathological conditions. Despite being second most abundant antibody in the circulation, the immunoregulatory function of IgA is relatively unexplored. Therefore, I have investigated whether monomeric IgA (mIgA) isolated from pooled of healthy donors interfaces with human Th17 response by studying the effect of mIgA on differentiation, amplification and function of Th17 cells. I have shown that mIgA inhibits differentiation and amplification of human Th17 cells and the production of their effector cytokine IL-17A. mIgA also suppresses IFN- γ responses under these experimental conditions. mIgA significantly enhanced Foxp3⁺ regulatory T cells (Tregs) among the memory CD4⁺ T cells, thus indicating reciprocal regulation of Tregs and Th17 cells by mIgA. The inhibitory effects of mIgA on Th17 cells are F(ab')₂-dependent and independent of Fc α RI (CD89) and DC-SIGN. The effect of mIgA on Th17 cells implicates suppression of

phosphorylation of STAT3. Additionally, I found that mIgA recognize CD4⁺ T cells as well as receptors for cytokines (IL-6R α and IL-1RI) that mediate Th17 responses. Thus, my results demonstrate immunomodulatory functions of naturally-occurring mIgA and potential therapeutic utility of mIgA in autoimmune and inflammatory diseases that implicate Th17 cells.

Table of Contents

I. INTRODUCTION	Page no
1. The immune system	
1.1 Overview	1
1.2 Innate immunity	2
1.3 Adaptive immunity	3
1.3.1 Activation of CD4 ⁺ T cells	5
1.3.2 CD4 ⁺ T cells differentiation	5
1.3.2.1 Th1 differentiation	7
1.3.2.2 Th2 differentiation	9
1.3.2.3 Th9 Cells	10
1.3.2.4 Follicular Helper (Tfh) T Cells	10
1.3.2.5 Regulatory T cells (Tregs) differentiation	11
1.3.2.6 Th17 cells differentiation	15
1.3.3 Reciprocal relationship between Th17 cells and Tregs	20
1.3.4 Th17 cells in the pathogenesis of autoimmune and inflammatory diseases	22
1.3.5 Therapeutic Approaches to target the Th17/Treg Axis	24
1.3.5.1 Targeting Th17-Related Cytokines and Receptors	25
1.3.5.2 Targeting Transcription Factors	27
2. Autophagy	
2.1 Overview	28
2.2 Types of autophagy	29
2.2.1 Macroautophagy	29
2.2.2 Microautophagy	30
2.2.3 Chaperon-mediated autophagy (CMA)	30
2.3 Molecular machinery of autophagy	32
2.3.1 The core autophagy proteins	32
2.4 The process of autophagy	35
2.4.1 Induction	35
2.4.2 Cargo recognition and selectivity	36
2.4.3 Autophagosome formation	36
2.4.4 Vesicle fusion and autophagosome breakdown	37
2.5 Signalling pathways regulating autophagy	38
2.5.1 PI3K/AKT/mTOR pathway	40
2.5.2 AMPK pathway	41
2.5.3 Beclin 1:hVps34 pathway	42
2.5.4 Ras/Raf/MEK1/2/ERK1/2- MAPK pathway	42
2.6 Physiological and pathological role of autophagy	43
2.6.1 Autophagy and liver diseases	43
2.6.2 Autophagy and cancer	44
2.6.3 Autophagy and neurodegeneration	45
2.6.4 Autophagy and ageing	46
2.7 Autophagy and Immunity	47

2.7.1	Autophagy and Innate Immunity	47
2.7.2	Autophagy in Antigen Processing and Presentation	48
2.7.3	Autophagy in T cells	50
2.8	Autophagy in Autoimmune and Autoinflammatory diseases	50
2.8.1	Autophagy and Systemic lupus erythematosus	50
2.8.2	Autophagy and Crohn's diseases	51
2.8.3	Autophagy and multiple sclerosis	51
3.	Intravenous immunoglobulin (IVIG)	
3.1	Overview	52
3.2	Composition	52
3.3	Pharmacology	53
3.4	Clinical indication of IVIG in autoimmune and auto-inflammatory diseases	53
3.5	Mechanisms of action of IVIG: immunomodulatory and anti-inflammatory effects	54
3.5.1	The effects of IVIG on soluble mediators	55
3.5.1.1	Autoantibody Neutralization and Inhibition of Autoantibody Production	55
3.5.1.2	Scavenging of complement components	56
3.5.2	Fc-mediated effects of IVIG	56
3.5.2.1	Blockade of FcγRs	56
3.5.2.2	Saturation of FcRn	56
3.5.2.3	Increased expression of inhibitory FcγRIIB	57
3.5.2.4	Sialylation requirement for IVIG-mediated anti-inflammatory effect	57
3.5.3	The effects of IVIG on immune cells	59
3.5.3.1	Effector functions of IVIG on monocytes and macrophages	59
3.5.3.2	Regulation of DC functions by IVIG	59
3.5.3.3	Modulatory effects of IVIG on B cells	60
3.5.3.4	Modulatory effects on T cells	60
4.	Immunoglobulin A (IgA)	
4.1	Overview	61
4.2	IgA receptors	63
4.2.1	Interaction with FcαRI	64
4.2.2	Interaction with pIgR	64
4.2.3	Interaction with Fcα/μR	65
4.2.4	Alternative IgA receptors	65
4.3	Role of IgA in Homeostasis and Immune Protection	66
II.	OBJECTIVES	68
III.	RESULTS	
Article I	Intravenous Immunoglobulin Induces Autophagy in Immune cells	70

Article II	Monomeric immunoglobulina from plasma inhibits human Th17 responses <i>in vitro</i> independent of Fc α R1 and DC-SIGN	99
IV.	DISCUSSION AND PERSPECTIVE	114
V.	BIBLIOGRAPHY	122
VI.	ANNEXES	167

List of figures

	Page no
Figure 1: Cellular components of innate and adaptive immune cells.	4
Figure 2: Polarized CD4+ T cell subsets.	6
Figure 3. IL12 and IL4 driven T helper differentiation.	8
Figure 4. Stepwise induction of tTreg cells and pTreg cells.	12
Figure 5. Basic mechanisms used by Treg cells.	14
Figure 6. Mechanisms of Th17 cell induction.	16
Figure 7. Functions of Th17 cytokines and chemokines.	19
Figure 8. A common requirement for transforming growth factor- β in the induced regulatory T cell and T helper 17 cell lineages.	21
Figure 9. Drugs targeting Th17 pathways and their use in human diseases.	25
Figure 10: Types of autophagy.	31
Figure 11: Schematic model of autophagy.	37
Figure 12: Signalling regulation of mammalian autophagy.	39
Figure 13. Regulation of the mTOR pathway by nutrients (amino acids, glucose), stress and insulin/IGF-1.	41
Figure 14. The role of autophagy in human disease.	44
Figure 15. Autophagy and its opposing role in cancer development.	45
Figure 16. ATG proteins in MHC-restricted antigen presentation.	49
Figure 17: A schematic representation of the proposed mechanisms of action of IVIG on cellular immunity.	55
Figure 18. Immunoglobulin A.	63

List of Tables

Table 1: Mammalian homologs of yeast ATG proteins.	33
Table 2: Comparison of different IVIG preparations.	53
Table 3. Examples for clinical use of IVIG.	54

Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cell mediated cytotoxicity
Ag	Antigen
AHR	Aryl hydrocarbon receptor
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANCA	Anti-neutrophil cytoplasmic antibodies
APC	Antigen presenting cell
ATG	Autophagy related protein
BCR	B cell receptor
Bcl	B-cell lymphoma/leukemia
BSA	Bovine serum albumin
CD	Cluster of differentiation
CD	Crohn disease
CIA	Collagen-induced arthritis
CIDP	Chronic inflammatory demyelinating polyneuropathy
CMA	Chaperon-mediated autophagy
CNS	Central nervous system
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTL	Cytotoxic T lymphocyte
CVID	Common variable immunodeficiency
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DCIR	Dendritic cell immunoreceptor
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalitis
ELISA	Enzyme link immunosorbent assay
ER	Endoplasmic reticulum
F(ab') ₂	Fragment antigen binding
Fc	Fragment crystallizable
FcεRI	Fc epsilon receptor I
FcγR	Fcγ receptor
FcRn	Neonatal FcR
FCS	Foetal calf serum
FDA	Food and Drug Administration
FIP	Focal adhesion kinase family-interacting protein
Foxp3	Forkhead box 3
GALT	Gut-associated lymphoid tissue
GBS	Guillain Barré syndrome
GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HSA	Human serum albumin
HSC	Hematopoietic stem cell
H ₂ O ₂	Hydrogen peroxide
IBD	Inflammatory bowel disease
IFN	Interferon

Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
iTreg	Induced regulatory T cell
ITP	Immune thrombocytopenic purpura
IVIG	Intravenous immunoglobulin
JNK	c-Jun amino-terminal kinase
LAG-3	Lymphocyte activation gene-3
LAMP	Lysosome-associated membrane protein
LPS	Lipopolysaccharide
MALT	Mucosal associated lymphoid tissues
MAPKs	Mitogen activated protein kinases
MHC	Major histocompatibility complex
mIgA	Monomeric Immunoglobulin A
MNN	Multifocal Motor Neuropathy
MO	Monocyte
M ϕ	Macrophage
MS	Multiple sclerosis
mTORC	Mammalian target of rapamycin complex
NAbs	Natural antibodies
NK cell	Natural Killer cell
NO	Nitric oxide
nTreg	Natural regulatory T cell
O ₂ ⁻	Superoxide anion
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
RA	Rheumatoid Arthritis
RORC/ROR γ t	Retinoic acid-related orphan receptor
SEM	Standard error of mean
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
STAT	Signal transducing and activating transcription factor
T-bet	T box transcription factor
TCR	T cell receptor
Tc cell	Cytotoxic T cell
Tfh	Follicular helper T cell
TfR	Transferrin receptor
TGF- β	Transforming growth factor β
Th cell	T helper cell
TIGIT	T cell immunoglobulin and ITIM domain
TLR	Toll like receptor
Treg	Regulatory T cell
TNF	Tumor necrosis factor
TSC	Tuberous sclerosis complex
ULK	UNC-51-like kinase

Introduction

1. The immune system

1.1 Overview

The immune system evolved to protect multicellular organisms from pathogens and maintaining homeostasis. The fully functional immune system involves many organs, molecules, cells, and pathways in such an interconnected and sometimes circular process that it is often difficult to know where to start! The complex host immune system is capable of recognising the self and the non-self components (antigens) to mount an array of defense mechanisms referred as 'immune response' against foreign components without harmful effects on the host. Immune system possesses diversity; to respond against enormous variety of pathogens as well as specificity; to mediate appropriate and efficient response against the particular pathogen. However, in certain conditions, the dysregulation of immune system either in the self and non-self recognition or an excessive immune response lead to various autoimmune and inflammatory diseases.

Immune system can be functionally separated into four related activities. ***Immune recognition*** is the primary task by which immune system recognizes foreign pathogen. Upon recognition, it mounts an appropriate ***effector response*** to eliminate the foreign pathogen. The effector response needs to be regulated to avoid immunopathology by means of ***immune regulation***. Further, immune system provides ***memory response*** for protection on later exposure to the same foreign invaders characterized by a more rapid immune reaction.

Although blood is not an immunological organ per se, it is the conduit for most immune cells circulating in the body, especially after an immunological stimulus such as vaccination. All blood cells arise from the hematopoietic stem cell (HSC) by a process haematopoiesis. Early in haematopoiesis, a multipotent stem cell differentiates along one of two pathways, giving rise to either a common lymphoid progenitor cell or a common myeloid. Lymphoid progenitor cells give rise to B, T, and NK (natural killer) cells and some DC. Myeloid progenitor cells generate progenitors of red blood cells (erythrocytes), many of the various white blood cells (neutrophils, eosinophils, basophils, monocytes, leukocytes, mast cells, DC, and platelets (1)). After haematopoiesis white blood cells circulate though out the body where they encounter antigens. Development and maturation of lymphocytes takes place in the thymus and bone marrow, the primary (central) lymphoid organs. Mature lymphocytes migrate to secondary lymphoid organs

that include the lymph nodes, spleen, and various mucosal associated lymphoid tissues (MALT) such as gut-associated lymphoid tissue (GALT), where they interact with antigens.

The immune system can be simplistically viewed as having two lines of defense: *innate immunity* and *adaptive immunity*. Innate immunity represents the first line of defense to an intruding pathogen. It is an antigen-independent (non-specific) defense mechanism that is used by the host immediately or within hours of encountering an antigen. The innate immune response has no immunologic memory and, therefore, it is unable to recognize or memorize the same pathogen should the body be exposed to it in the future. Adaptive immunity, on the other hand, is antigen-dependent and antigen-specific and, therefore, involves a lag time between exposure to the antigen and maximal response. The hallmark of adaptive immunity is the capacity for memory which enables the host to mount a more rapid and efficient immune response upon subsequent exposure to the antigen.

1.2 Innate immunity

The primary function of innate immunity is the recruitment of immune cells to sites of infection and inflammation through the production of cytokines. The innate immune response also promotes clearance of dead cells or antibody complexes and removes foreign substances present in organs, tissues, blood and lymph. Numerous cells are involved in the innate immune response such as phagocytes (macrophages and neutrophils), DC, mast cells, basophils, eosinophils and NK cells (Figure 1). Responses from innate immune system are triggered upon pathogen recognition by a set of pattern recognition receptors (PRRs) expressed on the surface of innate immune cells. PRRs identify two classes of molecules: pathogen-associated molecular patterns (PAMPs), which are expressed only by microbes, but not by host and damage-associated molecular patterns (DAMPs), which are associated with cell components that are released during cell damage or death (2). In addition to their phagocytic properties, neutrophils and macrophages also produce a variety of other toxic products such as hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) and nitric oxide (NO), which assist in the elimination of pathogens. Unlike neutrophils (which are short-lived cells), macrophages are long-lived cells that not only play a role in phagocytosis, but are also involved in antigen presentation to T cells.

DCs also phagocytose and function as professional antigen-presenting cells (APCs) and act as important messengers between innate and adaptive immunity. DCs develop in the bone marrow and migrate to the tissues in an immature form. They express high levels of PRRs and exhibit high antigen uptake capacity. DCs undergo maturation when they are exposed to a number of danger signals including the PAMP as well as various cytokines and tissue factors (3). Mature DCs express co-stimulatory molecules and produce variety of cytokines that facilitate the differentiation of CD4⁺ T cells. They can load antigenic peptides on both MHC class I and MHC class II molecules, allowing presentation to both CD8 and CD4 T cells (4, 5). Mast cells and basophils share many salient features with each other and both are instrumental in the initiation of acute inflammatory responses, such as those seen in allergy and asthma. Unlike mast cells, which generally reside in the connective tissue surrounding blood vessels, basophils reside in the circulation. Eosinophils are granulocytes that possess phagocytic properties and play an important role in the destruction of parasites that are too large to be phagocytosed. Along with mast cells and basophils, they also control mechanisms associated with allergy and asthma. NK cells (also known as large granular lymphocytes [LGLs]) play a major role in the rejection of tumours and the destruction of cells infected by viruses. Destruction of infected cells is achieved through the release of perforins and granzymes from NK-cell granules which induce apoptosis (programmed cell death) (6).

1.3 Adaptive immunity

The hallmarks of the adaptive immune response are flexibility and memory. T and B lymphocytes are the cellular elements of the adaptive immune system. In addition, there is a minor population of $\delta\gamma$ T cells and NKT cells which share properties of T cells and NK cells (Figure 3). T cells derive from hematopoietic stem cells in the bone marrow and, following migration, mature in the thymus. They are primarily responsible for cell mediated immune response. B cells develop in bone marrow and are involved in humoral immune responses. Unlike cells in the innate system, which use a fixed repertoire of inherited receptors, T and B cells undergo a recombination of antigen receptor genes to create novel and unique antigen receptors capable of recognizing virtually any antigen. B and T cells that have encountered antigen persist over the long term within an organism and provide rapid and specific responses to reinfection, a concept known as

immunologic memory. T cells are activated when they encounter an APC that has digested an antigen and is displaying antigen fragments bound to its MHC molecules. The MHC-antigen complex activates the TCR and the T cell secretes cytokines which further control the immune response. This antigen presentation process stimulates either activation of cytotoxic T cells (CD8+ T cells) or CD4+ T helper (Th) cells subset differentiation. Cytotoxic T cells are primarily involved in the destruction of cells infected by foreign agents. They are activated by the interaction of their TCR with peptide-bound MHC class I molecules. CD4+ T cells play an important role in establishing and maximizing the immune response. These cells have no cytotoxic or phagocytic activity, and cannot kill infected cells or clear pathogens. However, they “mediate” the immune response by directing other cells to perform these tasks. They are activated through TCR recognition of antigen bound to class II MHC molecules. Once activated, CD4+ T cells release cytokines that influence the activity of many cell types, including the APCs that activate them.

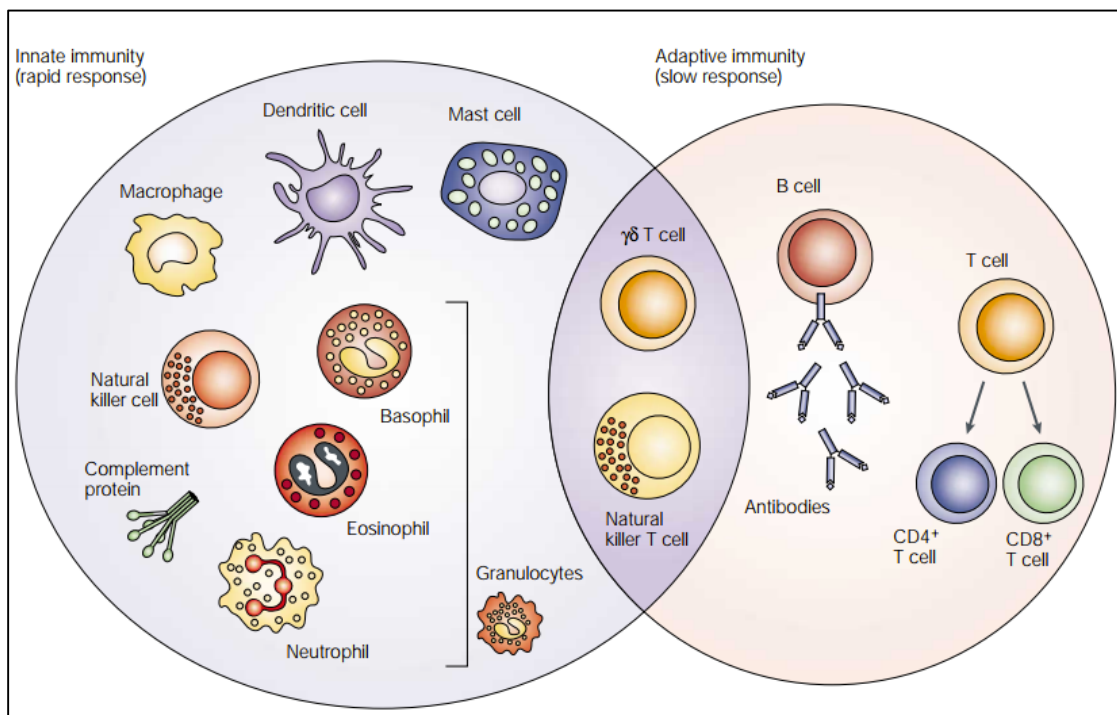


Figure 1. Cellular components of innate and adaptive immune cells. The innate immune response functions as the first line of defence against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells, and CD4+ and CD8+ T lymphocytes. Natural killer

T cells and $\gamma\delta$ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity. (Adapted from Dranoff G, Nat Rev Cancer. 2004)

1.3.1 Activation of CD4+ T cells:

Human CD4+T cells are critical regulators of immune system. CD4+ T cells are highly heterogeneous as they are generated in response to different pathogens and increasing in number of various subsets with specialized functions (7). Activation of CD4+T cells is the most critical step in developing immunity and requires 3 signals for their lineage commitment (8). The first signal is generated following the interaction between T-cell receptor (TCR) and the peptide presented in the context of MHC class II on an APC (9). However, this signal alone is insufficient and further signals in the form of accessory molecules and adhesion molecules are crucial for optimum T cell activation. T cells that do not receive costimulatory signal are likely to be rendered unresponsive or anergic. The second signal is generated following the interaction between the CD28 co-receptor on the T cell and B7 family of co-stimulatory molecules such as CD80 or CD86 on the APC. The third signal is generated by polarizing cytokines produced by the APC or other cells at the site of T cell activation. These cytokines direct differentiation of naïve CD4+T cells into a particular effector subset.

1.3.2 CD4+ T cells differentiation:

The affinity of the MHC/peptide/TCR interaction is thought to be one factors determining the differentiation of naïve CD4+ T cells. However, the local cytokine environment at the time of priming is critical, both in terms of effects on the responding T cells and on the APC (10). The type of APC is important, as are the costimulatory molecules expressed by the APC (11). TCR stimulation enhances IL-2 production from T cells, which signals in an autocrine fashion inducing various cytokine receptors.

Effector CD4+ T cells can be categorized into three major subsets based on the type of cytokine they produce and the major transcription factor (TF) they express. In the presence of interleukin (IL)-12, naïve CD4+ T cells differentiate into Th1 effectors. Th1 effectors express a transcription factor T-bet and secrete the cytokines IFN- γ and TNF- α ; these cells play an essential

role in inhibiting replication of intracellular pathogens such as viruses (12, 13, 8). Under the influence of IL-4, naïve CD4+ T cells differentiate into Th2 effectors. Th2 cells express TF GATA-3, secrete cytokines IL-4, IL-5, and IL-13 (14, 15); these cells are critical during infection by extracellular pathogens such as extracellular bacteria and helminthes. In the presence of IL-6/IL-21 and transforming growth factor (TGF)- β , naïve CD4+ T cells differentiate into Th17 cells. Th17 cells express a transcription factor ROR- γ t and produce cytokines IL-17 and IL-22 (16, 17); these cells are important for control of certain bacterial and fungal infections. Th1, Th2, and Th17 cells are considered to be the major effector CD4+ T cells (18-30). Tregs express TF FoxP3; these cells secrete anti-inflammatory cytokines like TGF- β and IL-10. Tregs maintain immune homeostasis by limiting the magnitude of immune response against pathogens and control inflammatory reactions (31). T follicular helper cells (Tfh) express a TF Bcl-6 and these cells are essential for the production of high affinity IgG antibodies (32).

In addition to the aforementioned Th subsets, existence of other Th subsets has been described, such as, Th9 and Th22 subsets, which secrete IL-9 and IL-22 respectively (33-35), Th3 (TGF- β -secreting CD4 T cells) (36, 37), Tr1 (IL-10 secreting CD4 T cells) (38) and Tfr cells (39), which has been implicated in the inhibiting the functions of Tfh cells (Figure 2) (40).

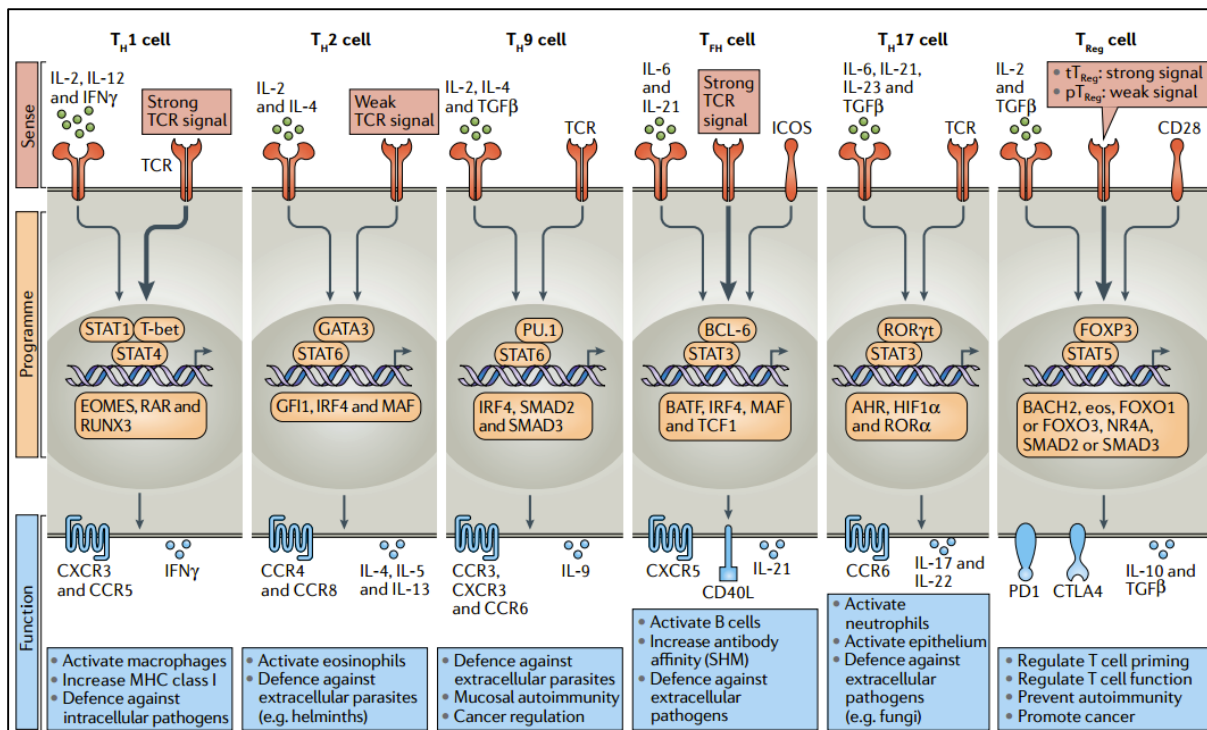


Figure 2. Polarized CD4⁺ T cell subsets. Each CD4⁺ T cell subset can be defined by their distinct abilities to sense (red), programme (orange) and function (blue) in the control of specific pathogens or immune pathologies. The inductive cytokines, polarizing transcription factors and cytokines or chemokine receptors that are characteristic of each subset are shown, along with their association with specific forms of immune defence. AHR, aryl hydrocarbon receptor; BATF, B cell-activating transcription factor; BCL-6, B cell lymphoma 6; CCR, CC-chemokine receptor; CD40L, CD40 ligand; CTLA4, cytotoxic T lymphocyte antigen 4; CXCR, CXC-chemokine receptor; EOMES, eomesodermin; FOXO, forkhead box O; FOXP3, forkhead box P3; GATA3, GATA-binding protein 3; GFI1, growth-factor independent 1; HIF1 α , hypoxia-inducible factor 1 α ; ICOS, inducible T cell co-stimulator; IFN γ , interferon- γ ; IL, interleukin; IRF4, interferonregulatory factor 4; MAF, macrophage-activating factor; NR4A, nuclear receptor 4A; PD1, programmed cell death 1; pTReg cell, peripherally derived regulatory T cell; RAR, retinoic acid receptor; ROR, retinoic acid receptor-related orphan receptor; RUNX3, runt-related transcription factor 3; SHM, somatic hypermutation; STAT, signal transducer and activator of transcription; TCF1, T cell factor 1; TCR, T cell receptor; TFH, T follicular helper; TGF β , transforming growth factor- β ; TH, T helper; TReg cell, regulatory T cell; tTReg cell, thymus-derived regulatory T cell. (Adapted from DuPage M, Bluestone JA, Nat Rev Immunol. 2016)

1.3.2.1 Th1 differentiation

Interleukin 12 (IL12) and interferon γ (IFN γ) are the critical cytokines initiating the downstream signalling cascade to develop Th1 cells (41). IL12 is secreted in large amounts by APCs after their activation through the pattern recognition receptors (42-44). The IL12, in turn, induces NK cells to produce IFN γ . Several transcription factors in coordination induce full differentiation of the Th1 cells. T-bet is the principal transcription factor, as it significantly enhances the production of IFN γ , and plays important role in suppressing the development of Th2 and Th17 (45, 46). T-bet expression was found to be strongly dependent on signal transducer and activator of transcription 1 (STAT1) (47, 48). STAT1, is in turn activated by IFN γ . T-bet suppresses development of Th2 cell by inhibiting the crucial IL4 gene and impairing the function of the Th2 master regulator GATA3 (49, 50). Th17 lineage is inhibited by the interaction of T-bet

with *Rorc* promoter, which encodes $ROR\gamma_t$, the principal transcription factor of Th17 (46). IL12-induced STAT4 is another important transcription factor involved in the Th1 cell differentiation (51). STAT4 induces $IFN\gamma$ production. However, STAT4 and T-bet do not function in a linear way in the differentiation of Th1 cell, with each having their unique signaling pathway. But for complete Th1 cell differentiation, these-lineage specific transcription factors need to operate in coordination with one another (52). In later stages of differentiation, IL12/STAT4 pathway upregulates IL-18R α . IL12 along with IL18 induces $IFN\gamma$ production independent of TCR activation, thus creating a pathway for enhancing Th1 response (Figure 3).

Th1 cells are involved with the elimination of intracellular pathogens and are associated with organ specific autoimmunity (53). They mainly secrete $IFN\gamma$, lymphotoxin α ($Lf\alpha$), and IL2. $IFN\gamma$ is essential for the activation of mononuclear phagocytes, including macrophages, microglial cells, thereby resulting in enhanced phagocytic activity (54). $Lf\alpha$ is a member of the TNF super family. $Lf\alpha$ is associated with autoimmune diseases. The depletion of $Lf\alpha$ has shown to inhibit the development of experimental autoimmune encephalitis (55, 56).

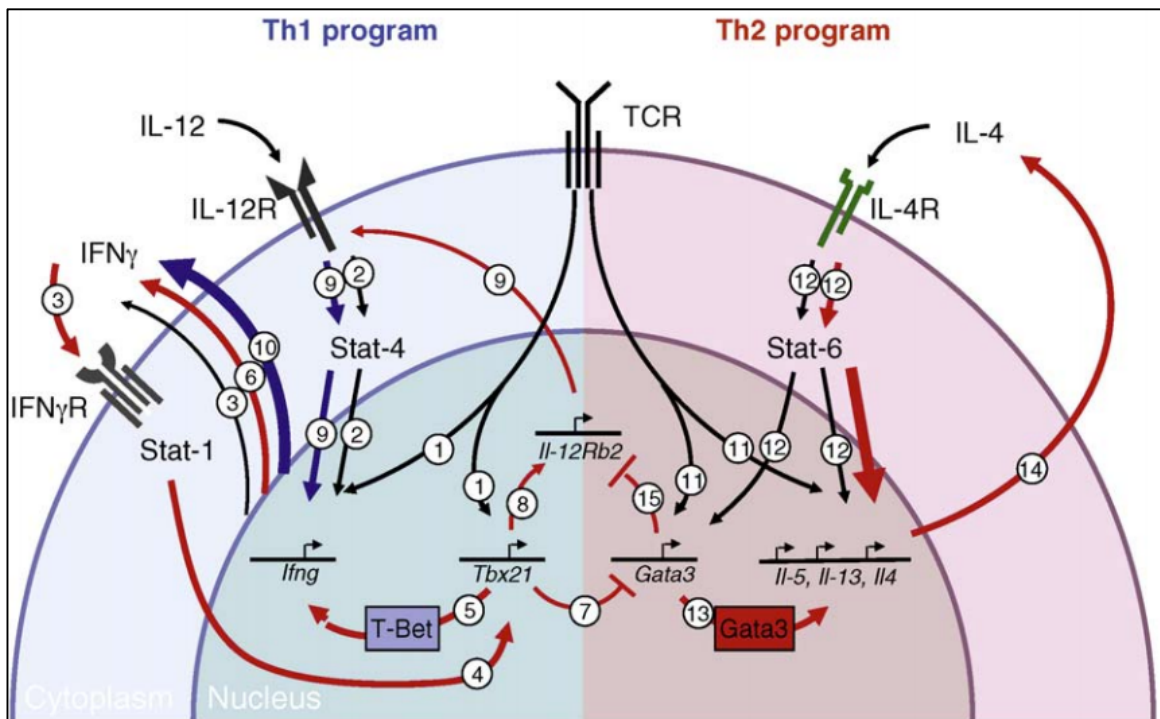


Figure 3. IL12 and IL4 driven T helper differentiation. Th1 induction by IL12: Initial TCR activation induces low-grade expression of the *Ifng* and the *Tbx21* genes (1). Signaling through

the IL12 receptor results in STAT4 mediated promotion of *Ifng* expression (2). Binding of the IFN γ receptor by low initial auto/paracrine produced IFN γ activates STAT1 (3), which strongly promotes expression of the *Tbx21* gene (4). T-bet then enhances the transcriptional competence of the *Ifng* gene (5) leading to increased production of this cytokine (6). In addition, T-bet prevents Th2 differentiation by inhibiting *Gata3* (7). Finally, T-bet promotes expression of the IL12 receptor $\beta 2$ chain (8), resulting in greater IL12 responsiveness (9) and yet further elevated production of IFN γ (10). Th2 induction by IL4: Initial TCR signaling induces low-level expression of the *Il4* and *Gata3* genes (11). IL4 receptor signaling strongly promotes expression of these two genes (12). *Gata3* reorganizes chromatin structure in the Th2 locus, encompassing the *Il4*, *Il5*, and *Il13* genes, enhancing their transcription competence (13). Increased IL4 production further enhances TH2-cell differentiation in a feed forward loop (14). Finally, *Gata3* prevents the Th1 differentiation program by inhibiting expression of the IL12 receptor $\beta 2$ chain (15) and of the *Stat4* gene (not depicted). Primary events are indicated with black arrows, secondary events with red arrows, and tertiary events with blue arrows. (Adapted from Amsen et al., 2009; *Curr Opin Immunol*).

1.3.2.2 Th2 differentiation

IL4 and IL2 are critical for Th2 differentiation. The major transcription factor involved in Th2 lineage differentiation includes the IL4-induced STAT6, which upregulates the expression of the master regulator GATA3 (GATA-binding protein) (57-59). Three distinct mechanisms of GATA3 involvement in Th2 differentiation have been postulated, including enhanced Th2 cytokine production, selective proliferation of Th2 cells through recruitment of Gfi-1, and inhibition of Th1 differentiation presumably by interacting with T-bet (60). Moreover, GATA3 was found to suppress Th1 differentiation by downregulating STAT4 (61). In GATA3 deficient mice, differentiation of naïve cells was diverted towards the Th1 lineage (62). Recent studies showed that GATA3 by itself cannot regulate all the Th2-specific genes, but instead needed the collaboration of STAT6 (63). STAT5 has an important role in the Th2 lineage commitment. It is readily activated by IL2 (64, 65). STAT5 activation is independent of IL4 signaling and does not induce GATA3 expression (66). For full differentiation of Th2 cells, the coordinated activity of STAT5 and GATA3 is required, since GATA3 alone cannot induce the production of IL4 (Figure 3).

Th2 cells mount immune response to extracellular parasites, including helminthes, and play major role in induction and persistence of asthma as well as other allergic diseases (53, 67). The key effector-cytokines include IL4, IL5, IL9, IL13, IL10, IL25, and amphiregulin. IL4 is a major cytokine involved in allergic inflammation. It is involved in IgE switching and secretion by B cells. IL4 also upregulates low-affinity IgE receptor (FcεRII) on B-lymphocytes and mononuclear phagocytes, and also high-affinity IgE receptor (FcεRI) on mast cells and basophils, with subsequent degranulation of the cells and release of several active metabolites, including histamine and serotonin (68). IL5 mainly targets eosinophils (69). IL9 participates actively in the immune pathogenesis of asthma (70).

1.3.2.3 Th9 Cells

Initially characterized as a subset of Th2 cells, ongoing researches tend to classify IL9 secreting-Th9 cells as a distinct subset of CD4⁺ T cells. TGF-β was found to divert the differentiation of Th2 towards the development of Th9 cells. Moreover, TGF-β in combination with IL 4 directly induces the differentiation of Th9 cells (71). IRF4 also plays an important role. IRF4 was found to directly bind to the IL9 promoter (72).

1.3.2.4 Follicular Helper (Tfh) T Cells

Tfh are C-X-C motif receptor-5 (CXCR 5⁺)-expressing cells and are located in follicular areas of lymphoid tissue, where they participate in the development of antigen-specific B-cell immunity (73, 74). IL6 and IL21 are the main cytokines involved in the differentiation process (75, 76). STAT3, activated downstream to cytokine signaling, is an important transcription factor of Tfh. Inducible costimulator (ICOS), member of CD28 family, is also required for Tfh development (77, 78).

1.3.2.5 Regulatory T cells (Tregs) differentiation

CD4⁺CD25⁺ regulatory T (Treg) cells play a pivotal role in the maintenance of immune homeostasis, where the X-linked master transcription factor forkhead box P3 (FOXP3) determines Treg cell development and function (79, 80). Tregs are primarily generated in the thymus (tTreg), but also may be generated extra thymically at peripheral sites (pTreg), or in the presence of signal from APC and TGF β (iTreg). tTregs also called nTregs (81). Naturally occurring thymus-derived CD4⁺CD25⁺ Tregs are a subset of T cells which have immunosuppressive properties and are ~2% of the total peripheral CD4⁺ T cells. Thymic induction of Foxp3 requires TCR signalling of increased strength. Besides TCR, engagement of common γ -chain (γ c) cytokine receptors, foremost IL-2R, is required for both tTregs and pTregs (82, 83). Interestingly, unlike conventional T cells, Tregs do not produce IL-2 due to chromatin inaccessibility of the IL-2 locus (84). Consequently, survival and growth of Tregs is dependent on paracrine IL-2. According to a two-step model of Treg differentiation, TCR engagement first leads to CD25 upregulation making precursor cells receptive to receiving IL-2 signals leading to Stat5 activation (85, 86). Finally, CD28 signaling induced upon binding of its ligands CD80 and CD86 has also a cell-intrinsic role in Foxp3 induction in the thymus (87, 88). Further, activation of TCR and costimulatory signalling recruitment of a number of transcription factors including NFAT, cRel, Creb, and Stat5 to the promoter and several conserved noncoding regulatory elements of the Foxp3 gene and its expression (89-91).

iTregs develop outside of the thymus in a tolerogenic environment. Induction of iTregs requires TGF- β and IL-2 (Figure 4) (92). Smad2 and Smad3, activated through TGF- β signaling pathways, are involved in the iTreg differentiation process by inducing FOXP3 (93, 94). Smad3 can differentially enhance iTreg development by upregulating FOXP3 expression and inhibit Th17 differentiation by blocking ROR γ t (95). STAT5-induced downstream to IL2 signaling is required for the differentiation of iTreg (96, 97). STAT5 was found to enhance FOXP3 expression and subsequently downstream to FOXP3 signaling and promote iTreg development. Additionally, programmed death-1- programmed death-1-ligand-1, ICOS-ICOSL, OX40-OX40L signaling expand Tregs.

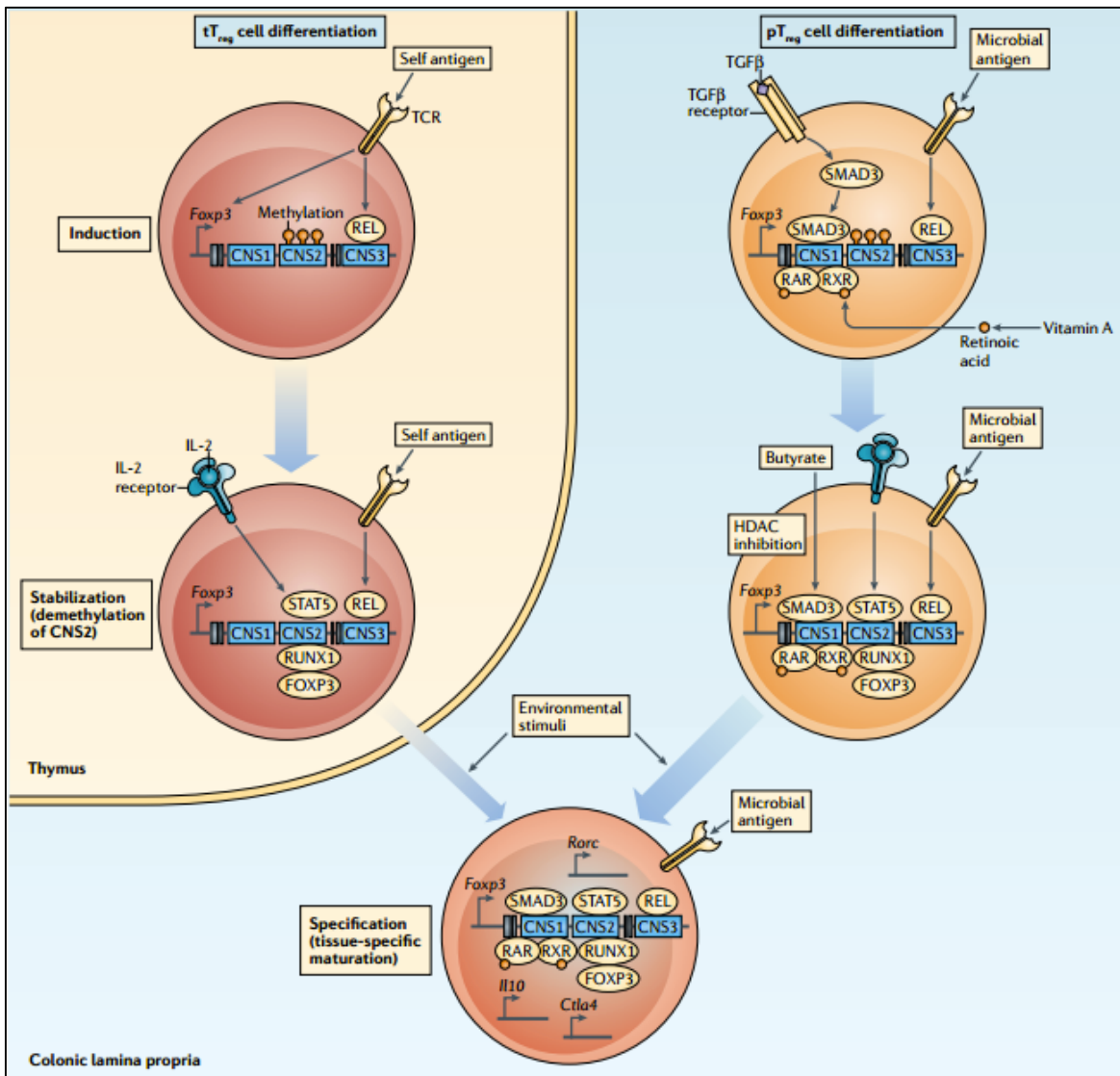


Figure 4. Stepwise induction of tTreg cells and pTreg cells. The thymus-derived regulatory T (tTreg) cell differentiation programme is initiated by strong T cell receptor (TCR) signals induced by self-antigens in the thymus, which results in nuclear entry of REL. In mice, REL, together with several other transcription factors (not depicted) bind to conserved non-coding sequence 3 (CNS3) and the forkhead box P3 (Foxp3) promoter to induce Foxp3 gene expression. These cells become a stable Treg cell lineage when CNS2 is demethylated, and signal transducer and activator of transcription 5 (STAT5) activated by interleukin-2 (IL-2) receptor signalling contributes to this demethylation. Demethylated CNS2 allows binding of FOXP3 and runt-related transcription

factor 1 (RUNX1), which results in stable Foxp3 expression. tTreg cells migrate to the intestines and further undergo functional maturation in response to environmental stimuli. Peripherally derived Treg (pTreg) cell differentiation from uncommitted naive CD4⁺ T cells occurs in the intestines but progresses in a stepwise manner similarly to tTreg cells. The differentiation is initiated by TCR signalling induced by microbial antigens together with transforming growth factor- β (TGF β) and retinoic acid-mediated signals. These signals promote REL binding to CNS3, as well as SMAD3 and retinoic acid receptor (RAR)-retinoid X receptor (RXR) binding to CNS1 of Foxp3. Similarly, to tTreg cells, pTreg cells establish their Treg cell character and lineage commitment by demethylation of CNS2, which is induced by IL-2 and STAT5 signalling. Microbiota-derived butyrate contributes to chromatin remodelling and lineage commitment by its histone deacetylase (HDAC) inhibitory activity. pTreg cells further acquire functionally specialized features such as expression of RAR-related orphan receptor γ t (ROR γ t; encoded by *Rorc*), IL-10 and cytotoxic T lymphocyte antigen 4 (CTLA4) to adapt to the intestinal environment. (Adapted from Tanoue et al., 2016; Nat Rev Immunol).

Tregs have emerged as key players in the development and maintenance of peripheral immune tolerance. In normal conditions, Tregs regulate ongoing immune responses and prevent autoimmunity. Imbalanced function or number of these cells, either enhanced or decreased, might lead to tumour development and autoimmunity, respectively. These cells thus play a major role in autoimmune diseases, transplantation tolerance, infectious diseases, allergic disease and tumour immunity. The cellular targets of Treg-mediated suppressor functions include CD4⁺, CD8⁺ T cells, DC, B cells, macrophages, monocytes, mast cells, NK cells and NKT cells (98-100). Tregs exert their suppressive functions through several mechanisms, some of which require cell-cell contact and other mediated through cytokines (Figure 5) (101). Their main effector cytokines include IL10, TGF- β , and IL35. IL10 is a potent inhibitory cytokine, with the ability to suppress proinflammatory response and thus limits tissue damage by the inflammatory process (102, 103). IL10 and TGF- β potently suppress IgE production, thereby showing their important role in attenuating allergic inflammation (104). Small molecules elaborated by Treg cells have also been implicated in restraining inflammation. Specifically, Foxp3-dependent expression of the ectonucleotidases CD39 and CD73 endows Treg cells with the ability to sequentially convert

highly inflammatory extracellular ATP, which accumulates under hypoxic conditions, into adenosine via an AMP intermediate (105).

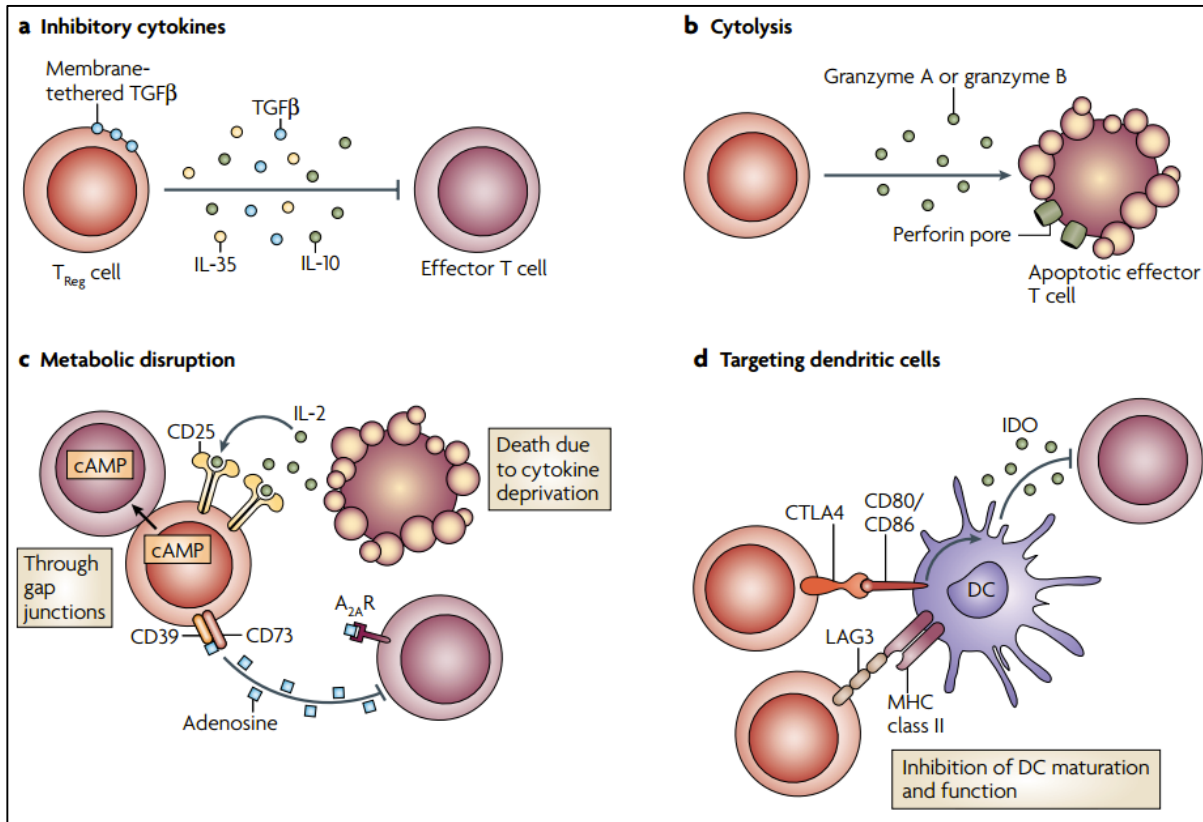


Figure 5. Basic mechanisms used by Treg cells. Depiction of the various regulatory T (Treg)-cell mechanisms centred around four basic modes of action. (a) Inhibitory cytokines include interleukin-10 (IL-10), IL-35 and transforming growth factor- β (TGF β). (b) Cytolysis includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. (c) Metabolic disruption includes high-affinity CD25 (also known as IL-2 receptor α)-dependent cytokine deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A (A_{2A}R)-mediated immunosuppression. (d) Targeting dendritic cells (DCs) 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 made by DCs (Adapted from Vignali et al., 2008; Nat Rev Immunol)

In addition to cytokine-mediated modulation of inflammatory responses, Treg cells display high amounts of CTLA4 and TIGIT (T cell immunoglobulin and ITIM domain), two Ig family members that are able to inhibit the immune stimulatory potential of DCs by reducing expression of the costimulatory molecules CD80 and CD86 and by inducing IL-10 production in DCs, respectively (106-108). Besides immunomodulation or inactivation, perforin- and granzyme-mediated cytotoxicity of immune effector and accessory cells by Treg cells has been considered as a means of suppression (109, 110). In addition to direct killing, an indirect way of elimination of effector T cells is through induction of apoptosis due to a growth factor withdrawal. In this regard, Treg cells are able to deprive effector T cells of IL-2 due to a much higher level of CD25 expression (111).

1.3.2.6 Th17 cells differentiation

Th17 cells are a uniquely pro-inflammatory lineage of effector/memory Th cells first identified by (and named for) production of IL-17A (hereafter referred to as IL-17). Th17 differentiation, survival, and expansion rely on a variety of cytokines and transcription factors that work in concert to drive the induction of increased Th17 numbers (Figure 6).

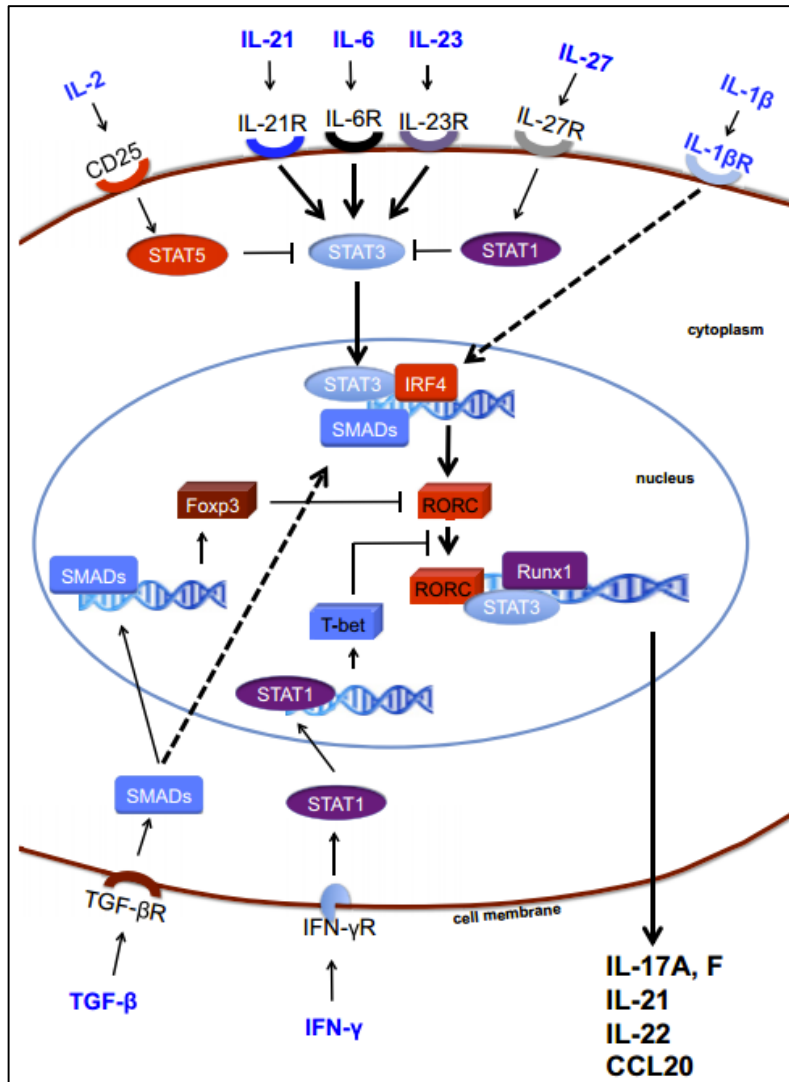


Figure 6. Mechanisms of Th17 cell induction. TGF- β is essential for the generation of both induced regulatory T cells and Th17 cells via the induction of FoxP3 and RORC. However, in the absence of inflammation, FoxP3 represses RORC and promotes iTregs. Signaling via inflammatory cytokines, such as IL-6, IL-21, and IL-23, results in STAT3 phosphorylation, relieves RORC from the suppression of FoxP3, and initiates Th17 programming. STAT3 in combination with IFN regulatory factor 4 (IRF4) further induces RORC expression. The transcription factors STAT3, RORC, and Runx1 bind to the promoter regions of the IL17, IL21, IL22, and CCL20 genes and induce IL-17, IL-21, IL-22, and CCL20. Th17 programming can be antagonized by cytokines, such as IFN- γ , IL-2, and IL-27. IL-2-mediated and IL-27-mediated activation of STAT5 and STAT1 inhibit STAT3, whereas T-bet induced by IFN- γ can block RORC (Adapted from Maddur et al., 2012; Am J Pathol).

The full differentiation of Th17 cells requires three different steps: induction, amplification and stabilization/maintenance. (1) The differentiation is initiated by the combined actions of IL-6 and TGF- β ; (2) the amplification of the Th17 response is driven through the production of IL-21 by Th17 cells; (3) the stabilization/maintenance of the Th17 phenotype is achieved by IL-23. Several studies found that a combination of the immunoregulatory cytokine TGF- β and the proinflammatory and pleiotropic cytokine IL-6 is required to induce IL-17 in naive T cells (112-115). However, TGF- β signaling pathways also play significant role in the development of iTreg. TGF- β promotes Th17 and iTreg development by inducing the expression of the transcription factors ROR γ t and FoxP3, respectively. In fact, both transcription factors are initially up-regulated after naïve CD4⁺ T-cells encounter TGF- β (115). Whether cells are shuttled towards a proinflammatory Th17 phenotype or a regulatory phenotype depends largely on the surrounding cytokine environment. Up-regulated Foxp3 initially binds to ROR γ t thereby inhibiting the development of Th17-cells and favoring Treg development (116-118). IL-6 has been identified as an important mediator driving the development of Th17 cells via activation of STAT3 (112, 114, 115). In the presence of IL-6, STAT3 activation releases FoxP3 inhibition. Eventually, this leads to the differentiation of Th17-cells and up-regulation of the receptor IL-23R.

Also, IL-21, a member of the IL-2 family of cytokines, is produced in overwhelming amounts by Th17 cells and could, in combination with TGF- β , induce Th17-differentiation (Korn et al., 2007; Ivanov et al., 2007). These findings point to a relevant function of IL-21, produced by newly generated Th17 cells, in amplifying the precursor frequency of differentiating Th17 cells (119-121).

IL-23 is another inflammatory cytokine that contributes to Th17 expansion and stability. A stronger connection between IL-23 and Th17 cells was established when investigators showed that IL-23 promotes the production of IL-17 by activated T cells (122) and that IL-23-expanded T cells are able to transfer EAE and CIA (collagen-induced arthritis) (123, 124). IL-23R is clearly not expressed on naive T cells, and after the identification of the factors (IL-6, IL-21, and TGF- β) required for the differentiation of Th17 cells, it became clear that IL-23 was not involved in the initial differentiation of Th17 cells. IL-23p19-deficient mice have limited numbers of Th17 cells and that prolonged culture of Th17 cells in vitro requires the addition of IL-23. IL-23 synergizes with IL-6 to enhance survival, and stabilization of Th17 cells (121, 125). IL-23 can amplify Th17

cells by inducing pro-inflammatory cytokines, such as IL-1 β , TNF- α , and IL-6 in innate immune cells (126).

Besides the master regulator ROR γ t, several other transcription factors need to collaborate for full differentiation of Th17 cells. As such, deficiency of ROR γ t does not lead to complete interruption of Th17 cytokine expression (118). STAT3, activated downstream to IL6, IL21, IL23 signaling plays an important role in the differentiation process. It enhances ROR γ t expression. STAT3 deficiency was found to cause enhanced expression of T-bet and FOXP3, which are involved in the development of opposing cell lineages (127). STAT3 binds to IL-17A and IL-17F promoters (128). ROR α , another member of the ROR family, also participates in the lineage commitment pathway. Together ROR α and ROR γ t synergistically enhance Th17 differentiation, and their absence completely aborted the development of Th17 cells (117). Runx1 also influences Th17 differentiation. Runx1 through the induction of ROR γ t, promotes differentiation. However, Runx1/FOXP3 interaction negatively regulates Th17 development (129). Moreover, T-bet in collaboration with Runx1 leads to the interruption of Runx1-mediated transactivation of Rorc, thereby suppressing Th17 development (130). Aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor, was found to promote Th17 differentiation, presumably through the inhibition of STAT1 and STAT5, which negatively regulate Th17 development. However, its absence did not cause complete abortion of Th17 differentiation, but was associated with inability to produce IL22 (131, 132).

Translation of the Th17 differentiation knowledge from mice to human met with discrepancies with regard to the cytokine cocktail required for the Th17 differentiation. TGF- β was believed to be dispensable for the differentiation of human Th17 cells from naïve T cells. One of the initial studies in human T cells reported that T cell receptor (TCR) cross-linking leads to IL-17 production (133). Other studies showed that Th17 differentiation can be achieved by a combination of IL-1 β and IL-6/IL-23 or IL-21 alone (134, 135). However, the problem with these early studies was that they did not separate effects on memory T cells versus naïve T cells. Also, the endogenous source of TGF- β was not rigorously controlled in those reports. It was only more recently studies done with rigorously sorted naïve T cells and with serum free medium suggested that TGF- β is indispensable for the differentiation of human Th17 cells from naïve T cells (136-138). Importantly, it was shown that Th17 differentiation can be achieved by using TGF- β and IL-21, whereas IL-1 β and IL-6 was important for amplification (138). Mechanistically, TGF- β and

IL-6/IL-21 induces the expression of IL-1R and IL-23R, making the cells receptive to IL-1 β and IL-23 (139, 136, 138).

Th17 is responsible to mount immune response against extracellular bacteria and fungi. They are also involved in the generation of autoimmune diseases (140, 141, 17). The key effector cytokines include IL17A, IL17F, IL21, and IL22. IL17A and IL17F signaling occurs through a common receptor, IL17RA, thereby suggesting similar functions (Figure 7) (142). IL-17A and IL-17F are the key cytokines for recruitment and activation of neutrophils. The effect of IL17 extends beyond T cell-mediated inflammatory response. IL17 leads to the induction of proinflammatory cytokines, including IL6, IL1, TNF α , and also proinflammatory chemokines ensuring the chemotaxis of inflammatory cells to sites of inflammation (17). IL21, along being an amplifying cytokine for TH17 development, has pleiotropic functions, including activating T cells, inducing B cells to differentiate into plasmocytes and memory cells, and activating NK cells (119, 143). IL22 is known to mediate both inflammatory response and exhibits tissue protective properties. In acute liver disease, IL22 was shown to be involved in limiting liver tissue damage (144). IL22 participates actively in mucosal host defense against bacterial pathogens, by inducing antimicrobial peptides and increasing cell proliferation (145).

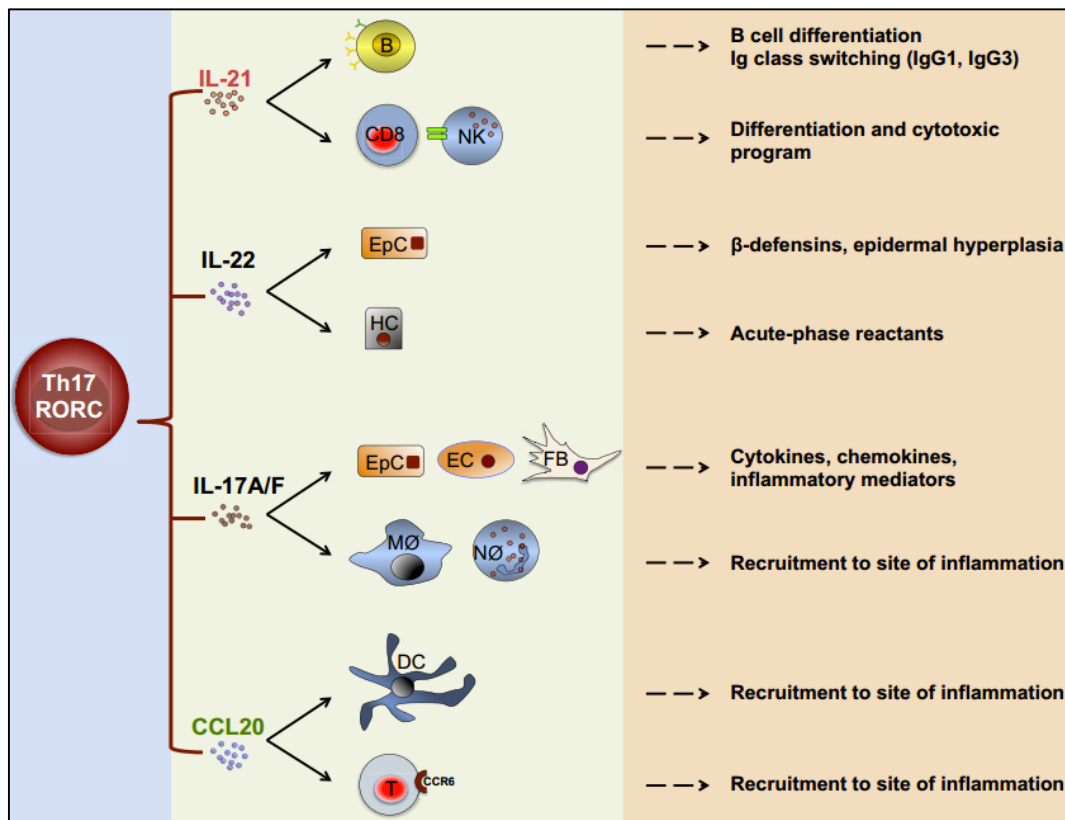


Figure 7. Functions of Th17 cytokines and chemokines. Th17 cells secrete several effector molecules, including IL-21, IL-22, IL-17A/F, and CCL20. These soluble factors act on both immune and nonimmune cells and mediate several functions, such as differentiation of cells; release of antimicrobial molecules, cytokines, and chemokines; and recruitment of cells to sites of inflammation. B, B cell; CD8, cytotoxic T cell; DC, dendritic cell; EC, endothelial cell; EpC, epithelial cell; FB, fibroblast; HC, hepatic cell; MØ, macrophage; NK, natural killer cell; NØ, neutrophil; T, T cell (Adapted from Maddur et al., 2012; Am J Pathol).

1.3.3 Reciprocal relationship between Th17 cells and Tregs:

The balance between factors promoting iTreg and Th17 development is critical in determining homeostatic versus inflammatory condition. Th17 and Treg developmental programs as well as their functions are reciprocally interconnected. TGF- β is a critical factor for both lineages. TGF- β is required for the differentiation and maintenance of Tregs and is also essential for the differentiation of Th17. In steady state (in the absence of inflammatory stimuli) produces TGF- β , which induces the generation of iTregs, which together with nTregs keep activated/effector memory cells in check. IL-6/IL-21, induced during an acute-phase response, inhibits the function of nTregs and prevents the generation of iTregs, but instead induces Th17 cells. The mechanism by which IL-6 and IL-21 act as switch factors relies on the control of the Foxp3/ROR γ t balance (116). TGF- β is required for the expression of both Foxp3 and ROR γ t, although the signaling cascades downstream of the TGF- β receptor might be different for the induction of Foxp3 versus ROR γ t. For example, whereas Smad4 appears to be essential for the induction of Foxp3, it is probably dispensable for the induction of ROR γ t (146). Both ROR γ t and ROR α physically associate with Foxp3 to antagonize each other's functions (116, 146). Both ROR α and ROR γ t have been identified as binding partners of FoxP3 (116, 147, 148), and binding of FoxP3 inhibits the transcriptional activity of ROR α and ROR γ t. Importantly, the repression of ROR γ t-mediated effects by FoxP3 depends on the local concentration of TGF β (146, 116). Th17 cell-promoting cytokines can act through STAT3-dependent pathways to reverse the FoxP3-mediated repression of ROR γ t and ROR α . TGF β signals initiated by IL-6 through STAT3 and sustained by IL-21 and IL-23, override FoxP3-mediated repression of ROR γ t (Figure 8) (116).

Additional evidence for a reciprocal developmental relationship between Foxp3⁺ Tregs and Th17 cells comes from studying the effects of retinoic acid, a vitamin A metabolite, on T cell differentiation. Retinoic acid could drive the generation of Tregs while abrogating the differentiation of Th17 cells, but not of Th1 cells (149). CD103⁺ DCs appear to be a relevant source of retinoic acid and, together with TGF- β , are efficient in inducing Foxp3⁺ Tregs (150, 151). Mechanistically, retinoic acid enhances the TGF- β -driven phosphorylation of Smad3 but reduces the TGF- β -induced upregulation of the IL-6R α subunit on T cells (152).

The reciprocal relationship between Tregs and Th17 cells is further strengthened by IL-2. IL-2 signaling is critically involved in Treg as well as Th17 cell differentiation. IL-2 is consumed preferentially by Treg since they express high affinity IL-2 receptors (101) (54). IL-2 also acts to stabilize Foxp3 induced by TGF- β (153, 154). TGF- β and IL-2, along with other proinflammatory cytokines such as IL-1 β , IL-6, IL-21, or IL-23, facilitate Th17 differentiation (155).

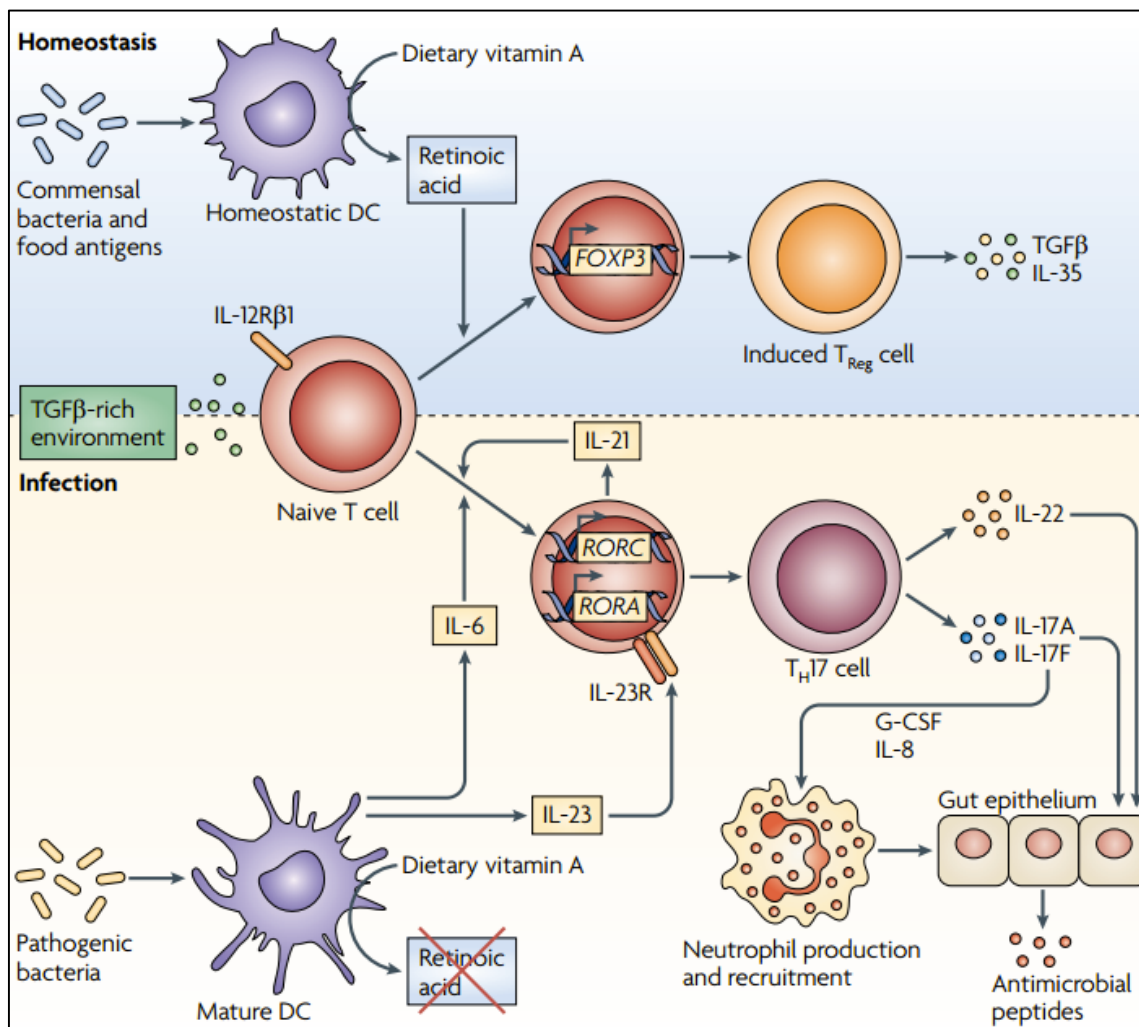


Figure 8. A common requirement for transforming growth factor- β in the induced regulatory T cell and T helper 17 cell lineages. Dendritic cells (DCs) that are conditioned in environments rich in transforming growth factor- β (TGF- β), such as the intestines, present microorganism-derived antigens to induce the differentiation of naive CD4⁺ T cells to either induced regulatory T (Treg) cells or T helper 17 (Th17) cells, depending on the dominance of retinoic acid or interleukin-6 (IL-6), respectively. During homeostasis, DCs loaded with antigens from commensal bacteria or food produce retinoic acid derived from dietary vitamin A, which favours the upregulation of forkhead box P3 (FOXP3) expression and the differentiation of induced Treg cells. Under conditions of microbial breach of the intestinal epithelial cell barrier, pro-inflammatory stimuli activate DCs, which mature and produce IL-6 and stop retinoic acid production, thereby inducing Th17 cell differentiation. IL-6 and IL-21 (induced by IL-6) suppress Foxp3 expression and upregulate expression of the retinoic acid-related orphan receptor genes RORC and RORA (which encode ROR γ t and ROR α , respectively), leading to expression of the inducible component of the IL-23 receptor (IL-23r) and further Th17 cell development. IL-17A and IL-17F produced by Th17 cells increase production of neutrophils in the bone marrow and their recruitment to the gut through intermediary cytokines (such as granulocyte colony-stimulating factor (G-CSF) and IL-8, respectively) and can also act to induce epithelial cell production of antimicrobial peptides. IL-22 produced by Th17 cells promotes enhanced epithelial barrier function and the production of antimicrobial peptides. collectively, therefore, the Th17 cell response acts both to clear invasive microorganisms and to restore barrier function. (Adapted from Weaver and Hatton, 2009; Nat Rev Immunol).

1.3.4 Th17 cells in the pathogenesis of autoimmune and inflammatory diseases:

The role of Th17 cells in autoimmunity and inflammation was demonstrated first in mice that were deficient for the p19 chain of the IL-23, in which the IL-17-producing T cells were significantly lower than in wild-type mice, highlighting the importance of the IL-23/Th17 axis in the pathogenicity of these autoimmune and autoinflammatory diseases (156). Since then, the study of the pathogenic role of Th17 subset cells has focused on autoimmune inflammatory diseases, such as multiple sclerosis (MS), rheumatoid arthritis (RA), psoriasis and others (157, 158).

Th17 cells were found to be the central effector lineage involved in the pathogenicity of murine EAE, an important model of MS (159). IL-17 expression is correlated with disease activity. Human blood-brain barrier endothelial cells were found to express receptors for IL-17 and IL-22, thus making it possible for IL-17 and IL-22 to disrupt blood brain barrier junctions (160). Further studies continued to correlate the number of Th17 cells and amount of IL-17 expression found in MS lesions with active disease (161, 162). IL-23 that expands IL-17-producing cells is critical for the induction of EAE. It has been shown that myelin antigen-specific Th17 cells directly interact with neuronal cells in demyelinating lesions (163, 164). Two independent studies have identified the critical nonredundant role of Th17-produced GM-CSF in the pathogenesis of EAE. ROR γ t drives the production of GM-CSF in Th17 cells under the influence of the IL-1 and IL-23 pathways. GM-CSF acts on CD103⁺ dermal DCs to stimulate the secretion of IL-23 and the activation of encephalitogenic T cells, creating a positive feedback loop during inflammation (165-167). In consensus, absence of GM-CSF confers resistance to EAE in mice, even in the presence of IL-17 and/or IFN- γ (165).

Psoriasis is a chronic, relapsing, and immune mediated inflammatory skin disease (168). Psoriasis is defined as a Th1/Th17/Th22-based inflammatory disease (169). IL-23, overproduced by dermal DCs and keratinocytes, stimulates Th17 cells within the dermis to elevate the amounts of IL-17, TNF- α , IL-22, and CCL20. These soluble mediators of Th17 cells might further enhance inflammation by their distinct actions. For example, CCL20 can attract CCR6⁺ cells to lesions, and IL-17 can induce IL-6 and IL-8 in human skin keratinocytes (170). Further, an experimental model of psoriasis-like skin inflammation indicates that IL-22 plays a central role in Th17 cell-mediated skin pathology (171).

RA is a systemic autoimmune disease characterized by progressively destructive joint inflammation, destruction of articular cartilage, and bone and synovial hyperplasia. In 2003 Murphy et al. saw that IL-23p19^{-/-} mice were protected from collagen-induced arthritis (CIA), a mouse model for RA (124). Blockage of IL-23 expression protected mice from joint and bone destruction, and an anti-IL-17 antibody has been shown to inhibit osteoclast formation in human rheumatoid arthritis samples (124, 172). PGE₂, a known positive regulator of Th17 differentiation, was shown capable of enhancing CIA severity through enhanced DC-derived IL-6 production,

thereby shifting the IL-23/IL-12 more towards IL-23 and IL-17 (173). Patients with RA exhibit high levels of IL-17, IL-17R, IL-1 β , and IL-6 among others in synovial fluid samples (172, 174, 175). IL-17 has also been found in large quantities in the synovial biopsies of RA patients (175).

Another disease exhibiting strong links to the Th17 lineage is systemic lupus erythematosus (SLE). SLE is a complex autoimmune disorder characterized by autoantibody and immune complex formation and has been strongly correlated to a dysregulated Th17/Treg balance (176, 177). IL-17 and Th17 cells have been detected at higher levels in several murine models of lupus compared to those found in healthy, wild type mice (178). It has been proposed in SLE that DCs commonly come into contact with apoptotic debris and therefore develop into a mature phenotype and produce higher amounts of IL-6; in turn the higher level of IL-6 production results in the differentiation of naïve CD4⁺ T cells into Th17 cells, when in fact they may have been converted into Tregs under normal conditions (179). SLE patients have elevated levels of inflammatory cytokines including IL-23, IL-17, and IL-6 and higher frequencies of lymph nodal Th17 cells (180, 181).

1.3.5 Therapeutic Approaches to target the Th17/Treg Axis

Although the role of Th17 lymphocytes in the pathogenesis of human diseases is quite different and multifaceted, both *in vitro* and *in vivo* studies have clearly demonstrated the pivotal role of IL-17 in the pathogenesis of various diseases. Various drugs with the potential to modify Treg and in particular Th17-responses have already proven efficacy and received approval for the treatment of certain autoimmune diseases while others are currently being tested in clinical trials. Generally, therapeutic approaches are multidirectional and comprise direct targeting of Th17-related cytokines, cytokine receptors, intracellular signaling pathways, as well as inhibiting Th17- and enhancing Treg-specific transcription factors (Figure 9).

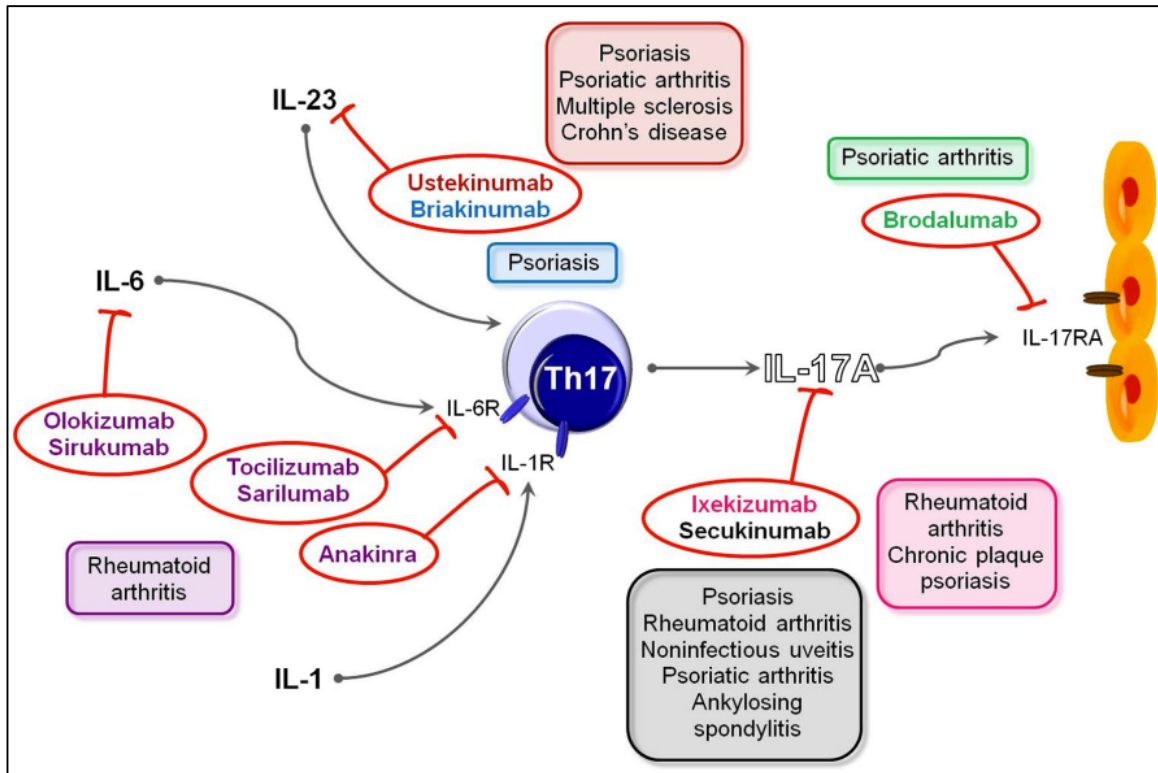


Figure 9. Drugs targeting Th17 pathways and their use in human diseases (Adapted from Tabarkiewicz et al., 2015; Arch Immunol Ther Exp (Warsz)).

1.3.5.1 Targeting Th17-Related Cytokines and Receptors

IL-17 is the most prominent effector cytokine of Th17-cells and is implicated in a variety of inflammatory diseases (182). Thus, neutralization of IL-17 can abrogate IL-17-mediated pathogenic effects in autoimmune settings. Several IL-17 neutralizing monoclonal antibodies have been developed in recent years including secukinumab (AIN457), ixekizumab (LY2439821) and brodalumab (AMG827) (183-185) for psoriasis, psoriatic arthritis (PsA) and ankylosing spondylitis (AS). On the other hand, in RA patients, IL-17 neutralization yielded inconsistent results (186-189). IL-17 neutralization in Crohn disease (CD) were terminated due to high rates of serious adverse events and fungal infections (190). It is most likely because of the protective role of IL-17 at mucosal surfaces where it confers host defense and contributes to the maintenance of immune homeostasis (191).

Another cytokine, IL-23, required for expansion and maintenance of Th17 cells. Its requirement has been recently illustrated for conversion of non-pathogenic Th17 cells with a protective role at mucosal barriers into highly pathogenic cells that promote inflammation (192, 193). IL-23 is a heterodimeric cytokine composed of two subunits, p19 and p40. The latter subunit is shared by the Th1-inducing cytokine IL-12 (194). Genetic abrogation of the p19 subunit of IL-23 in mice resulted in resistance to the development of EAE (159). Similarly, resistance against joint inflammation was observed in p19-lacking mice with collagen induced arthritis (CIA), a mouse model of RA (124). In this study, IL-23-deficiency was linked to the absence of IL-17-producing CD4⁺ T-cells (124). Therefore, blocking IL-23 and its cognate receptor IL-23R is another promising therapeutic strategy for the inhibition of Th17-responses in autoimmunity. Several antibodies for the application in humans have been developed so far, including the anti-IL-12/23p40 antibodies ustekinumab and briakinumab. Due to its favorable efficacy, ustekinumab has already been approved for the treatment of psoriasis (195, 196), PsA (197-200) and CD. However, briakinumab was associated with an increased risk of major adverse cardiovascular events (201), and its application for marketing approval was therefore withdrawn in 2011. Several anti-IL-23p19 antibodies (tildrakizumab, guselkumab, BI-655066, AMG 139, LY3074828) are in clinical development. These antibodies enable specific targeting of IL-23 without the cross-reactive effect on IL-12 (202).

Tocilizumab and sarilumab are monoclonal antibodies targeting the human IL-6R. In RA, clinical studies have demonstrated a significant reduction in Th17-cells in tocilizumab-treated patients compared to pre-treatment levels, while at the same time an increase in Tregs was seen (203, 204). Similarly, Tada et al. (205) reported the Foxp3/ROR γ t ratio rised upon tocilizumab treatment in RA patients. Tocilizumab is meanwhile not only approved for the treatment of RA, but also for polyarticular and systemic juvenile idiopathic arthritis (JIA) due to its favorable results in numerous clinical trials (206). In addition, sarilumab was demonstrated to significantly improve various disease-aspects in RA patients (207, 208) but its potential approval for RA is still pending.

1.3.5.2 Targeting Transcription Factors

The ROR family of nuclear hormone receptors generally comprises three different members: ROR α , ROR β and ROR γ . After ROR γ t was recognized as master transcription factor of the Th17-lineage, there has been a major research interest in potential inhibitors of the ROR γ t/Th17 axis. Digoxin is reported to inhibit the transcriptional activity of ROR γ t (209). Digoxin suppressed differentiation of murine Th17-cells without affecting other T-cell lineages (210) and inhibited IL-17 production of T-cells from EAE mice (209). Consistently, digoxin was effective in attenuating EAE (210) as well as in reducing the incidence of arthritis and joint inflammation in CIA mice, where it significantly reduced Th17-cells and increased Treg numbers (211). Another study in the CIA mouse model identified the herbal medicine compound ursolic acid (UA) to effectively inhibit ROR γ t function and to decrease IL-17 expression in developing and differentiated Th17-cells (212). TMP778 and TMP920 were identified as inverse agonists of ROR γ t (213). Both molecules were shown to potently suppress Th17-cell generation and IL-17 secretion by differentiated Th17-cells *in vitro* (213). Two independent murine studies reported TMP778 administration to reduce imiquimod-induced cutaneous inflammation (a murine model of psoriasis) and the severity of EAE (213, 214). Lately, Takaishi et al. (215) described attenuation of psoriasis-like lesions in two independent psoriasis mouse models after oral administration of the novel ROR γ t antagonist A213. They suggested that this effect was based on neutralization of IL-17-producing cells (215), however, A213 also led to a systemic attenuation of Tregs.

Differentiation of Th17-cells is triggered by an intracellular signaling cascade involving IL-6-dependent phosphorylation and activation of STAT3 (146). IL-6-dependent STAT3 activation downregulates TGF- β -induced Foxp3-expression and promotes differentiation of naïve CD4⁺ T-cells into Th17-cells with simultaneous suppression of Treg development (146). Therapeutic approaches aimed at the modulation of IL-6 signaling are thus not limited to direct targeting of IL-6 and its receptor IL-6R, but also comprise inhibition of the JAK/STAT pathway. Tofacitinib is a targeted synthetic small molecule that potently inhibits IL-6 induced phosphorylation of STAT1 and STAT3 in human whole blood cellular studies (216) and in synovial tissue extracts from RA patients (217). Moreover, tofacitinib potently suppressed the generation of pathogenic Th17-cells with an IL-23/STAT3 signature by abrogating IL-21, IL-22 but also IL-23R expression (218). Tofacitinib is approved for RA (219), and it is under

investigation as a possible treatment option for other inflammatory disorders, including psoriasis (220, 221), PsA (222) and inflammatory bowel disease (IBD) (223). The STAT3 inhibitor STA-21 is another compound that interferes with STAT3 signaling leading to an improvement of the clinical course of arthritis in IL-1Ra–KO mice. STA-21 enhanced Treg function and numbers while acting suppressive on Th17-cells and osteoclast formation (224).

Several other biological and synthetic compounds were shown in animal models to skew the Th17/Treg balance towards the regulatory direction by blocking STAT3 phosphorylation. These compounds include the green tea-derived component epigallocatechin-3-gallate (EGCG) (225), the herbal compound celastrol (226), grape seed proanthocyanidin extract (GSPE) (227), the gastrointestinal protective drug rebamipide (228), halofuginone (229) and the antidiabetic drug metformin (230). Among these, GSPE and metformin were also reported to increase the phosphorylation of STAT5 thus additionally enhancing Treg development (227, 230).

Targeting the bona fide Treg marker Foxp3 is a reasonable choice to influence the Th17/Treg equilibrium and thus, numerous studies investigated the effect of Foxp3 modulation. Inhibition of ROR γ t by Foxp3, however, is abolished under the influence of TGF- β plus IL-6, leading to the generation of Th17-cells (116). Consequently, tocilizumab treatment was shown to increase the Foxp3/ROR γ t ratio in patients with RA (205). A similar effect was observed in studies evaluating the effect of TNF- α blockade on Foxp3. Etanercept treatment induced transcriptional levels of Foxp3, STAT3 and STAT4 mRNA in responding psoriasis patients (231) and increased the Foxp3/ROR γ t ratio in RA patients (232). More recently, adalimumab was reported to expand functional Foxp3⁺ Tregs via binding monocyte membrane TNF, and it unexpectedly enhanced the expression of TNF-RII on Treg-cells (233).

2. Autophagy

2.1 Overview

The word ‘*autophagy*’ is derived from the Greek roots “auto” (self) and “phagy” (eating). Autophagy is an evolutionarily conserved, fundamental self-cannibalism process which acts in favour of cell survival under numerous physiological and metabolic stresses, originated by accumulation of damaged organelles, toxic protein aggregates and pathogenic invasion through their capture and auto digestion. Autophagy is a degradation pathway in which cytoplasmic content

is engulfed and degraded by the lysosome. Autophagy has long been recognized as a response to nutrient deprivation to provide energy and anabolic building blocks to maintain energy homeostasis. Christian de Duve, who was awarded the Nobel Prize for his work on lysosomes, first used the term *autophagy* in 1963. Until the early 1990s, autophagy was predominantly studied using morphological and biochemical methods. In 1992, Ohsumi's group demonstrated that autophagy occurred in *Saccharomyces cerevisiae* (baker's yeast). The discovery of yeast autophagy genes was followed by the identification of mammalian orthologs with similar roles and provided a series of reagents for characterizing the molecular machinery of the system as well as its roles in physiology and disease (234). In 2016, Yoshinori Ohsumi awarded Noble Prize in Physiology or Medicine for his discoveries on mechanisms of autophagy. Pathophysiological role of autophagy ranges from neurodegeneration, cancer, aging, atherosclerosis and metabolic disorders encompassing diabetes, obesity and others. to various immunological deficits (235, 236). Thus, autophagy provides new pools of fatty acids, amino acids and nucleosides for multiple anabolic purposes, driving an incessant flow of biomolecules in the cells in a regeneration-degradation cycle (237).

2.2 Types of autophagy

Three main type of autophagy have been described (238).

- a) Macroautophagy
- b) Microautophagy
- c) Chaperon-mediated autophagy (CMA)

2.2.1 Macroautophagy: Of these types of autophagy, macroautophagy has the largest known impact on human health and disease (239) and is often termed as autophagy. In macroautophagy, a double-membrane organelle called the 'autophagosome' has a central role. Macroautophagy is a bulk degradation pathway not only for complete portion of cytosol, soluble proteins or aggregations, but also for damaged organelles sequestered in a de novo synthesized phagophore (autophagic membrane), which upon closure forms a double membrane vesicle called autophagosome. The sequestered products are degraded with the help of lysosomal hydrolases released after fusion of these vesicles to lysosomes (240).

2.2.2 Microautophagy: Microautophagy is mostly studied in yeast and its understanding in eukaryotic cells is still very less. Microautophagy is a constitutive mechanism that bypasses the requirement of cytosol engulfment by autophagosomes, instead, lysosomes directly entrap cytoplasm, causing pinocytosis of small cytosol content (241). It is responsible for selective removal of organelles and constant turnover of cellular components. In this pathway, the vacuole invaginates and internalizes cytosolic components, resulting in the budding of the vesicle into the vacuole lumen and its subsequent degradation (242). This form of lysosomal proteolysis is unresponsive to nutrient deprivation, glucagon, amino acids or stress exposure stimuli unlike macroautophagy and remains active in resting state. However, these stimuli result in inducing morphological changes in pinocytic bodies without any clear outcome of biomolecules degradation (243). It involves the direct engulfment of organelles, such as peroxisome and the nucleus (242). Because of its remarkable selectiveness, autophagic degradation of different organelles gives different kinds of microautophagy, for example, pexophagy for peroxisomes' degradation, mitophagy for mitochondria degradation, etc. (244, 245).

2.2.3 Chaperon-mediated autophagy (CMA): CMA is characterized by direct and selective translocation of substrate proteins through lysosomes into the lumen without prior formation of intermediate vesicles. This form of autophagy is responsible for selective degradation of almost 30% of total soluble proteins harbouring a unique pentapeptide KFERQ in their amino acid motifs (246). This motif is recognized by a cytosolic chaperon, heat shock protein of 70 kDa (hsc70), and its co-chaperons (Bag1, Hip, Hop and Hsp40), resulting in selective targeting of proteins to lysosomes for degradation (247). The substrate protein–chaperone complex docks at the lysosomal membrane through interaction with the cytosolic tail of a single-span membrane protein, the lysosome-associated membrane protein type 2A (LAMP-2A), which acts as a receptor for this autophagic pathway (248). Multimerization of LAMP2A is required for substrate translocation, though involvement of some unfoldases in lysosomal membrane can't be denied (249). The Hsc73 is a critical requirement here for complete translocation but, it is still unclear how this “pulling motor” protein obtains ATP to drive translocation in the lysosomal lumen (249, 250). Advantage of this system in studying important steps of selective autophagy is that one can isolate lysosomes from culture cells and reconstitute it *in vitro* (251).

Different types of autophagy and sequential steps from autophagosome formation to degradation are represented in Figure.10

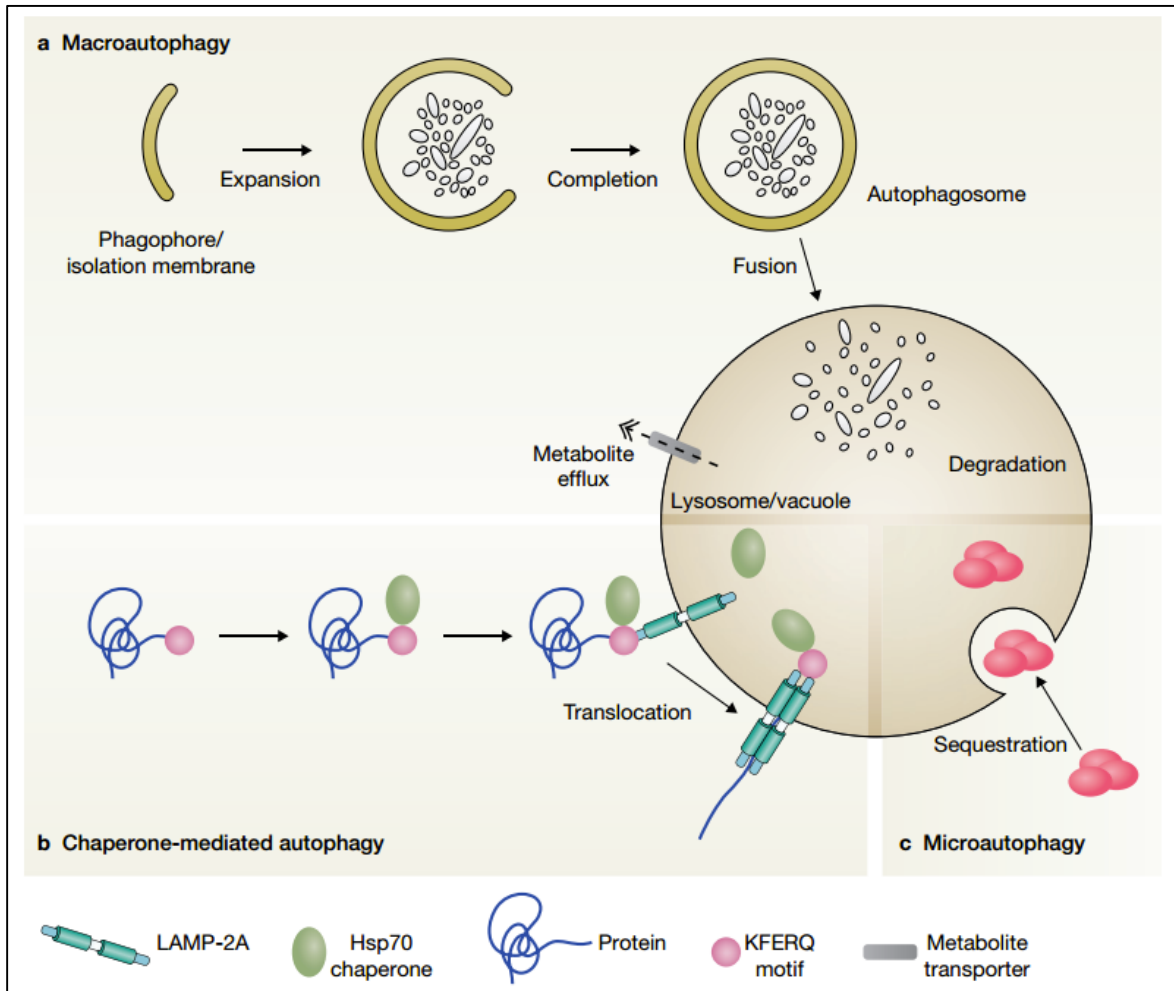


Figure 10: Types of autophagy. (a) Macroautophagy is characterized by the sequestration of structures targeted for destruction into double membrane vesicles called autophagosomes. Complete autophagosomes first fuse with endosomes before finally exposing their content to the hydrolytic interior of lysosomes. The resulting metabolites are transported into the cytoplasm and used either for the synthesis of new macromolecules or as a source of energy. (b) During chaperone-mediated autophagy, proteins carrying the pentapeptide KFERQ-like sequence are recognized by the Hsc70 chaperone, which then associates with the integral lysosome membrane protein LAMP-2A, triggering its oligomerization. This event leads the translocation of the bound protein into the lysosome interior through a process that requires Hsc70. (c) Microautophagy

entails the recruitment of targeted components in proximity with the lysosomal membrane, which subsequently invaginates and pinches off (Adapted from Boya et al., Nat Cell Biol. 2013).

2.3 Molecular machinery of autophagy

Until the early 1990s, autophagy was predominantly studied using morphological as well as biochemical methods mostly in *Saccharomyces cerevisiae*. Genetic screens in *Saccharomyces cerevisiae* have led to the discovery of numerous core autophagy proteins, which are essentially defined as products of genes whose transcription is necessary to observe accumulation of autophagic bodies in yeast vacuoles under starvation conditions (252, 253). The identification of the ATG genes in genetically tractable yeast led to molecular analysis of autophagy in higher eukaryotes, starting with identification of ATG5 and ATG12 (254) and demonstration of the conserved ATG12 - ATG5 conjugation system. The most critical finding in higher eukaryotes was the identification of the mammalian ATG8 homolog, MAP1LC3 (also known as LC3) (255), followed by the development of LC3-based assays for monitoring autophagy in mammals and other higher eukaryotes. However, it is critical to follow autophagy flux throughout the autophagy pathway along with lipidation of LC3 to get better insight of autophagy machinery.

2.3.1 The core autophagy proteins

Insights into the molecular control of autophagy can only be possible through understanding the functions of core autophagy proteins. Identification of various mammalian homologs of ATG proteins have revolutionized the field to expand at a surprisingly fast pace and revealed the significance of autophagy in human health and disease. One subset, including ATG1 to ATG10, ATG12 to ATG14, and ATG16 to ATG18, and the resultant core machinery for autophagosome formation among these ATG proteins, is required for efficient autophagosome formation, (256, 257). Interestingly, the higher complexity of autophagy regulation in mammals might be reflected by the existence of multiple mammalian homologs for single Atg proteins in yeast (Table 1). ATG proteins are often listed in six co-ordinating functional groups to perform key processes in autophagosome formation (258).

Yeast	Mammals	Known or anticipated function	Useful protein mutations (not a comprehensive list)
Atg1	ULK1/2	ULK complex; initiation of autophagy	
Atg2	Atg2A/B	ATG18-interacting protein(s)	
Atg3	Atg3	E2-like enzyme in Atg8 conjugation; regulation of mitochondrial homeostasis	K243R (no Atg12 conjugation)
Atg4	Atg4A-D	Cysteine protease; activation as well as delipidation of ATG8; redox-regulated	C74A in Atg4B (dominant negative)
Atg5	Atg5	Autophagosome formation/elongation; induction of apoptosis (N-terminal portion)	K130R (no Atg12 conjugation) Deletion of amino acids 191–196 (no cleavage by calpain 1/2)
Atg6	Beclin 1	Essential for autophagosome formation; target for viral proteins to interfere with autophagy function/efficacy	
Atg7	Atg7	E1-like enzyme in Atg8 and Atg12 conjugation	
Atg8	LC3A/B/C; GABARAP; GABARAPL1–3	Ubiquitin-like modifier(s); conjugation to autophagosomal membrane; closure and determination of size of autophagosome; autophagy-independent functions	G→A mutation of glycine exposed after cleavage by Atg4 (no conjugation to autophagosomal membrane)
Atg9	Atg9A/B	Transmembrane protein; possibly “membrane shuttle”	
Atg10	Atg10	E2-like enzyme in Atg12 conjugation	
Atg12	Atg12	Ubiquitin-like modifier	G140A (no conjugation to substrates, e.g., Atg5 and Atg3); F108A (no binding to Atg3)
Atg13	Atg13	ULK complex; initiation of autophagy	
Atg14	Atg14L (Barkor)	ER targeting of Vps34 complex	
Atg16	Atg16L1/L2	Atg16L1: Atg12-Atg5/Atg16L1 complex, essential for autophagosome formation Atg16L2: uncertain, no role in basal autophagy despite Atg12-Atg5/Atg16L2 complex formation	Atg16L1: deletion of the coiled-coil domain (no homodimerization) or amino acids 1–78 (no binding to Atg12-Atg5) abolishes autophagosome formation
Atg17	FIP200	ULK complex; initiation of autophagy	
Atg18	WIPI1–4	PI3P binding, possible function(s) in omegasome formation and/or later stages of autophagy	

Table 1: Mammalian homologs of yeast ATG proteins.

I. ULK complex: The ULK (UNC-51-like kinase) complex is the functional equivalent of the Atg1 complex in yeast. The ULK complex consists of Atg13, FIP200, Atg101, and one of the two Atg1 homologs, ULK1 or ULK2. ATG13 is conserved in both yeast and mammals. FIP200 (the focal adhesion kinase family-interacting protein of 200 kD) is a putative functional homolog of ATG17 and ATG13 and facilitates its interaction with ULK1/2 (259). The ULK complex is negatively regulated by mTORC1 (mammalian target of rapamycin complex 1), which phosphorylates ULK1/2 and Atg13 (260, 261). Also, acetylation of ULK1/2 plays important role in autophagy regulation (262). ATG101, is another mammalian protein that can form complex with ULK1 and ATG13 and is found to be essential for autophagy.

II. Vps34 complexes: Phosphatidylinositol 3-phosphate (PI3P) and PI3P-binding proteins are important factors in autophagy. The majority of cellular PI3P is generated by class III PI3 kinases (PI3K), and Vps34 appears to be the only class III PI3K in eukaryotic cells. The Vps34 complex consists of class III PI3K or hVps34, its regulatory protein kinase p150 or hVps15, Beclin 1 (Atg6 in yeast), and the recently discovered mammalian homolog of Atg14, known as Atg14L or Barkor (Beclin 1-associated ATG key regulator) (263, 264). Additional complex of Vps38 homolog, ultraviolet irradiation resistance-associated gene (UVRAG), mediates endocytosis and regulates autophagy (263, 265). Mammals also possess a third class III PtdIns3K complex comprising of KIAA0226/Rubicon.

III. ATG9 and its cycling system: Atg9 and its mammalian ortholog (mAtg9 or Atg9L1) are the only transmembrane Atg proteins. Under normal conditions, Atg9L1 traffics between the trans-Golgi network (TGN) and late endosomes (266). Atg9 is intimately linked to membranous structures and involved in every step of autophagosome biogenesis from membrane generation to expansion.

IV. PtdIns 3-phosphate [PtdIns(3)P] binding proteins: WD repeat domain phosphoinositideinteracting (WIPI) proteins WIPI1 and WIPI2 are orthologs of the yeast PI3P-binding protein Atg18 (267, 268). In yeast, PtdIns3P recruits the Atg18:Atg2 complex to autophagic membrane through an Atg18-PtdIns3P interaction (269). DFCP1 (double FYVE domain-containing protein) and Alfy (autophagy-linked FYVE protein) bind PI3P with high affinity via their FYVE domains (270, 271). DFCP1 translocates to the site of autophagosome formation in a PI3P-dependent manner and is essential for the generation of omegasomes on the ER (268, 270)

V. Atg12 conjugation system: The Atg12 conjugation system is one of two ubiquitin-like (UBL) systems with essential functions in autophagosome formation (272). The conjugation of Atg12 to Atg5 normally occurs soon after the individual proteins have been synthesized. Atg12 is activated by the E1-like enzyme Atg7 and then conjugated to Atg5 by the E2-like enzyme Atg10 (273, 274). The Atg5-Atg12 conjugate mainly persist within an approximately 240 kDa complex generated by binding to Atg16L1 and homodimerization of Atg16L1 (275).

VI. Atg8 conjugation system: The second UBL system plays essential roles in autophagosome formation as well as in recognition of specific cargoes. Multiple mammalian Atg8 homologs (Table 1) have been identified and can be divided into two subfamilies: LC3s and

GABARAPs (γ -aminobutyric acid receptor-associated proteins). LC3 is first cleaved by a protease, Atg4B, leaving a glycine residue at the C terminus. In response to the induction of autophagy, the C terminus of LC3 is conjugated to the polar head of phosphatidylethanolamine (PE), a component of the phospholipid bilayer, in a reaction that requires Atg7 (E1-like), Atg3 (E2-like) and the Atg12-Atg5:Atg16L complex (E3-like). The lipidated form of LC3 (LC3 II) is associated with the outer and the inner membranes of the autophagosome, whereas unconjugated LC3 (LC3 I) is cytosolic, and is the major form of LC3 present under normal conditions (255, 277, 278). A unique property of Atg8s among all Atg proteins examined thus far is that Atg8s remain associated with autophagosomes even after fusion with lysosomes. Because Atg8s are also incorporated into the inner membrane, Atg8s are degraded along with the inner membrane upon formation of the autolysosome (255, 277).

2.4 The process of autophagy

The autophagy process is divided into different steps, such as induction, cargo recognition and selection, vesicle formation, autophagosome-vacuole fusion, and breakdown of cargo followed by release of degradation products back into the cytosol. Different sets of Atg proteins are involved in these steps and form core autophagic machinery.

2.4.1 Induction: Basal-level autophagy is very low under normal conditions. Therefore, an efficient mechanism to induce autophagy is crucial for organisms to adapt to stress and extracellular cues. The most typical trigger of autophagy is nutrient deprivation and lack of any type of essential nutrients can induce autophagy. Nitrogen starvation is the most potent stimulus (279, 280), but withdrawal of other essential factors such as carbon, auxotrophic amino acids and nucleic acids, or even sulphate can induce autophagy in yeast, however, less efficiently (281). A central inhibitor of autophagy is the serine/threonine protein kinase mTOR. The Unc-51-like kinase 1 (ULK1) and -2 (ULK2), and FIP200, which forms a complex with ULKs and mammalian Atg13, localizes to the phagophore upon starvation (259, 260). It is suggested that mammalian Atg13 and FIP200 are phosphorylated by ULKs (260), and ULKs also undergo autophosphorylation, which is conducive to a conformational change and autophagy induction (282). mTOR interacts with, phosphorylates, and inactivates ULKs and Atg13 under nutrient-rich conditions. Inactivation of mTORC1 by starvation or rapamycin, results in partial de-

phosphorylation of ULK1/2 and Atg13, thereby activating the ULK complex. Activated ULK1/2 then phosphorylates FIP200 and Atg13, which are essential for autophagy activity (283, 260). Atg101, a newly identified protein component in the ULKs-Atg13-FIP200 complex, binds and stabilizes Atg13, and is required for autophagy in mammals (284).

2.4.2 Cargo recognition and selectivity: In multicellular organisms, an important function of autophagy is the clearance of cytosolic ubiquitinated substrates or aggregate-prone proteins. In selective autophagy, cargos are recognized through interactions with specific receptor proteins. Recent studies suggest that this degradative process is mediated through the mammalian protein p62/sequestosome 1 (SQSTM1) (284, 285). p62 directly binds both poly- or mono- ubiquitin via its ubiquitin-associated (UBA) domain and the mammalian Atg8 homolog, LC3 (286, 287), and links the ubiquitinated cargos to the autophagy machinery for autophagic degradation. The structural and functional similarity between a C-terminal motif in mammalian p62 and yeast Atg19 (288) gives rise to an intriguing hypothesis that p62 is an Atg19 analog in higher eukaryotes and acts as a receptor for ubiquitinated proteins or organelles in selective autophagy.

2.4.3 Autophagosome formation: Multiple Atg proteins are recruited to the phagophore to participate in autophagosome formation, and this step requires the highly regulated coordination of all of these proteins (Figure 11). Autophagosomes can either be generated de novo from pre-existing intracellular structure or originate from various intracellular membrane structures mainly ER (Endoplasmic reticulum) (289), especially rough ER (290, 291), mitochondria during starvation (292), Golgi bodies and plasma membrane (293). The nucleation and assembly of the initial phagophore membrane require the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, which is composed of the PtdIns3K Vps34, a myristoylated serine/threonine kinase Vps15 (p150 in mammalian cells), Atg14 and Beclin 1. The function of Beclin 1 in autophagy is regulated by Bcl-2 (B-cell lymphoma/leukemia-2), an antiapoptotic protein that inhibits autophagy by binding and sequestering Beclin 1 under nutrient-rich conditions; dissociation of Beclin 1 from Bcl-2 is required for autophagy induction. The PtdIns3K complex is specifically recruited to the phagophore to phosphorylate PtdIns, generating PtdIns(3)P which is essential for enlistment of a class of phospholipid-binding proteins to initiate autophagy (263, 264). PtdIns(3)P stabilizes ULK1 at omegasomes. The PtdIns3K complex further recruits two interrelated ubiquitin-like (Ubl) conjugation systems, Atg12–Atg5–Atg16L and LC3 II, to the phagophore (294, 295), which play

an essential role in regulating the membrane elongation and expansion of the forming autophagosome. Recent studies demonstrate that self-multimerization of Atg9 may facilitate membrane tethering and/or fusion (266, 296). Atg9 is the only identified integral membrane protein required for autophagosome formation.

2.4.4 Vesicle fusion and autophagosome breakdown: Autophagosome-lysosome fusion is mediated by the same machinery that is involved in homotypic vacuole membrane fusion. In mammalian cells, the fusion event requires the lysosomal membrane protein LAMP-2 and the small GTPase Rab7 (297, 298). UVRAG also plays important role in maturation independently of Beclin1. It activates Rab7 promoting fusion with endosomes and lysosomes (299). Rubicon is another Beclin1 partner, which blocks maturation (300).

After fusion, degradation of the inner vesicle is dependent on a series of lysosomal/vacuolar acid hydrolases, including cathepsin B, D (a homolog of proteinase A), and L in mammalian cells (301). The resulting small molecules from the degradation, particularly amino acids, are transported back to the cytosol for protein synthesis and maintenance of cellular functions under starvation conditions.

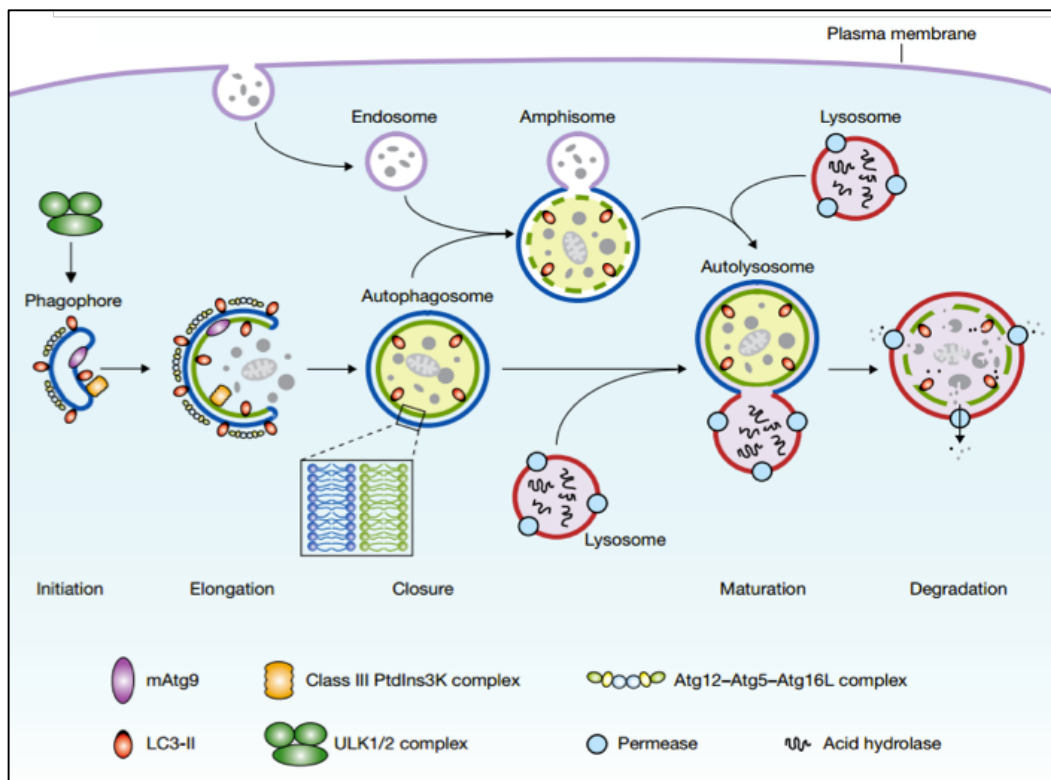


Figure 11: Schematic model of autophagy. Mammalian autophagy is initiated by the formation of the phagophore, followed by a series of steps, including the elongation and expansion of the phagophore, closure and completion of a double-membrane autophagosome (which surrounds a portion of the cytoplasm), autophagosome maturation through docking and fusion with an endosome (the product of fusion is known as an amphisome) and/or lysosome (the product of fusion is known as an autolysosome), breakdown and degradation of the autophagosome inner membrane and cargo through acid hydrolases inside the autolysosome, and recycling of the resulting macromolecules through permeases. So far, there is no evidence for a PAS that exists in mammalian cells, and so the mammalian phagophore could be equivalent to the yeast PAS, or derived from the PAS. The core molecular machinery is also depicted, such as the ULK1 and ULK2 complexes that are required for autophagy induction, class III PtdIns3K complexes that are involved in autophagosome formation, mammalian Atg9 (mAtg9) that potentially contributes to the delivery of membrane to the forming autophagosome and two conjugation systems, the LC3-II and Atg12–Atg5–Atg16L complex, which are proposed to function during elongation and expansion of the phagophore membrane (Adapted from Yang Z and Klionsky DJ. *Nat Cell Bio.* 2010).

2.5 Signalling pathways regulating autophagy

Eukaryotic cells have developed a mechanism through which autophagy induction is tightly coupled to the regulation of cell growth. Cellular signalling pathways, particularly kinases are involved the execution of autophagy (Figure 12).

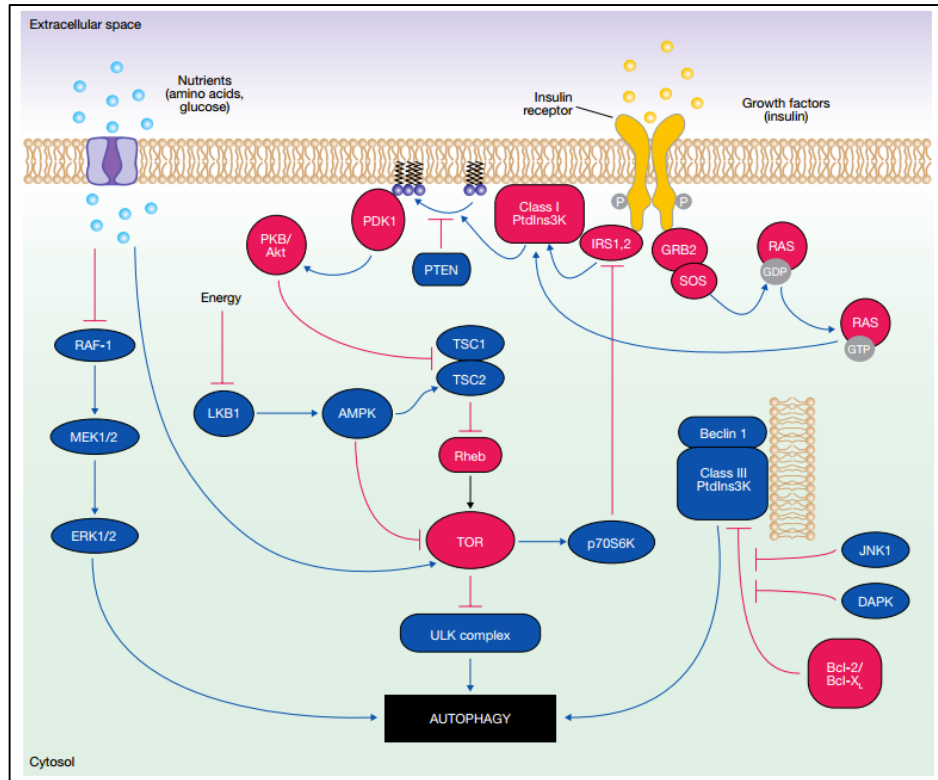


Figure 12: Signalling regulation of mammalian autophagy. In the figure, the blue components represent the factors that stimulate autophagy, whereas the red ones correspond to inhibitory factors. Autophagy is regulated by a complex signalling network of various stimulatory (blue arrows) and inhibitory (red bars) inputs. TOR plays a central role in autophagy by integrating the class I PtdIns3K signalling and amino acid-dependent signalling pathways. Activation of insulin receptors stimulates the class I PtdIns3K complex and small GTPase Ras, leading to activation of the PtdIns3K–PKB–TOR pathway. PKB phosphorylates and inhibits the tuberous sclerosis complex 1/2 (TSC1–TSC2), leading to the stabilization of Rheb GTPase, which in turn activates TOR, causing inhibition of autophagy. Amino acids inhibit the Raf-1–MEK1/2–ERK1/2 signalling cascade, leading to inhibition of autophagy. Energy depletion causes the AMP-activated protein kinase (AMPK) to be phosphorylated and activated by LKB1. AMPK phosphorylates and activates TSC1–TSC2, leading to inactivation of TOR and autophagy induction. p70S6K kinase is a substrate of TOR that may negatively feedback on TOR activity, ensuring basal levels of autophagy that are important for homeostasis. JNK1 and DAPK phosphorylate and disrupt the association of anti-apoptotic proteins, Bcl-2 and Bcl-XL, with Beclin 1, leading to the activation of the Beclin 1-associated class III PtdIns3K complex and stimulation of autophagy. Beclin 1 is

shown bound to the phagophore membrane (Adapted from Yang Z and Klionsky DJ. Nat Cell Bio. 2010).

2.5.1 PI3K/AKT/mTOR pathway: mTOR is a serine/threonine protein kinase with a large molecular size near 300 kDa that belongs to the phosphatidylinositol kinase-related kinase (PIKK) family. mTOR is involved in cell growth, nutrient sensing, protein synthesis and cell cycle progression (302, 303). It integrates the metabolic status-information of cells and modulates hormonal signalling. Limited amino acids block mTOR and start autophagy. The activity of mTOR is inhibited under nutrient starvation, which has been known as a crucial step for autophagy induction in eukaryotes (260, 261). The mTOR pathway involves two functional complexes: mTORC1, which regulates autophagy, consisting of the mTOR catalytic subunit, raptor (regulatory associated protein of mTOR), GβL (G protein β -subunit-like protein; also known as mLST8), and PRAS40 (proline-rich Akt substrate of 40 kDa); and mTOR complex 2 (mTORC2) consisting of mTOR, rictor (rapamycin-sensitive companion of mTOR), GβL, SIN1 (SAPK-interacting protein 1), and PROTOR (protein observed with rictor) (304-311) (Figure 13a). Availability of cellular amino acids, especially branched chain amino acids such as leucine, regulates mTOR1 activity (Figure 13b). Amino acid signaling is mediated by the RagA family small GTPases in mammalian cells (312, 313). Another important link of nutrient signalling to mTORC1 involves MAP4K3 (mitogen-activated protein kinase kinase kinase 3) in mammalian cells that is evolutionarily conserved and belongs to the Ste20 family protein kinase (314). Amino acid uptake at the plasma membrane is performed by a group of membrane transport proteins classified into a family of solute-linked carrier (SLC) proteins. A recent study revealed that cellular uptake of L-glutamine by SLC1A5 and its subsequent rapid efflux by SLC7A5/SLC3A2, a bidirectional transporter that regulates the simultaneous efflux of L-glutamine and transport of leucine, are important for mTORC1 activation (315) (Figure 13b). There are two classes of phosphoinositides and phosphatidylinositol kinases (PtdIns3K). Class III PtdIns3K is critical for autophagy induction, and class I PtdIns3K blocks autophagy. This can further be supported by the fact that overexpression of PTEN stimulates autophagy by facilitating hydrolysis of PtdIns(3,4)P2 and PtdIns(3,4,5)P3, (316). PtdIns3K inhibitors like wortmannin, LY 294002 and 3- methyladenine repress both classes of PtdIns3K enzymes, thus downregulate

autophagy (317). Insulin inhibits autophagy by activating class I PtdIns3K at the membrane. This leads to PtdIns(3,4,5)P₃ production promoting membrane recruitment and activation of protein kinase B (PKB)/ AKT through PDK1 (3-phosphoinositide-dependent protein kinase1) (317). c-Jun amino-terminal kinase 1 (JNK1) activates autophagy in mTOR independent way via Beclin1 activation by death associated protein kinase (DAPK) in some cases (318). The mTOR is consists of rapamycin-sensitive mTORC1 (catalytic unit) and mTORC2. Several mTOR partners are known- raptor, rictor, GβL, PROTOR etc. Phosphorylation and activation of AKT (Ser473) downregulates FoxO3 transcription factor (Forkhead Box O3), an autophagy inducer in myocytes. (319).

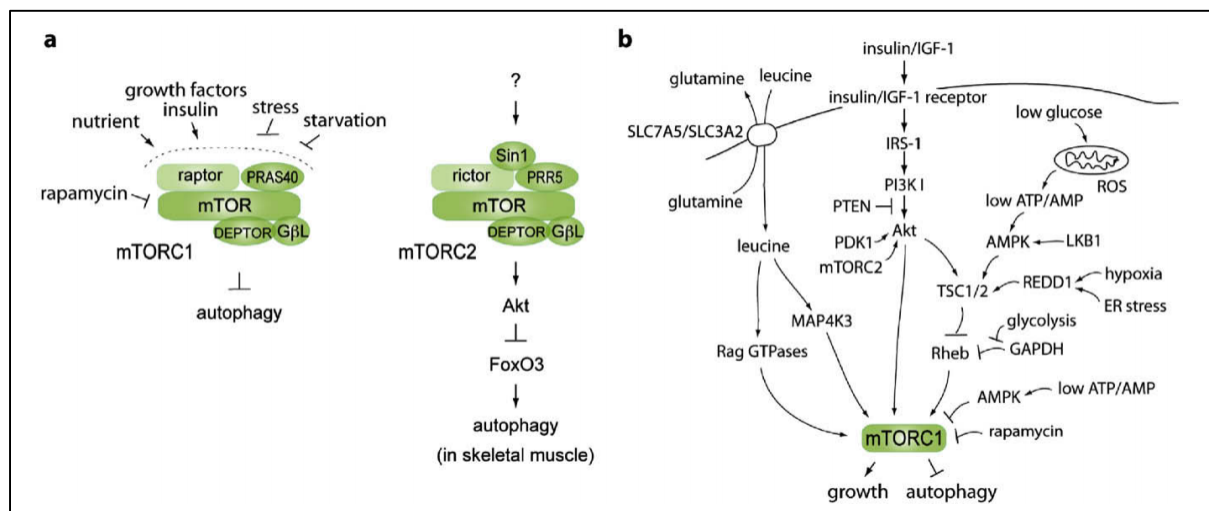


Figure 13. Regulation of the mTOR pathway by nutrients (amino acids, glucose), stress and insulin/IGF-1. (a) Two mTOR complexes, mTORC1 and mTORC2, and their components. mTORC1 is the protein complex responsible for autophagy induction in response to nutrient starvation, stress and reduced growth factor signaling. mTORC2 regulates autophagy via Akt-FoxO3 in skeletal muscle cells in response to a fasting condition. (b) The signalling pathways upstream of mTORC1 that regulate cell growth and autophagy in response to nutrient levels, growth factors, and stress (Adapted from Jung CH., et al, FEBS Lett. 2010).

2.5.2 AMPK pathway: AMP-dependent protein kinase (AMPK) senses increased cytosolic AMP-to-ATP ratio, activates LKB1 kinase, a protein kinase phosphorylating AMPK (320, 321). As a consequence, the activated AMPK inhibits mTORC1 through phosphorylation and activation of

tuberous sclerosis complex 2 (TSC2), a negative regulator of mTORC1 (322). A recent study revealed that AMPK can inhibit mTORC1 independent of TSC2 by phosphorylating raptor at Ser863 (323). Thus, there are two separate pathways that transmit AMPK signalling to mTORC1 (Figure 13b). Another glucose-sensing pathway has been proposed to involve glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that negatively regulates Ras homolog enriched in brain (Rheb)-mTORC1 signaling independently of TSC1/2 under low glucose influx (324). This GAPDH-dependent pathway has a distinct feature relative to the above two AMPK-dependent pathways given that glycolytic influx, rather than energy state, is a signal that regulates mTORC1 (Figure 13b). Recent reports, however, suggest a more direct role for AMPK in autophagy regulation. Similar to mTORC1, AMPK was shown to interact with ULK1 (325). Indeed, it has been demonstrated that AMPK directly phosphorylates ULK1 although there are controversies regarding the sites of phosphorylation (326, 327). Also, the interaction between ULK1 and AMPK was not affected by glucose starvation. However, mTORC1-mediated phosphorylation of ULK1 at Ser757 disrupted the ULK1-AMPK interaction (326). Thus, AMPK ensures the induction of induce autophagy upon exposure to several stresses like hypoxia, glucose deprivation, ischemia, heat shock, metabolic stress or oxidative stress (328, 329), and promotes cell survival in response to bioenergetic stress.

2.5.3 Beclin 1:hVps34 pathway: As discussed previously, the trimer Beclin 1:hVps34:hVps15 can interact with various different partners to control the formation and maturation of autophagosomes. Bcl-2/Bcl-XL binds Beclin 1 through a BH3 domain that mediates the docking of the latter to the BH3-binding groove. The constitutive Bcl-2/Bcl-XL:Beclin 1 interaction is disrupted by signals that promote autophagy (330-332). Importantly, c-Jun N-terminal kinase 1 (JNK-1) phosphorylates three amino acids in the N-terminal loop of Bcl-2 to trigger its release from Beclin 1 in response to starvation (333, 334). In a reciprocal manner, the BH3 domain of Beclin 1 can be phosphorylated by death-associated protein kinase (DAPK), which has the effect of reducing its affinity for Bcl-XL (319).

2.5.4 Ras/Raf/MEK1/2/ERK1/2- MAPK pathway: Ras is both positive and negative regulator of autophagy. It activates class I PI3K leading to AKT/mTOR activation and inhibits autophagy (335) while activation of class III PI3K results in activation of downstream Raf, and MEK1/2. This

in turn, phosphorylates ERK1/2 and this trail of signals stimulate autophagy (336). Mitogen activated protein kinases (MAPKs) are serine/threonine kinases that mediate responses to various extracellular stimuli. The best-studied MAP kinases are ERK, p38 and JNK. p38 has dual role autophagy (337). The induction of autophagy by p38 was often accompanied by an increase in Atg proteins, such as beclin 1 and Atg5 (338-341). However, p38 was also shown to negatively regulate autophagy. A recent report, demonstrated that p38 inhibits autophagy by competing with transmembrane protein mAtg9 for binding to p38 interacting protein (p38IP) (342). JNK has been implicated in the induction of autophagy by various stimuli, including starvation (343), cytokine stimulation (344), T-cell receptor activation (343), neuronal excitotoxic stimuli (345) and ER stress (346). JNK has been shown to induce upregulation of p62, Atg5 and Atg7 in response to resveratrol, oxidative stress and oncogenic transformations (347-349). Besides its role in posttranslational modification of proteins that regulate autophagy, JNK can also regulate the expression of beclin 1 transcript via phosphorylation of c-Jun (350-352). However, role and regulation of individual MAPKs in autophagy are very complicated and vary from one experimental model to another.

2.6 Physiological and pathological role of autophagy

Autophagy has been implicated in pathophysiology of various diseases and age-related disorders including certain myopathies, neurodegenerative disorders, and cancer.

2.6.1 Autophagy and liver diseases: Autophagy is involved in protein and organelle quality control at basal level, protecting hepatocytes from toxic effects of alcohol, drugs, etc. Various gain of function point mutations in proteins like α -antitrypsin (α -ATZ) causes improper folding, making these proteins non-functional and aggregating in hepatocyte ER (353). α -ATZ deficiency often results in chronic inflammation and carcinogenesis (354). Depletion of ATG5 in hepatocytes results in insoluble proteins and increased accumulation of cytoplasmic inclusion bodies. These alterations are speculated to sequester autophagy proteins reducing autophagy flux in hepatocytes and result in various ailments.

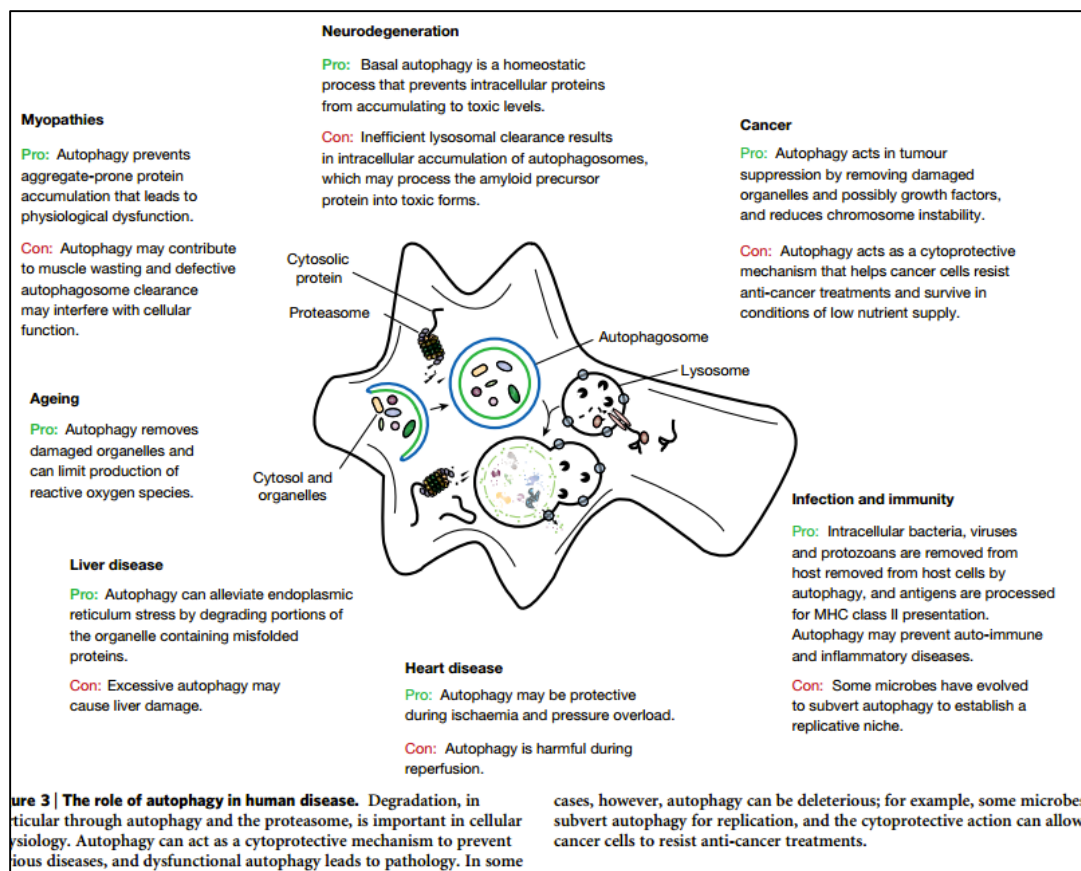


Figure 14. The role of autophagy in human disease. Degradation, in particular through autophagy and the proteasome, is important in cellular physiology. Autophagy can act as a cytoprotective mechanism to prevent various diseases, and dysfunctional autophagy leads to pathology. In some cases, however, autophagy can be deleterious; for example, some microbes subvert autophagy for replication, and the cytoprotective action can allow cancer cells to resist anti-cancer treatments (Adapted from Mizushima N., et al. Nature. 2008).

2.6.2 Autophagy and cancer: Cancer is one of the first diseases genetically linked to autophagy malfunction (355, 356). The ATG gene beclin 1 is monoallelically deleted in 40–75% of cases of human breast, ovarian, and prostate cancer. UVRAG, Ambra1 and Bif-1 that enhance the autophagic activity of Beclin 1/ class III PI3K complex, also play role in the cell growth control and/ or tumour suppression (357, 358). Beyond a role for ATG genes in tumour suppression, there is accumulating evidence that autophagy signalling regulation is tightly linked to oncogenic signalling. Several commonly activated oncogenes (for example, those encoding class I PI3K,

PKB, TOR, Bcl-2) inhibit autophagy, whereas commonly mutated or epigenetically silenced tumour suppressor genes (such as those encoding p53, PTEN, DAPK, TSC1/TSC2) stimulate autophagy (359). However, autophagy may also promote tumour cell survival during metabolic stress in the tumour microenvironment, under conditions of hypoxia and low nutrients (Figure 14). Accordingly, genetic or pharmacological inhibition of autophagy was shown to enhance the cytotoxicity of cancer chemotherapy agents and to promote tumour regression (360, 361). Thus, autophagy can act both positively and negatively with regard to cancer cell survival; autophagy probably functions to prevent cancer initially, but once a tumour develops, the cancer cells utilize autophagy for their own cytoprotection (Figure 15).

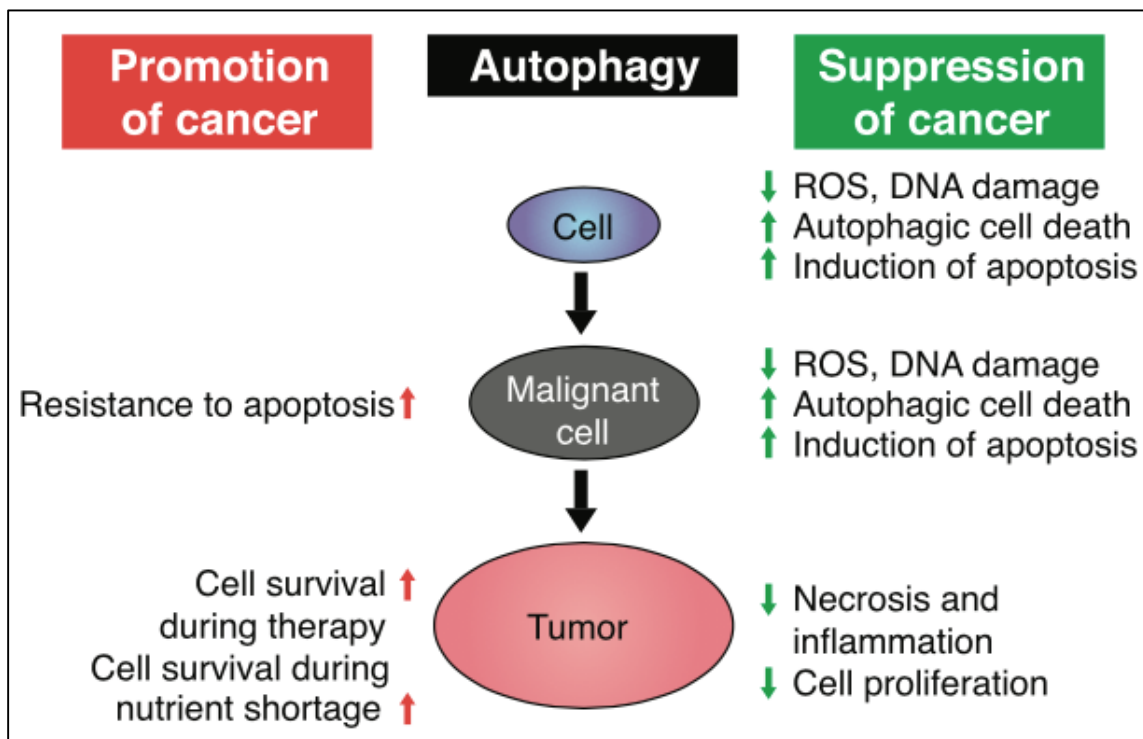


Figure 15. Autophagy and its opposing role in cancer development. While the homeostatic function of autophagy is required for the control of tumorigenesis in healthy tissues, its pro-survival role can be exploited by malignant cells to support tumor establishment and further development (Adapted from Ravikumar B., *Physiol Rev.* 2010).

2.6.3 Autophagy and neurodegeneration: Ideally, autophagy is meant to get rid of cellular/protein aggregates however, some aggregates are not cleared properly due to defective

autophagy proteins. Insufficient autophagy or excessive autophagy both hinders the balance of cellular microenvironment. Recent studies reveal that degradation of disease-related mutant proteins is highly dependent on autophagy, in addition to the ubiquitin–proteasome system. In neurons, because of insufficient autophagy flux, these aggregates are not cleared away and gets accumulated, resulting in neuronal cell death which leads to deficient nerve activity, dementia, Alzheimer’s, Huntington’s and Parkinson’s diseases. Defect in autophagosome maturation often results in toxicity and is lethal to neuron (362, 363). Induction of autophagy by inhibition of TOR attenuates the accumulation of mutant huntingtin aggregates and protects against neurodegeneration in fly and mouse models of Huntington’s disease (364). Further studies provided compelling evidence that activation of autophagy is a beneficial physiological response in nearly all neurodegenerative diseases (365, 366). Furthermore, autophagy may eliminate protein aggregates through a selective mechanism (367, 368). One possible autophagy receptor is p62/SQSTM1, a multifunctional adaptor protein that contains an LC3- interacting region (LIR) and a ubiquitin-associated (UBA) domain (369).

2.6.4 Autophagy and ageing: A common feature of all ageing cells is a progressive accumulation of damaged proteins and organelles (such as defective mitochondria) and decreased autophagic activity could be important for this. Early studies showed that autophagy function declines with age *in vivo* in rodents and *in vitro* in isolated hepatocytes (370, 371). The first experimental study implicating autophagy genes in ageing showed that knockdown of *bec-1* (*C. elegans* Beclin 1 orthologue-1), inhibits a lifespan-extending phenotype in mutants lacking the insulin signalling gene, *daf-2* (372). Subsequent studies in *Drosophila* confirm a critical role for autophagy in promoting longevity, based on the observation that *Atg7*-deficient flies are short-lived (373), whereas promoting basal levels of autophagy enhance longevity in adult flies (374). Current efforts to avoid the decline of autophagy function with age include the practice of using an anti-lipolytic drug, such as spermidine (natural polyamine) (375), that mimics the beneficial effect of caloric restriction on autophagy (376).

2.7 Autophagy and Immunity

Autophagy participates in nearly all aspects of immunity, affecting both innate and adaptive immunity processes. Autophagy may contribute to the function of the immune system exerting “specialized roles,” such as capturing, processing, or delivering microbes or endogenous immunologically active molecules, or “generic roles” by controlling the general functionality of immune cells. There are complicated feedback loops between the autophagy pathway/proteins and immunity/inflammation; the autophagy proteins function in both the induction and suppression of immune and inflammatory responses, and, vice versa, immune and inflammatory signals function in both the induction and suppression of autophagy (377-384).

2.7.1 Autophagy and Innate Immunity: Autophagy serves as a mechanism for the removal of intracellular pathogens, in a regulated manner. Autophagy proteins have diverse functions to benefit the host in the removal of invading pathogens, through xenophagy, phagolysosomal maturation, the recruitment of molecules that damage pathogen-derived membranes (385, 386). Beyond targeting intracellular pathogens for degradation, other functions such as the control of downstream inflammatory signalling by ROS production, production of antimicrobial peptides and cell-cell receptor-ligand interactions (387), have further beneficial effects in infected host cells. However, the autophagy pathway also has a crucial role in resistance to infection by intracellular pathogens. The genetic deletion, mutation or knockdown of autophagy genes prevents viral, fungal and bacterial infection. For example, in humans, recent studies support a link between autophagy and resistance to tuberculosis (388-390). Other intersections between autophagy and innate immunity come from studies on pattern recognition receptors, including pathogen associated molecular patterns: Toll-like receptors, Nod-like receptors, RLRs; DAMPs (damage-associated molecular patterns): HMGB1 (high mobility group box1), IL1B/IL-1 β ; SLRs (SQSTM1-like receptors): SQSTM1, NBR1 (391, 392), immunity-related GTPases: immunity-related GTPase family, M (IRGM) (393); and inflammasomes (394-396). In addition, autophagy may be involved in neutrophil-mediated, antibody-dependent cellular cytotoxicity (397). Although autophagy is a component of host defense, various pathogens have evolved several ways to adapt to host autophagy (Figure 14) (380, 398, 399). Bacteria can inhibit lysosomal fusion or maturation by residing in phagosomes, or camouflage themselves to avoid autophagic recognition.

2.7.2 Autophagy in Antigen Processing and Presentation: The immune control by T lymphocytes depends on the ability of antigen-presenting cells (APCs) to display peptide-MHC complexes at their surface. Dysregulation of this process results in autoimmunity. Classically, MHC class I molecules present intracellular antigens to CD8⁺ T cells, and MHC class II molecules present extracellular antigens to CD4⁺ T cells. However, professional APCs, such as dendritic cells, can process extracellular antigens for MHC class I presentation by a pathway called cross-presentation (400). Vice versa, some nuclear and cytosolic antigens can be presented by MHC class II after intracellular processing (401, 402). Autophagy has been implicated both in intracellular and extracellular antigen processing for MHC class II presentation to CD4⁺ T cells (Figure 16) (402). Autophagosomes fuse with lysosome to form autolysosomes, and can also fuse with late endosomes, such as multivesicular bodies, and MHC class II loading compartments, resulting in MHC class II presentation of the autophagic cargo (403). Recent studies indicate self-derived (404), pathogen-derived (405, 406), and tumor antigens (407) can be presented by autophagy. Autophagy might assist with extracellular antigen processing through pathogen associated molecular patterns, i.e., toll-like receptors (408) and NOD-like receptors (409). Some studies also suggest that autophagy might even assist MHC class I antigen presentation to CD8⁺ T cells (410, 411).

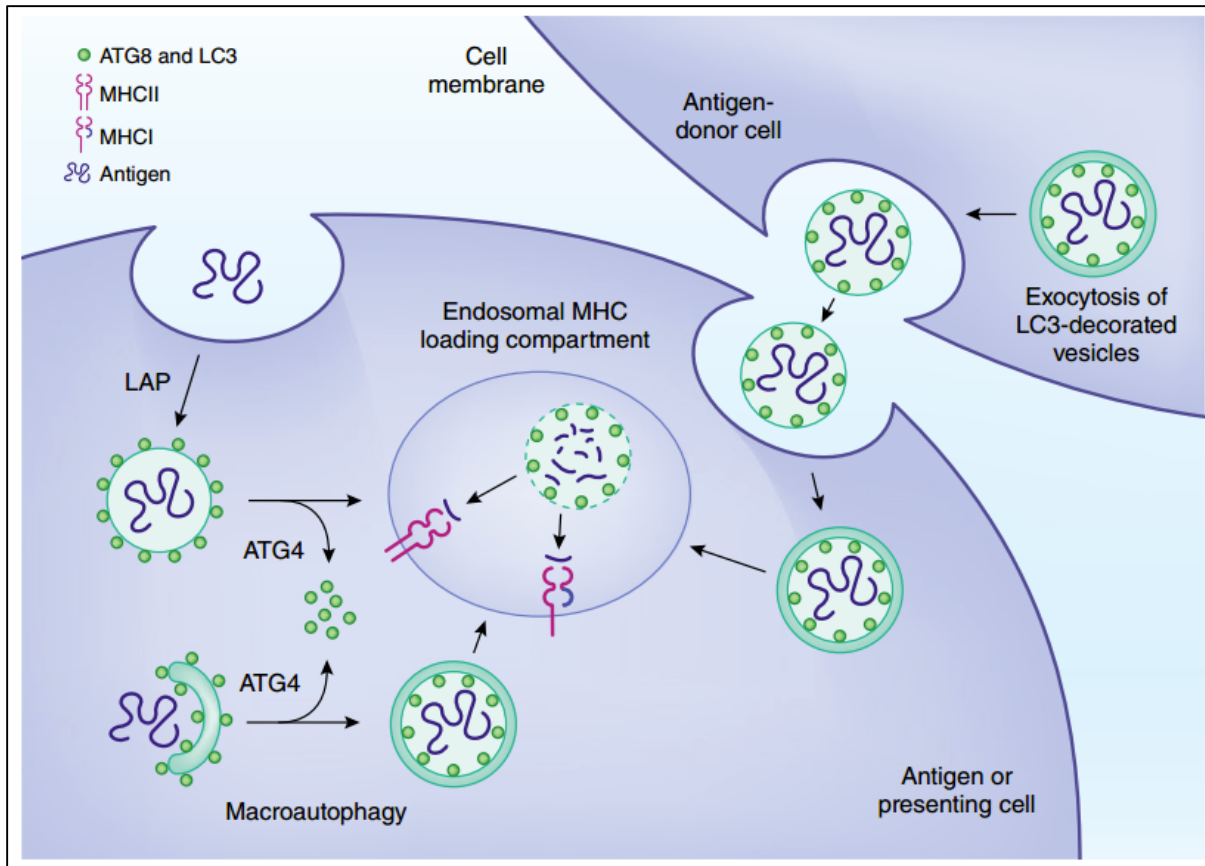


Figure 16. ATG proteins in MHC-restricted antigen presentation. Autophagy-related proteins contribute, by at least three pathways, to the loading of vesicular antigens onto MHC molecules. Autophagosomes can fuse with endosomal MHC loading compartments and deliver cytoplasmic constituents for loading onto MHC class II (MHCII). Loading of MHC class I (MHCI) might also occur in these vesicular compartments, especially under conditions in which the classical processing of classical MHC class I antigens, including import into the ER by TAP, is inhibited. LAP can deliver extracellular antigens to this loading compartment. LC3 might need to be deconjugated from the outer autophagosomal and the LAP phagosome membrane by ATG4 for efficient fusion with the endosomal MHC loading compartment. Finally, signal peptide-independent but autophagy dependent exocytosis seems to efficiently transfer antigens from donor cells to antigen presenting cells, mainly for cross-presentation by MHC class I molecules (Adapted from Shibutani ST., Nat Immunol. 2015).

2.7.3 Autophagy in T cells: Autophagic pathways intersect with a variety of T cell functions, including the development of CD4⁺ T cell self-tolerance. Once T cells depart from the thymus, autophagy plays a role in their survival and homeostasis. Multiple autophagy proteins including Beclin 1, Atg3, Atg5, and Atg7 are essential for the development, maintenance, and survival of T cells (412-417). Using a model in which Atg5^{-/-} thymi were transplanted into wild-type or TCR transgenic mice, blocking autophagy in the thymus led to altered positive and negative selection of CD4⁺ T cells, as well as to colitis and multi-organ inflammation. Several studies have investigated the roles of autophagy proteins in T cell function. Mice with Atg3-, Atg5-, or Atg7-deficient T cells all show reduced thymic cellularity (412, 416, 417). Naive peripheral CD4⁺ and CD8⁺ cells deficient in Atg3, Atg5, and Atg7 all show reduced peripheral numbers, increased apoptosis, and impaired proliferation upon stimulation (412, 415-417). In addition, autophagy plays a role in regulating ER and calcium homeostasis in T cells and that impaired autophagy can lead to ER stress and impaired calcium homeostasis (412, 418).

2.8 Autophagy in Autoimmune and Autoinflammatory diseases

One central role of autophagy is sensing cytoplasmic danger signals, including the sensing of nucleic acids, which are known inducers of inflammatory responses and are frequently targeted by autoimmune responses. An imbalanced function of autophagy has recently been linked to the causation of autoimmune and/or autoinflammatory diseases.

2.8.1 Autophagy and Systemic lupus erythematosus: SLE is a systemic autoimmune disease associated with production of autoantibodies, formation of immune complexes and persistent inflammation that can target essentially every organ system. Life-threatening disease manifestations in SLE result from inflammatory tissue damage in the kidneys, the lungs, the vascular system and the central nervous system. Autophagy is now considered a core pathogenic contributor to the abnormal immunity in SLE, affecting both innate and adaptive immunity (419, 420). Autophagy has been implicated in multiple malfunctions relevant to SLE patients; including removal of dead cells, scavenging of intracellular DNA and RNA, regulation of type I interferon (IFN) responses and control of the long-term survival of B cells and T cells. Genome-wide association studies have linked nucleotide polymorphisms in autophagy related genes to

susceptibility for SLE (419-421). At least five single nucleotide polymorphisms (SNPs) near to and in the *Atg5* locus are associated with SLE initiation and/or development. Beside abnormalities in innate immunity, improper activation and survival of T cells and B cells are critical elements in driving multiple organ inflammation and sustaining the production of autoantibodies in SLE. T cells from SLE patients have multiple abnormalities, including aberrant T cell activation and prolonged T cell survival. *Atg7*^{-/-} B cells have reduced survival in culture and fail to efficiently develop into plasma cells, suggesting that *Atg7* promotes B cell survival and contributes to their persistence in autoimmune conditions (422).

2.8.2 Autophagy and Crohn's diseases: Crohn disease (CD) has widely been described as an autoimmune disease, and a newer view describes it as a disease of immune deficiency (423, 424). This suggests an autoinflammatory rather than autoimmune origin of inflammation in CD (425). The discovery of the autophagy genes *ATG16L1* and *IRGM* as risk factors for CD put autophagy in the spotlight in its pathogenesis (426-431). High amounts of the proinflammatory cytokine IL-18 and IL-1 β were released from *Atg16L1*-deficient macrophage (432). Mice lacking *Atg16L1* are highly susceptible to dextran sulphate sodium-induced acute colitis, which can be alleviated by injection of anti-IL-1 β and IL-18 antibodies, indicating the importance of *Atg16L1* in the suppression of intestinal inflammation (432, 433). Also, *ATG16L1*-deficient mice die within the first day of delivery, however, *ATG16L1* hypomorphic mice, which express a very low level of *Atg16L1*, are viable and grossly normal but exhibit structurally abnormal Paneth cells with cytoplasmic lysozyme staining. These data suggest *ATG16L1* has a role in maintaining the normal function of Paneth cells (434, 435). In addition to these cell-intrinsic mechanisms, a link between autophagy and the immune system can also contribute to the pathogenesis of CD.

2.8.3 Autophagy and multiple sclerosis: Studies linking autophagy to risk for central nervous system (CNS) inflammatory disease, such as multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), have mostly focused on autophagic processes regulating adaptive immunity, including lymphocyte survival and antigen presentation. T cell activation in both the periphery and in the CNS is required for the induction of MS, and defects in culling out autoreactive T cells would inevitably render individuals susceptible to prolonged autoreactivity.

Similar to MS patients, increased expression of the autophagic gene Atg5 was correlated with immune-mediated myelin injury in EAE (436), an animal model that partially mimics MS (437).

3. Intravenous immunoglobulin (IVIG)

3.1 Overview:

Intravenous immunoglobulin (IVIG) is a therapeutic preparation of pooled normal polyspecific IgG obtained from the plasma pools of thousands of healthy blood donors. IVIG is mainly used in two clinical situations. It is administered at a low dose (400 mg/kg) at regular intervals as antibody replacement therapy in primary immune-deficiencies such as X-linked agammaglobulinemia and common variable immunodeficiency (CVID), or acquired immunodeficiencies where IgG plasma concentrations have become very low. On the other hand, at high dose (1-2 g/kg) IVIG is used as an immunomodulatory treatment in auto-immune or autoinflammatory diseases, including Kawasaki disease and immune thrombocytopenia (ITP) and chronic inflammatory demyelinating polyneuropathy (CIDP) and others. IVIG is a safe preparation with no long-term side effects. The adverse effects include headache, chills, nausea, fatigue, myalgia, arthralgia and back pain (438, 439).

3.2 Composition:

Preparations of IVIG consist at least 96% of IgG molecules with a distribution of IgG subclasses corresponding to that of normal serum. Subclass distribution may vary between preparations, with some products having less than physiological levels of IgG3 and/or IgG4 (Table 2) (438). IVIG also contains small, but variable, amounts of other proteins and products, notably, and depending on the commercial preparation, albumin, IgA, IgM, sugars, salts, trace amounts of solvents, detergents and buffers that may contribute to tolerability difficulties (440). IVIG has a high content of self-reactive Nabs (Natural antibodies) which can bind to various self-antigens (439). However, in IVIG preparations monomeric IgG predominant with variable quantities of dimer (5-10%) (441). The monomer and/or dimer content may vary between preparations.

Comparative analysis of several preparations of IVIg

	Baxter			LFB	Octapharma	CSL-Behring	
	Endobuline 50 mg/ml	Gammagard 50 mg/ml	Kiovig 100 mg/ml	Tegeline 50 mg/ml	Octagam 50 mg/ml	Sandoglobuline 1,2,3,6,12 g	Sandoglobuline 120 mg/ml
IgG	>95%	>90%	>98%	>97%	>95%	>96%	>96%
IgG1	50 à 80%	56.9%	>56.9%	58.8%	62.6%	65.2%	64.5%
IgG2	20 à 50%	16%	>26.6%	34.1%	31.1%	28.3%	32.4%
IgG3	/	3.3%	>3.4%	5.4%	6.1%	4.15%	2.3%
IgG4	1 à 3%	0.3%	>1.7%	1.7%	1.2%	2.4%	0.8%

Table 2: Comparison of different IVIG preparations. (Adapted from Seite et al., 2008; Autoimmunity Rev)

3.3 Pharmacology:

Dose regimen of IVIG is according to the therapeutic goals. Administration of IVIG as a replacement therapy consists of a dose of 200-400 mg/ kg body weight in immunodeficient patients. These injections repeated every 3 to 4 weeks, as long as protection is necessary. However, achieving an anti-inflammatory effect during active phases of autoimmune and inflammatory disease by using IVIG requires infusion of a much higher dose of 1-2g/kg body weight. High dose is given in single injection or divided into five daily doses of 400 mg/kg with additional maintenance dose at 4-6 week intervals (438, 442). The plasma level of IgG obtained by replacement dose in immunodeficiency patients is around 12-14 mg/ml and high dose therapy in autoimmune and auto-inflammatory patients is around 20-35 mg/ml (443). The half-life of IVIG after intravenous infusion or intramuscular injection is approximately 3 weeks. This can, however, vary depending on the immune status of the patient.

3.4 Clinical indication of IVIG in autoimmune and auto-inflammatory diseases:

Today, the use of IVIG in the treatment of autoimmune and systemic inflammatory diseases largely takes over its initial indication for patients with immune deficiency. In 1980, Imbach et al. administrated IVIG as a replacement therapy in a boy affected by secondary hypogammaglobulinemia due to long-term immunosuppressive treatment of immune thrombocytopenia (ITP). Unexpectedly, platelet counts dramatically increased after IVIG infusion, and additional studies in patients with ITP but without hypogammaglobulinemia confirmed these results (444). Since then the therapeutic use of IVIG in a variety of autoimmune and inflammatory disorders has considerably increased. The Food and Drug Administration (FDA) approved

therapeutic indications for IVIG in KD, Chronic inflammatory demyelinating polyneuropathy (CIDP), ITP and Multifocal Motor Neuropathy (MNN). In addition to its use in diseases where the therapeutic efficacy has been confirmed by randomized trials, IVIG is also used in an off-label manner for over 100 conditions. Off-label use accounts for an estimated 50-70% of the current global consumption of IVIG (445) (Table 3).

Replacement (low-dose therapy)	Anti-inflammatory (high-dose therapy)	
	Licensed	Off-label
Primary immunodeficiencies (CVID and others) HIV infection Bone marrow transplantation B cell lymphocytic leukemia Multiple myeloma	Idiopathic thrombocytopenia purpura Guillain-Barré syndrome Kawasaki disease Chronic inflammatory demyelinating polyneuropathy (CIDP)	Autoimmune neutropenia Autoimmune hemolytic anemia Anti-Factor VIII autoimmune disease Multiple sclerosis Myesthenia gravis Stiff person syndrome Multifocal neuropathy Systemic vasculitis (ANCA positive) Polymyositis Dermatomyositis Rheumatoid arthritis Systemic lupus erythematosus Antiphospholipid syndrome Toxic epidermal necrolysis Autoimmune skin blistering diseases Steroid-dependent atopic dermatitis Graft-vs-host disease Sepsis syndrome

Table 3. Examples for clinical use of IVIG. (Adapted from Ravetch et al., 2008; Annu Rev Immunol)

3.5 Mechanisms of action of IVIG: immunomodulatory and anti-inflammatory effects

Even though IVIG is widely used and has proven to be an effective treatment for many diseases, the precise immunomodulatory mechanism(s) have remained elusive. Several mechanisms by which IVIG may exert its anti-inflammatory effects have been proposed over the past decades (Figure 17). These mechanisms are not necessarily mutually exclusive, and may act in concert to modulate the immune system. There is evidence that both, the F(ab')₂ fragment and the Fc domain, are involved in the immunomodulatory mechanisms of IVIG.

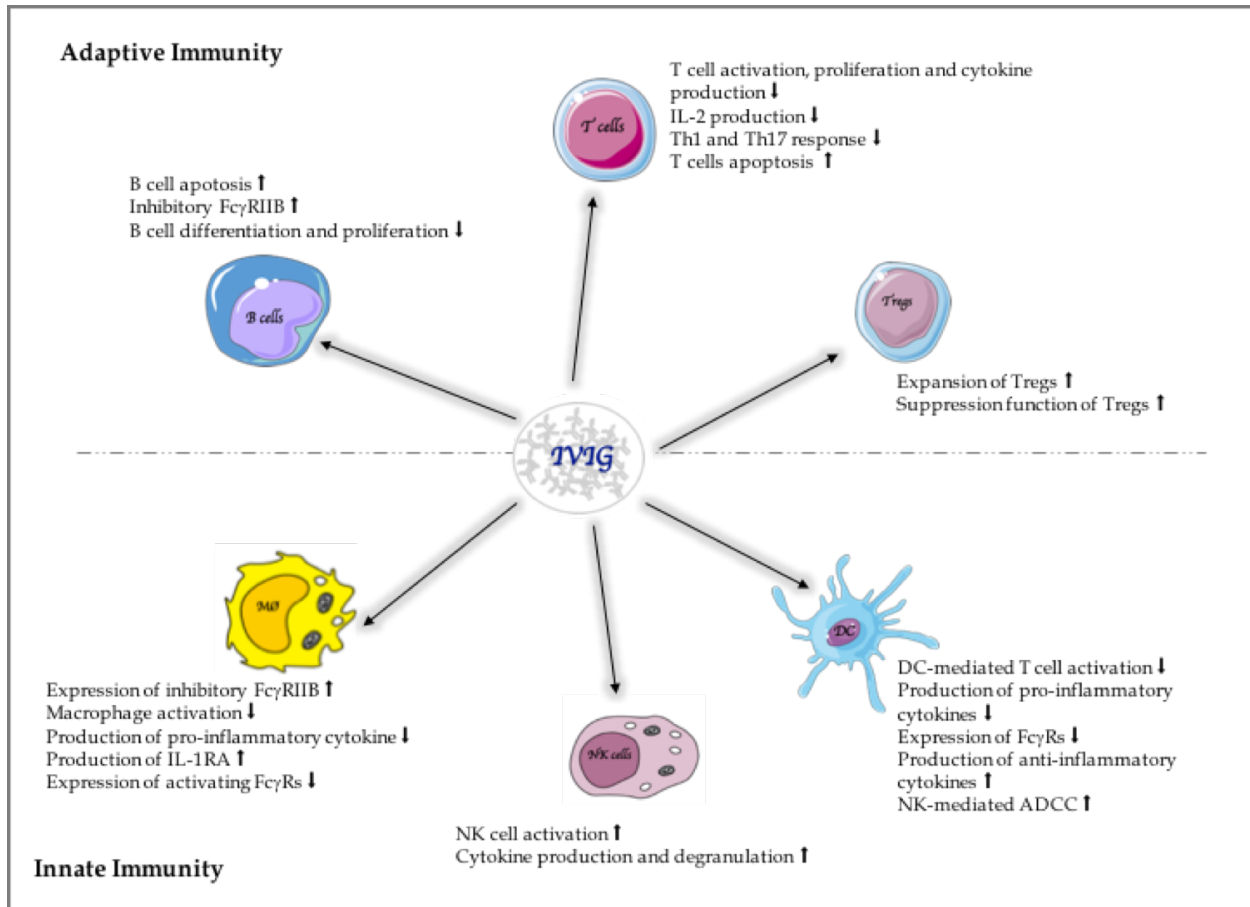


Figure 17: A schematic representation of the proposed mechanisms of action of IVIG on cellular immunity.

3.5.1 The effects of IVIG on soluble mediators

3.5.1.1 Autoantibody Neutralization and Inhibition of Autoantibody Production

IVIG preparations contain anti-idiotypic antibodies, i.e., antibodies that are able to interact specifically with the variable region (antigen recognition site) of autoantibodies. This interaction has the potential to neutralize an autoantibody and to hamper its production via binding to auto reactive B lymphocytes. Such a neutralizing or inhibitory activity has been shown, amongst others, for autoantibodies directed against factor VIII, DNA, intrinsic factor, thyroglobulin, anti-neutrophil cytoplasmic antibodies (ANCA) and others (446, 447).

3.5.1.2 Scavenging of complement components

Given the potent pro-inflammatory activity of the complement components, several studies have been suggested that the neutralization of complement molecules constitutes one of the potential pathways by which IVIG is able to block tissue inflammation. IVIG was shown to bind to several activated complement components such as C3a and C3b via its F(ab')₂ fragments, thereby attenuating complement-mediated tissue damage (448-451). Interestingly, IVIG-mediated neutralization of C5a was found to be important in the maintenance of a high FcγRIIB to FcγRIII expression ratio on macrophages cell line.

3.5.2 Fc-mediated effects of IVIG

3.5.2.1 Blockade of FcγRs

The effector functions of antibodies are crucially dependent on their interaction with the complement system as well as with Fc receptors. Phagocytes are activated by IgG antibodies which bind to their specific Fcγ receptors (FcγRs) on their surface. Traditionally, these receptors are divided into 2 groups, based on their activating or inhibitory properties. Monocytes, macrophages, and DCs express several members of the FcγRs family on their cell surface and can co-express activating and inhibitory FcγRs (452). In autoantibody-mediated diseases like ITP, Fc fragment-mediated competitive blockade of activating FcγR by IVIG might inhibit: binding of opsonized antigens, induction of effector functions and secretion of pro-inflammatory cytokines by macrophages, and degranulation of granulocytes (453, 454).

3.5.2.2 Saturation of FcRn

The neonatal Fc receptor (FcRn) is a specialized receptor that binds serum IgGs and protects them from degradation. IgGs are picked up from plasma by endothelial and myeloid cells through pinocytosis and bind to FcRn present in the acidic endosomes. FcRn binds IgG at acidic pH of (<6.5) but not at neutral or higher pH. IgGs bound to FcRn are recycled to the cell surface and released into the circulation. Without this protective mechanism, IgG would pass to the lysosome and be degraded. These specialized receptors account for the long half-life of serum IgG of 2-3 weeks. Thus, one possible mechanism postulated for IVIG is that the large dose of IgGs

administered saturates FcRn receptors and accelerates the degradation of circulating pathogenic autoantibodies (455). However, later studies showed that FcRn is not essential for IVIG-mediated improvement in humanized mouse model of ITP (456, 457).

3.5.2.3 Increased expression of inhibitory FcγRIIB

The inhibitory low- affinity FcγRIIB is widely co-expressed with activating FcγRs on a variety immune cells, including macrophages and B cells. Cross-linking of FcγRIIB leads to a dominant-negative signal, resulting in cell inactivation. Several studies addressed the hypothesis that FcγRIIB may play a role in the immune modulation of IVIG by promoting inhibitory signals within the immune cells. This was first reported in 2001 in a study showing that FcγRIIB was up-regulated in the splenic macrophages of ITP mice after administration of IVIG and that FcγRIIB KO mice failed to respond to IVIG therapy (458), which was confirmed by another study (459). The requirement of the inhibitory FcγRIIB for IVIG-mediated protective effect was reported in a variety of murine models, such as nephrotoxic nephritis (460), K/BxN serum-induced arthritis (461, 462), and in the T cell-mediated experimental autoimmune encephalomyelitis (EAE) (463).

3.5.2.4 Sialylation requirement for IVIG-mediated anti-inflammatory effect

Human IgG antibodies share a conserved N-glycosylation site within the CH2 domain of their Fc moieties, linked to the asparagine 297 (Asn297). Several studies demonstrated that the potent anti-inflammatory activity of IVIG is a direct result of α -(2,6) sialylated Fc fragments (464). Under both therapeutic and preventive application of IVIG, it has been demonstrated that the presence of both sialic acid on IVIG and the inhibitory FcγRIIB on macrophages was required for IVIG beneficial effects (465). Tetra-Fc sialylation further enhanced the anti-inflammatory activities of IVIG (466, 467). This drug candidate revealed enhanced antiinflammatory activity up to 10-fold higher than IVIG. The importance of sialylation was confirmed in another study where administration of sialic-acid enriched IVIG (SA-IVIG), but not non-SA-IVIG, to OVA-sensitized and intranasal OVA challenged mice attenuated airway hyperresponsiveness and inflammation (468).

Ravetch and colleagues have hypothesized that the anti-inflammatory mechanism of the sialylated IgG fraction of IVIG is mediated through binding to a C-type lectin receptor, the SIGNR1, which is expressed primarily on splenic marginal zone macrophages in mice or on DCs in human (named DC-SIGN [dendritic cell (DC)- specific ICAM-3 grabbing non-integrin]) (461, 469). Indeed, the sialylation of IgG Fc leads to a conformational change that would decrease the interaction with FcγRs but trigger DC-SIGN binding (470). Using their K/BxN arthritis model in mice, they further established that this interaction induced the production of IL-33 from the splenic marginal zone macrophages, leading to expansion of IL-4-secreting basophils. Effector macrophages then respond by increasing expression of the inhibitory FcγRIIB, which shifts the response away from the damaging pro-inflammatory cascade (471). In line with the dominant expression of SIGNR1 on mouse splenic marginal zone macrophages, IVIG activity was also abrogated in splenectomized mice as well as with SIGNR1-blocking Abs or in mice deficient in SIGNR1 (461). However, the involvement of SIGNR1 in IVIG anti-inflammatory effects may not be limited to its expression on splenic marginal zone macrophages. Indeed, a study in splenectomized mice have shown that IVIG still mediates the suppression of antibody-dependent platelet depletion (472). Despite the lack of involvement of splenic resident cells, they showed that IVIG activity was still fully dependent on terminal sialic acid residues and SIGNR1, and therefore hypothesized that SIGNR1-positive cell populations outside the spleen are crucial for IVIG activity in murine ITP (473).

However, in contrast to studies previously mentioned, several studies suggested that sialylation is not relevant for anti-inflammatory effects of IVIG in human as well as animal models (474). Although IL-33 was induced in autoimmune patients following IVIG therapy (475, 476), it was not produced from DC-SIGN⁺ DC or splenocytes. Also IL-33 induction is not associated with basophil expansion (475). The sialylation levels of therapeutic IVIG did not determine the response to in KD (477) and in ITP patients. IVIG treatment led to modification in the ratio of activating/inhibitory FcγRs primarily via reducing FcγRIIA (478). Furthermore, neither DC-SIGN nor sialylated IgG Fc fragments have been shown to be involved in the anti-inflammatory activity of IVIG on human DCs (479, 480). The effect of IVIG on the inhibition of human T cells proliferation was not affected after neuraminidase treatment that removes sialic acid (481). IVIG treatment did not following FcγRIIB mRNA expression in circulating human

monocytes from Kawasaki patients (482) nor increased Fc γ RIIB expression in monocytes in children with ITP (483).

In addition, several studies suggested that sialylation was not relevant for the effects of IVIG in a murine model of ITP (484) and arthritis models (485). In an EAE model, it was also demonstrated that sialylation was dispensable for IVIG-mediated amelioration. By using ITP model, it was suggested that the genetic background of mice and dose of IVIG are the important factors that determine the role of Fc γ RIIB in IVIG-mediated protection (486).

3.5.3 The effects of IVIG on immune cells

3.5.3.1 Effector functions of IVIG on monocytes and macrophages:

IVIG suppresses activation of monocytes and macrophages in both mice and humans by altering transcription of various inflammatory genes (482) and lowering circulating levels of inflammatory cytokines, such as TNF- α , IL- β (438, 487, 488). IVIG induces production of anti-inflammatory cytokines like IL-1 receptor antagonist (IL-1RA), TGF- β and IL-10 (487, 489-491). In patients, gene expression profiles indicated IVIG-mediated suppression of a diverse array of activating genes associated with inflammation including chemokines (482, 492-495). IVIG abrogated type I IFN response signatures in monocytes of ITP patients (478). Additionally, IVIG contains high affinity natural IgG to various cytokines and these antibodies can exert direct neutralization effect on innate inflammatory cytokines (496).

3.5.3.2 Regulation of DC functions by IVIG:

As a professional antigen presenting cells, DC play major role in immune tolerance and autoimmunity. IVIG treatment of DC *in vitro* inhibits the differentiation and maturation of DC and reduces the capacity of mature DC to secrete pro-inflammatory cytokines upon stimulation, while enhancing the production of IL-10 (497, 498). Interaction of IVIG via F(ab')₂ has been found to inhibit TLR-induced phosphorylation of ERK1/2, an intracellular signaling molecule that mediated inflammatory response (479). In addition, IVIG treatment can

promote the interaction between NK cells and DCs to enhance antibody-dependent cell mediated cytotoxicity (ADCC) of DCs by NK cells and hence reduce the antigen presenting pool (499).

3.5.3.3 Modulatory effects of IVIG on B cells

IVIG inhibits the proliferation, production of pathogenic autoantibodies and antigen-presenting functions of B cells (500-506). A direct interaction of IVIG with B cells was shown to occur through CD22, resulting in modulation of BCR-mediated signals and induction of apoptosis in mature human B cells (501). IVIG inhibit B cell differentiation (507, 508), IL-6 and TNF- α production (509) and induces B cell apoptosis via binding with FAS-L (510). IVIG also causes downregulation of specific autoreactive B cells (511-513).

3.5.3.4 Modulatory effects on T cells

IVIG has prominent regulatory effects on T cells, which occupy central stage in the process on immune response. IVIG inhibits human T cell activation, proliferation and cytokine production under mitogenic and allogenic stimulation *in vitro*. IVIG can interact with T cells to inhibit IL-2 production at a post-transcriptional level. IVIG can also induce T cell apoptosis via Fas (CD95) and activation of caspases (514). In addition, IVIG is known to impair antigen presentation and expression of co-stimulatory molecules on APC leading to indirect inhibition of T cell response. It is well known that both Th1, Th17 cells and Tregs have an important role in the regulation of autoimmune diseases. The differentiation of Th17 and Treg cells is believed to be reciprocal in nature. Notably, the restoration of imbalance between pathogenic T (Th1 and Th17) cells and Treg cells by IVIG therapy is observed in EAE and autoimmune patients (515-517).

Several studies showed that IVIG enhances and restores the functions of Tregs both in experimental models and in patients. Protection of EAE by IVIG in a murine model has been associated with increase of peripheral CD4⁺CD25⁺Foxp3⁺ Tregs (515) and these results have been confirmed by other studies as well (516, 517). IVIG suppressed allogeneic T-cell responses to promote skin allograft acceptance by direct activation of Tregs (518). Tregs have been analyzed in patients with various autoimmune rheumatic diseases, before and after IVIG treatment (519).

Patients had shown a significant increase in number of Tregs after IVIG therapy. Similarly, patients with KD showed lower Treg frequency during the acute phase of disease as well as lower FoxP3 expression (520), and both could be restored after IVIG therapy.

Thus, the ability of IVIG to influence Treg induction has been shown both in animal models and in human diseases and several mechanisms have been proposed. For example, IVIG induces Tregs by the induction of cyclo-oxygenase dependent prostaglandin E2 (PGE2) from human DCs (476, 521, 522). Inhibition of PGE2 synthesis prevented DC-mediated Treg expansion by IVIG *in vitro* and significantly diminished IVIG-mediated Treg expansion *in vivo* in EAE model. This Tregs expansion is mediated in part via interaction of IVIG and F(ab')₂ fragments of IVIG with DC-SIGN. Further, in autoimmune patients, IVIG therapy induces PGE2 (476, 522). However, PGE2 induction in the circulation did not correlate with Tregs expansion (476). In a mouse model of OVA-induced airway hyperresponsiveness (AHR), Massoud et al. reported that sialylated IgGs induce Tregs and attenuated airway hyperresponsiveness after binding to Dendritic cell immunoreceptor (DCIR) (523). The importance of Tregs in the immune modulation induced by IVIG is also underlined by the studies of De Groot et al. (524), who identified *in silico* conserved Tregs epitopes named Tregitopes in both the IgG Fc and F(ab')₂ regions that bind to multiple MHC Class II determinants.

Recently several studies shown that IVIG inhibits human Th17 cell differentiation and amplification in both experimental conditions and autoimmune patients (525-529). IVIG also inhibit effector cytokines production under *in vitro* differentiation and amplification conditions. The inhibitory effects of IVIG on Th17 cells are F(ab')₂-dependent and involve interference with the expression of RORC and STAT3 activation (525).

4. Immunoglobulin A (IgA)

4.1 Overview

Immunoglobulin A (IgA), as one of the major serum immunoglobulins and the predominant antibody class in the external secretions that bathe mucosal surfaces, plays key roles in immune protection (530). IgA is also the most heterogeneous among the Ig, and IgA displays a T-shaped structure, which differs from the common Y-shape of other Igs (532). Serum IgA, at

concentrations of about 2–3 mg/ml, is the second most prevalent antibody in serum after IgG. Since serum IgA is metabolized about five times faster than IgG, the production rates of serum IgA and IgG must be similar (533). Circulating IgA are predominantly monomers (mIgA) while the majority of IgA in the secretions are polymeric, mainly in the form of dimers (dIgA) comprising two IgA monomers linked by a J (joining) chain (530). Secretory IgA (SIgA) are synthesized by local plasma cells before being transported to mucosal surfaces through epithelial cells by the polymeric Ig receptor (533).

Unlike mouse IgA, which comprises only one class, human IgA comprises two subclasses, IgA1 and IgA2, that basically differ by the absence of a 13-amino acid sequence in the hinge region of the IgA2 molecule (Figure 18) (534). Two allotypic variants of IgA2, named IgA2m(1) and IgA2m(2), have been well characterized. The most notable difference relates to the fact that while IgA2m(2) has the disulphide bridges linking light and heavy chains typical of most immunoglobulins, these are generally lacking in IgA2m(1).

IgA1 contains both N- and O-linked glycans. In N-glycans, the first sugar added, N-acetylglucosamine (GlcNAc), is linked to a nitrogen atom in a N-X-S/T sequence motif, whereas O-glycans have GalNAc bound to the oxygen atom of serine or threonine. Both subclasses of IgA are N-glycosylated, but only the IgA1 subclass has O-glycans. IgA1 O-glycosylation sites are located in the hinge region, with 3–6 O-glycans per heavy chain. IgA1 O-glycans are core 1 structures that normally consist of GalNAc with a β 1,3-linked galactose (Gal). The final extension of the core 1 structure is an addition of sialic acid to Gal, GalNAc, or both (534-536). Upon completion of glycosylation, IgA1 moves out of the Golgi apparatus and the monomers are processed into polymeric forms by addition of joining the (J) chain at the tail end of the heavy chain (537-539).

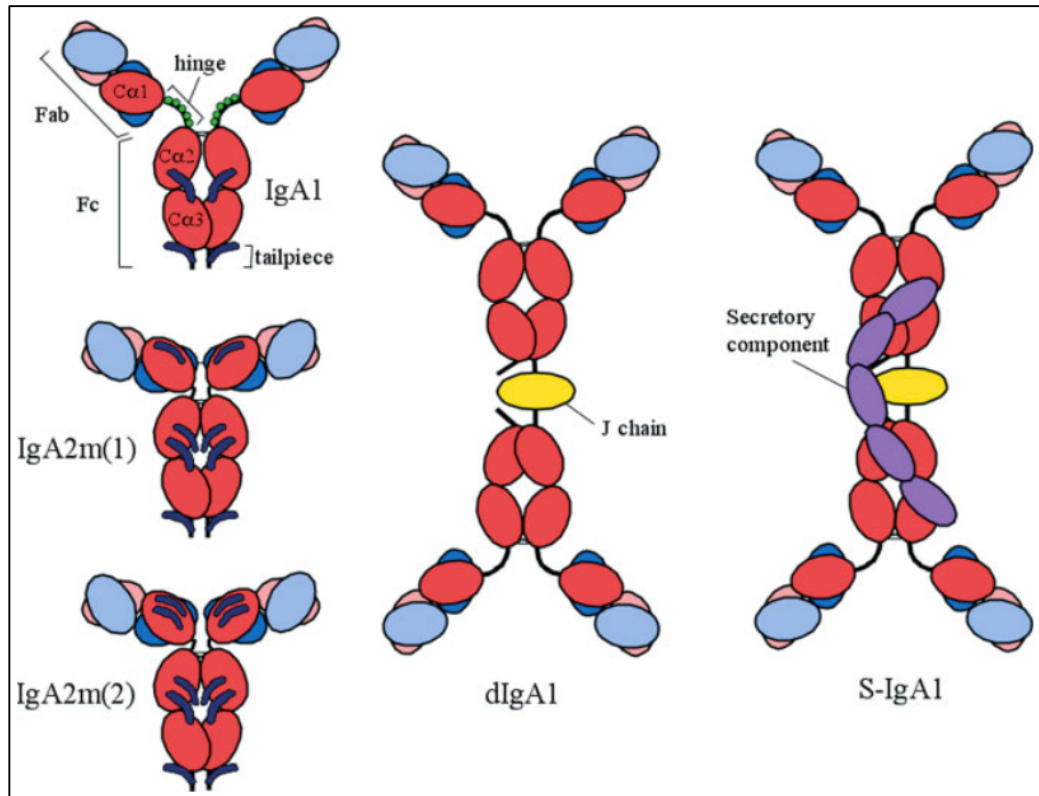


Figure 18. Immunoglobulin A. Schematic representation of the monomeric forms of human IgA1, IgA2m(1) and IgA2m(2), and the dimeric (dIgA1) and secretory (S-IgA1) forms of IgA1. Constant regions of the heavy chain are shown in red and the variable domains of the heavy chain in pink. For the light chains, constant regions are shown in mid-blue and variable regions in pale blue. J chain is shown in yellow and secretory component in purple. On the monomeric forms of IgA, O-linked sugars (on the IgA1 hinge) are shown as green circles, while N-linked oligosaccharides are shown in dark blue (Adapted from Woof JM, and Kerr MA. *J Pathol.* 2006).

4.2 IgA receptors:

Serum and secretory forms of IgA interact with various host receptors. Receptors specific for IgA are expressed on a diverse range of cell types. Currently, three bona fide receptors have been identified for IgA. The first one, FcαRI, is a receptor specific for IgA, capable of binding both human IgA1 and IgA2 subclasses (540, 541). The second one is polymeric Ig receptor (pIgR) is critical for the transport of polymeric IgA into mucosal secretions on epithelial cells (542, 543).

The third receptor type is the recently described Fc α / μ R (544). The two alternative IgA receptors are the asialoglycoprotein receptor and the transferrin receptor (545, 546).

4.2.1 Interaction with Fc α RI

A key mediator of IgA effector function is Fc α RI (CD89) (547), and it is present on neutrophils, monocytes, eosinophils and some macrophages and dendritic cells. These mechanisms include phagocytosis, release of cytokines and activated oxygen species, and antibody-dependent cell-mediated cytotoxicity. The Fc regions of both IgA1 and IgA2 bind to Fc α RI through the receptor's membrane distal domain, one of two Ig-like domains making up the extracellular portion of its ligand-binding chain. An interaction site on IgA lying at the interface of the C α 2 and C α 3 domains (548, 549)

The Fc α RI α chain associates with a homodimer of a signal transducing polypeptide termed Fc γ chain, which also associates with other Fc receptors. The γ chain carries an immunoreceptor tyrosine-based activation motif (ITAM), comprising a sequence of two tyrosine-containing boxes (Tyr-X-X-Leu) separated by seven amino acids. When the Fc α RI α chain is engaged by ligand and effectively becomes cross-linked, the γ chain ITAM tyrosines are phosphorylated that culminates in the assembly of a multimolecular adapter complex (550, 551). Binding of antigen-complexed IgA to Fc α RI elicits a wide range of biological responses depending on the cell type involved. These processes include antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, release of cytokines, superoxide generation, calcium mobilization, degranulation, and antigen presentation (552).

4.2.2 Interaction with pIgR

dIgA is transported into mucosal secretions by the pIgR expressed on the basolateral surface of cells making up the mucosal epithelial barrier. pIgR specifically binds and transports polymeric Igs. As dIgA is the predominant polymeric Ig at the mucosal surface, it forms the receptor's chief ligand and cargo. Upon binding, the pIgR-dIgA complex is internalized and transferred across the cell through a series of vesicles to the apical (luminal) surface. In addition, Cys467 in domain 5 of pIgR forms a disulphide bond with Cys311 of IgA C α 2 domain (553).

Over recent years, there has been a growing appreciation that pIgR is not only capable of delivering polymeric IgA into mucosal secretions, but it can also transport locally-formed pIgA-containing immune complexes from the lamina propria out across the epithelium (554, 555). Thus, antigens originating from the diet, environment or gut microbiota or generated during mucosal infections can be trapped by pIgA and excreted into the lumen. More than this, pIgR can mediate transcytosis of whole viruses and bacteria that have been recognized by specific pIgA (550-552).

4.2.3 Interaction with Fc α / μ R

Human Fc α / μ R, a receptor that binds IgA and IgM (556), is expressed in follicular DCs in tonsils (557), and on m ϕ , plasma cells, and Paneth cells in the lamina propria and germinal centers of the intestine (558). Although the function of human Fc α / μ R is not yet completely clear, these locations are consistent with a role in coordinating the immune response, at least in certain mucosal tissues. Fc α / μ R interacts only with polymeric forms of IgA and IgM (544), but the presence of the J chain does not seem to be essential (545). The N-linked glycans of IgA are not required for interaction, and based on mapping mutagenesis experiments, the C α 2 – C α 3 domain interface of the IgA heavy chain seems critical for the interaction (544).

4.2.4 Alternative IgA receptors

Asialoglycoprotein Receptor (ASGP-R)

The liver plays an important role in maintaining homeostasis through regulation of IgA catabolism. The ASGP-R expressed on hepatocytes (559, 560) recognizes terminal Gal residues on serum glycoproteins, including IgA, and conveys bound ligand for intracellular degradation. The major pathway for IgA2 clearance was recently shown to be mediated by the liver ASGP-R. Notably, only a small percentage of IgA1 is cleared through this pathway (561).

Transferrin Receptor (TfR)

Transferrin receptor, TfR (or CD71) has more recently been described as a receptor for IgA, selectively binds human IgA1 (polymeric rather than monomeric) but not IgA2 (562). CD71 is expressed on fetal mononuclear haematopoietic cells and certain lymphocytic and myeloid cells,

however is not detected on adult mononuclear or polymorphonuclear cells. The receptor is also present on mesangial cells, and up-regulated in IgA nephropathy patients has led to suggestions that this receptor may be associated with IgA deposition in the kidneys (546, 563). Interstitial-type DCs were also shown to express CD71. In immature but not activated monocyte-derived DCs, the receptor can mediate endocytosis of IgA complexes (564).

4.3 Role of IgA in Homeostasis and Immune Protection

SIgA antibodies serve a variety of functions in the immune system both under steady-state conditions and infection. It was proposed that high-affinity SIgA antibodies (from T cell dependent pathways) protect intestinal mucosal surfaces against colonization and invasion by pathogenic microorganisms, and low-affinity SIgA antibodies (from T cell-independent pathways) are important to confine commensal bacteria in the intestinal lumen (565).

On the other hand, serum IgA plays rather a paradoxical role in immunity with its involvement in inhibition of immune response and also as a pathogenic actor in inflammatory diseases depending on the type and structural form of IgA. The major role of serum mIgA in physiology is its powerful anti-inflammatory effects toward the immune system. It has been demonstrated that in the absence of antigen, serum IgA down-regulates IgG-mediated phagocytosis, chemotaxis, bactericidal activity, oxidative burst activity, and cytokine release (566-572).

The Fc α RI plays an essential role for both inflammatory and anti-inflammatory functions of IgA. Inflammatory responses are mediated following cross-linking of Fc α RI by IgA immune complexes which require its association with the FcR γ subunit to initiate ITAM-dependent cellular responses. The interconnected signalling pathways couple FcR γ -chain ITAM phosphorylation to different cellular processes by the activation of several transcription factors. Fc α RI activation may thus trigger specific signalling and functional responses (552).

ITAMs can also propagate inhibitory signals when they are in a conformation named inhibitory ITAM (ITAMi) (573, 574). With Fc α RI, monomeric serum IgA transduces inhibitory signals through the Fc α RI–FcR γ -chain complex. At the molecular level, an initial very low-intensity Fc α RI activation step promotes a Syk-dependent recruitment of the tyrosine phosphatase Src homology region 2 domain containing phosphatase-1 (SHP-1) to the FcR γ ITAM. Thus, IgA-induced both activating and inhibiting signals depend on Fc α RI–FcR γ -chain ITAM, but differ with

respect to the recruitment of tyrosine kinases versus tyrosine phosphatases, respectively. The cross-linking of Fc α RI during infection with IgA-opsonized pathogens induces proinflammatory responses, whereas naturally occurring serum IgA (which is not complexed with an antigen) induces inhibitory signals through the Fc α RI to dampen excessive immune responses.

SIgA also showed anti-inflammatory effects upon interaction with DC through the SIGNR1 (575). SIGNR1 is a mouse homologue of DC-SIGN, a C-type lectin receptor that was recently described as a receptor for human SIgA on the cell surface of DC. This interaction is dependent on sugars, particularly mannose residues, Ca⁺² ions and the presence of the secretory component. This interaction rendered DC tolerogenic characterized by the secretion of IL-10 and gaining the ability to expand Tregs.

Objectives of my thesis

Circulating antibodies (immunoglobulins) play an important role in maintaining immune homeostasis in healthy individuals. IVIG, a therapeutic preparation of polyclonal immunoglobulins obtained from the plasma pools of several thousand healthy donors is increasingly used in the treatment of several autoimmune and inflammatory diseases. Several mutually non-exclusive mechanisms of action are proposed to explain the therapeutic benefits of IVIG therapy. IVIG has been shown to interact with various immune cells to exert immune modulatory activities.

Autophagy is an important biological process involving lysosomal degradation of damaged cellular components and misfolded proteins. There are several evidences that support the involvement of autophagy in the regulation of autoimmune and auto-inflammatory responses and in mediating prominent anti-inflammatory effects. Disorders such as systemic lupus erythematosus, inflammatory bowel diseases, rheumatoid arthritis, psoriasis, multiple sclerosis and myositis were suggested to be associated with defects in autophagy process. Moreover, in some of these diseases, the therapeutic use of IVIG is well documented. Therefore, I hypothesised that IVIG modulates autophagy to achieve therapeutic effects in autoimmune and inflammatory patients and thus my thesis aimed at:

1. Investigating the autophagy in IVIG-treated autoimmune patients
2. Identifying the types of immune cells that are susceptible to IVIG-mediated autophagy
3. Dissecting the mechanism by which IVIG modulates autophagy

Although Th17 cells are implicated in the protection against fungi at mucosal surfaces and certain bacterial infections, aberrant activation of Th17 cells and their effector cytokines are implicated in the pathogenesis of various autoimmune and inflammatory diseases. Importantly, targeting Th17 responses have given promising results in experimental models of autoimmune diseases and in patients. Recent data from our lab and others show that beneficial effects of therapeutic IVIG is associated with inhibition of Th17 responses, indicating that circulating immunoglobulins have regulatory functions on Th17 cells. However, despite being the second most abundant antibody in the circulation, the immunoregulatory function of IgA is relatively unexplored. Therefore, I investigated the role

of monomeric IgA (mIgA) isolated from pooled plasma of healthy donors on human Th17 response with following aims:

1. Delineating the role of mIgA on differentiation of human Th17 cells.
2. Deciphering the effect of mIgA on human Th17 cells amplification and effector cytokine secretion.
3. Dissecting the mechanism(s) by which mIgA modulate human Th17 response.

Results

Intravenous Immunoglobulin Induces Autophagy in Immune cells

(Article I)

Das M, Stephen-Victor E, Gilardin L, Lecerf M, Bhatt B, Balaji KN, Benveniste O, Kaveri SV, Bayry J

Intravenous Immunoglobulin Induces Autophagy in Immune cells

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Abstract

Intravenous Immunoglobulin (IVIG), a therapeutic preparation of pooled normal IgG, is increasingly used in the treatment of diverse autoimmune and inflammatory diseases. However, the means by which IVIG acts as immune regulator to achieve beneficial effects in patients is not clear and several mutually non-exclusive mechanisms have been proposed. Autophagy is an important biological process involving lysosomal degradation of damaged cellular components and misfolded proteins. There are several evidences that support the involvement of autophagy in the regulation of autoimmune and auto-inflammatory responses and in mediating prominent anti-inflammatory effects. Therefore, in view of well-established anti-inflammatory action of IVIG in diverse pathologies, we hypothesized that IVIG induces autophagy in immune cells that explains in part the mechanism(s) by which IVIG therapy benefits patients with autoimmune and inflammatory diseases. We report that IVIG therapy in autoimmune patients leads to induction of autophagy in peripheral blood mononuclear cells (PBMC). Further dissection of this process by using healthy donors' PBMC revealed that IVIG-mediated autophagy is restricted to inflammatory cells like monocytes, dendritic cells, and M1 macrophages but not in cells associated with Th2 immune response like M2 macrophages. Furthermore, IVIG-mediated autophagy induction is F(ab')₂-dependent and requires endocytosis of IgG. Mechanistically, IVIG induces autophagy by activating AMPK, beclin-1, class III PI3K and p38MAPK and by inhibiting mTORC. Accordingly, inhibition of PI3K, p38MAPK and AMPK abrogated IVIG-mediated induction of autophagy. Induction of autophagy by IVIG is of potential significance to its immunoregulatory action in autoimmune and inflammatory diseases.

Introduction

Intravenous immunoglobulin (IVIG) is a therapeutic preparation of normal human polyclonal IgG obtained from pools of plasma of several thousand healthy blood donors. In addition to its use in primary and secondary immune deficiencies, IVIG is increasingly used for the treatment of large number of autoimmune and inflammatory diseases (1, 2). Despite its therapeutic use for more than three decades, the immune-regulatory mechanisms responsible for the beneficial effects of IVIG have not been fully elucidated.

Several mutually non-exclusive mechanisms have been proposed to explain the immune-regulatory role of IVIG in autoimmune and inflammatory diseases. IVIG acts on different cells of the immune system, including dendritic cells (DCs), monocytes/macrophages, granulocytes, natural killer cells, B-cells and various subset of effector T-cells to inhibit their activation and enhancing anti-inflammatory processes (3-10). In addition, IVIG also modulates complement and cytokine network. Together, IVIG exerts immune-regulatory effects in autoimmune and inflammatory conditions without suppressing the immune system, which provides a strong immunological rationale for its therapeutic use.

Macroautophagy, hereafter referred to as autophagy, is an important biological process involving lysosomal degradation of unnecessary or dysfunctional cellular components and misfolded proteins. During this process, targeted cytoplasmic constituents are isolated from the rest of the cell within a double-membraned vesicle known as an autophagosome. The autophagosome then fuses with a lysosome and its cargo is degraded and recycled (11, 12). A series of autophagy-related proteins (ATG) and several kinases play an important role in the regulation of autophagy. While mammalian target of rapamycin (mTOR) and AKT negatively regulate autophagy process, kinases such as phosphoinositide 3-kinase (PI3K) class III, AMP-dependent protein kinase (AMPK), extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK) positively regulate components of autophagy machinery. p38 mitogen-activated protein kinase (p38 MAPK) has a dual role in regulating autophagy (13).

Autophagy was initially investigated as a process involved in the 'cell survival' and nowadays is recognized as fundamental in the physiology of eukaryotic cells. Autophagy plays a critical role in the maintenance of cellular organization (14) and in the quality control of intracellular proteins, as demonstrated by the accumulation of ubiquitinated proteins in autophagy-deficient hepatocytes (15) and neuronal cells (16, 17), causing defects in cell function and toxicity (18).

Defective autophagy process is linked to severe pathologies, including metabolic disorders, neurodegenerative diseases and cancer (14, 19, 20). Autophagy also plays a fundamental role in the regulation of innate and adaptive immune responses (21), lymphocyte differentiation, survival and homeostasis (21-24). There are several evidences that support the involvement of autophagy in autoimmune and auto-inflammatory disorders including the discovery of polymorphisms in autophagy-related genes. Disorders such as systemic lupus erythematosus, inflammatory bowel diseases, rheumatoid arthritis, psoriasis, multiple sclerosis and myositis (25-33) were suggested to be associated with defects in autophagy process. Moreover, in some of these diseases, the therapeutic use of IVIG is well documented (1, 34-36). Therefore, in view of prominent anti-inflammatory effects of autophagy, we explored whether IVIG modulates autophagy in immune cells that explains in part the mechanism(s) by which IVIG therapy benefits patients with autoimmune and inflammatory diseases.

Materials and Methods

Reagents and antibodies

The mAbs anti-CD3 and anti-CD28 and the cytokines IFN- γ , M-CSF and IL-13 were from R&D Systems (Lille, France). CD14 and CD4 magnetic beads, GM-CSF and IL-4 were from Miltenyi Biotec (Paris, France). MAbs to LC3II, β -actin, GAPDH, PI3K class III, Beclin-I, mTOR, p-mTOR, p38MAPK, p-p38MAPK, AMPK and p-AMPK were from Cell Signaling Technology (Ozyme, Saint Quentin Yvelines, France). Plasma-derived human serum albumin (HSA) was from Laboratoire Française de Biotechnologies (Les Ulis, France).

Autoimmune Patients

Heparinized blood samples of patients with inflammatory myopathies were obtained before and 36-48 hours following initiation of IVIG therapy. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and processed for immunoblot. We analyzed five patients aged between 50 ± 15 years and include two men. They had polymyositis, Dermatomyositis, immune-mediated necrotizing myopathy (IMNM) and ant-isynthetase syndrome. They were treated with 2g/kg of IVIG for 48 hours. The study was approved by CPP-Ile-de-France VI, Groupe Hospitalier Pitié-Salpêtrière, Paris.

Purification of immune cells from healthy donors

PBMCs were obtained from the buffy bags of healthy donors by Ficoll density gradient centrifugation. Buffy bags were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (Institut National de la Santé et de la Recherche-EFS ethical committee convention 15/EFS/012). Monocytes and CD4⁺ T cells were isolated from the PBMCs by positive selection using human CD14 and the CD4 MicroBeads respectively. The cell purity was more than 97%.

Generation of DCs, M1 and M2 macrophages

DCs were generated from monocytes by differentiating them in the presence of GM-CSF (1000 IU/10⁶ cells) and IL-4 (500 IU/10⁶ cells) for 5 days.

To obtain macrophages, monocytes were cultured in the presence of M-CSF (200 ng/10⁶ cells) for 6 days. These macrophages were further polarized into either M1 or M2 macrophages by culturing in the presence of LPS (200 ng/10⁶ cells) and IFN- γ (40 ng/10⁶ cells), and IL-4 (40 ng/10⁶ cells) and IL-13 (40 ng/10⁶ cells) respectively for 3 additional days.

Cell line

THP-1, a pro-monocytic cell line, were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (both from Life Technologies) at 37 °C in 5 % CO₂ incubator. THP1 cells were treated with IVIG (25 mg/ml) or equimolar concentrations of HSA for 24 hours. In case of inhibitor treatment, cells were pre-treated with inhibitors for 1 hrs followed by culture with IVIG for 24 hours. Cells were treated with bafilomycin for last 45 mins.

Therapeutic IVIG

Sandoglobulin® was used throughout the study. IVIG was dialyzed against large volumes of PBS followed by RPMI-1640 at 4 °C for 18 hours. F(ab')₂ fragments were generated by digesting IVIG with pepsin (Sigma Aldrich) at 50:1 ratio for 18 hours in 0.2 M sodium acetate buffer (pH 4.1). F(ab')₂ fragments were extensively dialyzed against sterile PBS followed by RPMI-1640 medium at 4 °C and filtered through 0.22 μ m membrane. Purity of F(ab')₂ fragments were verified by SDS-PAGE and Coomassie blue staining. Peripheral blood monocytes or monocyte-derived DC and macrophages (M1 and M2) were treated with various concentrations of IVIG (10 mg/ml and 25 mg/ml) or equimolar concentrations of F(ab')₂ fragments of IVIG for indicated time points. CD4⁺ T cells were stimulated with plate bound

anti-CD3 (1 mg/ml) and soluble anti-CD28 (1 mg/ml) mAbs and cultured in X-VIVO medium. CD4⁺ T cells were treated with different dose of IVIG for 5 days. All cells were treated with bafilomycin (50 nM) for last 45 mins. The equimolar concentrations of HSA (0.15 mM) was used as an irrelevant protein control.

As a positive control for autophagy, immune cells were cultured in Hank's Balanced Salt Solution (HBSS) for 4 hours to induce autophagy by starvation.

Immunoblotting analysis

Treated cells were pelleted and lysed in RIPA buffer (Sigma) containing protease and phosphatase inhibitors (Roche Biotech) on ice for 30 min. Whole-cell lysates were collected. Proteins were quantified and equal amount of proteins from each sample was separated on SDS-PAGE. The proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes by a semi-dry Western blotting method. The membrane was blocked using 5% non-fat dry milk powder in TBST (20 mM Tris-HCl [pH 7.4], 137 mM NaCl, and 0.1% Tween 20) for 60 min. The blots were incubated overnight at 4 °C with primary antibody diluted in TBST-5 % BSA followed by incubation with HRP-conjugated anti-rabbit IgG secondary antibody for 2 hours. After washing in TBST, blots were developed with an ECL detection system (PerkinElmer) according to manufacturer's instructions. β -actin or GAPDH was used as loading control.

Immunofluorescence

Treatment conditions were mentioned in Cell line section

Treated THP1 cells were collected, fixed with 4 % paraformaldehyde and washed with PBS. After blocking with blocking buffer (1X PBS, 5 % FBS and 0.3 % Triton X-100), cells were stained at 4°C with specific antibodies diluted in antibody dilution buffer (1X PBS, 1 % BSA and 0.3 % Triton X-100) for overnight. Cells were washed thoroughly three times and incubated with daylight 488-conjugated secondary antibody for 2 hours. The nuclei were stained with DAPI for 10 mins. Cells were spread on coverslip and mounted with glycerol as a medium. Confocal images were taken using Zeiss LSM 710 meta confocal laser scanning microscope using a Plan-Achromat 63 \times /1.4 Oil DIC objective (both from Carl Zeiss). Images were analyzed using ZEN 2009 software.

Treatment of monocytes with pharmaceutical inhibitors

Different pharmaceutical inhibitors i.e., 3-methyladenine (3MA, for PI3K class III), SB202190 (for p38MAPK) and compound C (Comp C, for AMPK) were used in this study. Cytochalasin D (Cyto D) was used to block endocytosis. The concentration of inhibitors was chosen after careful titration experiments assessing the vitality of the monocytes. Monocytes were pre-treated with 3MA (1 mM and 5 mM), SB202190 (10 μ M), compound C (5 μ M) and cytochalasin D (10 μ M) for 1 hour with inhibitors followed by culture with IVIG for 24 hrs. The cells were then processed for immunoblot.

Statistical analysis

Statistical analyses were performed by two-way non-parametric Mann–Whitney test or one-way ANOVA (Kruskal–Wallis test) as indicated using Prism 6 software. $P < 0.05$ was considered as statistically significant.

Results

IVIG therapy induces autophagy in autoimmune patients

Earlier studies have indicated that IVIG therapy benefits patients with inflammatory myopathies (35, 36). Therefore, we first investigated if IVIG therapy induces autophagy in these patients. PBMC from paired blood samples of the patients (pre- and post-IVIG therapy) were subjected to western blot for the analysis of LC3II expression, one of the well-characterized markers of autophagy (37). Prior to IVIG therapy, patients' PBMC showed minimal expression of LC3-II. However, IVIG therapy led to significantly enhanced expression of LC3-II in the patients' PBMC (Figure 1A-B) indicating that IVIG treatment induces autophagy in autoimmune patients.

IVIG induces autophagy in peripheral blood mononuclear cells of healthy donors

In order to further delineate the process of autophagy induced by IVIG, we resorted PBMC of healthy donors. PBMC were cultured with different doses of IVIG (10 and 25 mg/ml) for 24 hours and analyzed for the expression of LC3-II. Untreated PBMC showed minimal expression of LC3-II and its expression was not altered by equimolar concentrations of HSA, an irrelevant protein control for IVIG. However, treatment of PBMC with IVIG led to significantly enhanced expression of LC3-II (Figure 2A-B). Further the effect of IVIG on autophagy was dose-dependent. High expression of LC3-II was observed at 25 mg/ml, a concentration of IVIG correspond to level of IgG reached in autoimmune patients immediately following therapy.

IVIG induces autophagy in monocytes, dendritic cells, CD4⁺ T cells, M1 macrophages but not in M2 macrophages

We then explored if ability of IVIG to induce autophagy is universal for all types of immune cells. To address this question, we exposed peripheral blood monocytes and monocyte-derived DC, M1 macrophages and M2 macrophages to different doses of IVIG (10 and 25 mg/ml) for 24 hours and activated CD4⁺ T cells for 5 days. We found that among innate immune cells, IVIG-mediated autophagy as analyzed by LC3-II expression by immunoblot, is restricted to inflammatory cells like monocytes, DC and M1 macrophages but not in cells associated with Th2 immune response like M2 macrophages (Figure 3A-B, D-E). In addition, CD4⁺ T cells, the cells of the adaptive immune compartment were also susceptible to IVIG-induced autophagy (Figure 3C). We further confirm that inability of IVIG to induce M2 macrophages is not due to technical errors or defects in the cells to undergo autophagy as starvation condition

could induce autophagy in M2 macrophages (Figure 3E). These results thus suggest that IVIG exhibits selective ability to induce autophagy and that all immune cells are not susceptible to IVIG-mediated autophagy.

To further authenticate IVIG-induced autophagy, we resorted to fluorescence microscopy to visualize LC3-II puncta in IVIG-treated THP-1 cells, a human monocytic cell line. We found significantly higher characteristic LC3 puncta formation in cells upon treatment with IVIG (Figure 3F), thus confirming the western blot results.

Induction of autophagy by IVIG is F(ab')₂-dependent

IgG consists of Fc and Fab regions. While some mechanisms of IVIG were dependent on Fc-fragments, other mechanisms were mediated via F(ab')₂ fragments. Therefore, we investigated the role of F(ab')₂ fragments to dissect the mechanisms of IVIG-induced autophagy. All the experiments for the dissection of mechanisms was performed on monocytes. Monocytes were cultured with equimolar concentrations of either IVIG or F(ab')₂ fragments for 24 hours. Interestingly, F(ab')₂ fragments of IVIG induced significantly higher expression of LC3-II. Notably, the autophagy induction by F(ab')₂ fragments was comparable to that of intact IVIG (Figure 4A-B). These results thus demonstrate that autophagy induction by IVIG is F(ab')₂-dependent.

IVIG-mediated autophagy is associated with induction and activation of AMPK, beclin-1, class III PI3K and p38MAPK, and inhibition of mTORC activation

Several kinases and signaling molecules play an important role in the regulation of autophagy (11, 13). Therefore, to gain further insight on IVIG-mediated autophagy, we probed the expression and activation status of various signaling molecules of autophagosome formation that are modulated by IVIG. We found that IVIG significantly reduced phosphorylated form of mTORC without modulating total mTORC expression (Figure 5A and B). On the other hand, IVIG induced Beclin-1 and class III PI3K and significantly enhanced phosphorylation of p38MAPK and AMPK (Figure 5A, C-F).

Inhibition of Class III PI3K, AMPK and p38MAPK activation leads to abrogation of IVIG-induced autophagy

To validate the role of Class III PI3K, AMPK and p38MAPK kinases in IVIG-mediated

induction of autophagy, monocytes were pre-treated with different pharmaceutical inhibitors i.e., 3-methyladenine (3MA, for PI3K class III), SB202190 (for p38MAPK) and compound C (comp C, for AMPK) followed by culture with IVIG for 24 hours. In line with the data on activation of these kinases by IVIG (Figure 5), inhibition of Class III PI3K, AMPK and p38MAPK kinases led to abrogation of IVIG-mediated autophagy as analyzed by LC3-II protein expression in western blot (Figure 6A-C). These results were further confirmed by LC3-II puncta analysis in IVIG-treated THP-1 cells wherein pre-treatment with 3MA, SB202190 and comp C significantly abolished IVIG-mediated induction of autophagy (Figure 6D, E). These results thus demonstrate that IVIG induces autophagy through activation of PI3K class III, p38MAPK and AMPK kinases.

Endocytosis of IgG is mandatory for IVIG-mediated autophagy in innate immune cells

Several reports have shown that innate immune cells internalize IVIG by endocytosis (38, 39). We therefore investigated whether signaling on the surface of innate immune cells by IgG is sufficient or it requires endocytosis of IgG for inducing autophagy. To probe this hypothesis, monocytes were pretreated with cytochalasin D, an inhibitor of endocytosis followed by culture with IVIG for 24 hrs. As shown in Figure 7A and B, cytochalasin D treatment abrogated IVIG-mediated LC3-II protein expression indicating that endocytosis of IVIG is mandatory for the induction of autophagy in innate cells like monocytes.

Discussion:

Several reports have demonstrated that a number of immunological processes are highly dependent on autophagy, including pathogen recognition and destruction, antigen presentation, lymphocyte development and effector functions of immune cells (21-24). Various pro-inflammatory cytokines, including IFN- γ TNF- α , IL-1 and IL-23 induce autophagy (40, 41), while Th2 and regulatory cytokines, including IL-4, IL-13 and IL-10 are inhibitory (42, 43). Although autophagy is regulated by cytokines, autophagy itself can directly influence the transcription, processing and secretion of a number of cytokines and chemokines (44). For example, autophagy can modulate the secretion of members of the IL-1 cytokine family, IL-23 and, as a consequence, IL-17 (40). The regulation of inflammatory cytokine secretion by autophagy is critical for the prevention of autoimmune and auto-inflammatory diseases (45-48). In fact, malfunctions in the autophagic pathway have been linked to several autoimmune diseases. It is well established that compromised autophagy is linked with Crohn disease (CD), a chronic inflammatory condition that is a common form of IBD. Genetic variants of important autophagy genes such as ATG16L1, immunity-related GTPase family M protein (IRGM), and ULK1, have been reproducibly associated with susceptibility and clinical outcome of the disease, demonstrating the important role of autophagy in CD (26-28). Several studies revealed that defective Paneth cell function (49), impaired bacterial defense (50, 51) and aberrant antigen presentation (52, 53) have been linked to ATG16L1 variant. Further, genetic association studies have demonstrated autophagy as an important process in SLE, as several mutations have been identified in autophagy-related genes that confer susceptibility to this disease, including IRGM (29, 30). It is well documented that autophagy is important in T-cell development, function and homeostasis. Defects in autophagy genes alter the activity of T cells and enhances T cell death in the context of SLE (54, 55).

Autophagy is controlled by products of numerous Atg and several macromolecular signaling complexes including mTOR (13). Inhibition of mTOR is essential for autophagy to initiate, and allows the translocation of a complex containing Atg1/unc-51-like kinase (ULK)1/2, Atg13, FIP200 and Atg101 from cytosol to the endoplasmic reticulum (56). This leads to the recruitment of the type III PI3K, VPS34, in a complex with other proteins, including beclin-1, to the developing autophagosome (11, 13). Rapamycin that inhibits mTOR and enhances autophagy has been successfully used in the treatment of autoimmune and auto-inflammatory diseases (57-59). In lupus-prone mice, rapamycin treatment prevented development of

nephritis, inhibiting lymphoproliferation and MCP-1 expression in kidneys,¹³³ reducing autoantibody production and enhancing survival of mice (60, 61). Recent report showed that rapamycin decreases the severity of myositis as curative and preventative treatment in murine model of experimental myositis. The improvement in the disease phenotype was associated with decrease in the frequency of effector T cells and a relative increase in Tregs (62) In view of these data from rapamycin and well demonstrated beneficial effects of IVIG therapy in inflammatory myopathies led us to conclusion that IVIG might induce autophagy in immune cells and hence represents a novel mechanisms of action of IVIG in autoimmune and inflammatory conditions. In fact, we found that IVIG therapy induces autophagy in immune cells and was associated with inhibition of mTOR phosphorylation.

IVIG has been demonstrated to modulate the functions of both innate and adaptive immune cells. Thus, IVIG was reported to suppress the activation of various innate cells such as DCs, macrophages and monocytes and secretion of inflammatory cytokines while enhancing anti-inflammatory mediators like IL-10 and IL-1RA. In addition, IVIG inhibits Th1 and Th17 cells, the effector T cells that are pathogenic in autoimmune diseases and reciprocally enhances regulatory T cells (4, 8). The present data demonstrate that IVIG exerts autophagy in various types of immune cells including innate cells and T cells that are previously shown to be modulated by IVIG. In view of diverse role of autophagy in regulating inflammation and autoimmune response, we propose that autophagy might be considered as a central process for various anti-inflammatory actions of IVIG. Although the induction of autophagy by IVIG was higher at concentrations corresponding to high-dose therapy in autoimmune diseases, autophagy was significantly induced by IVIG even at concentrations (10 mg) corresponding to circulating levels of IgG in healthy individuals. As IVIG is a pool of IgG from the plasma of healthy individuals, these results thus point towards a role for circulating normal IgG in the maintenance of immune homeostasis under physiological conditions.

While mTOR acts as negative regulator of autophagy, beclin-1 and several kinases like PI3K, p38MAPK and AMPK are critical for initiating autophagy although several studies also suggest that p38MAPK has dual role in autophagy (63-65). In this regard, the role of AMPK is of particular importance as phosphorylation of AMPK under the conditions of low cellular energy and essential amino acid deprivation directly antagonizes mTOR leading to initiation of autophagy (66, 67). Emerging evidences imply that AMPK is a key modulator of immune response. AMPK can reduce the severity of inflammation and tissue damage in various models

of autoimmune diseases (68-70). Also, AMPK suppresses endotoxin-induced IL-6 and TNF- α in neutrophils (71, 72) and can drive the induction of Tregs (73). In this study we show that IVIG-induced autophagy is associated with induction and activation of AMPK, beclin-1, class III PI3K and p38MAPK. It is not clear at this stage whether inhibition of mTOR by IVIG is mediated via AMPK or due to direct suppressive effects on mTOR. However, abrogation of IVIG-induced autophagy upon inhibition of AMPK implies that mTOR inhibition by IVIG was not due to its direct effect on this molecule rather AMPK-mediated process.

Exploration of mechanisms by which IVIG induce autophagy have revealed that F(ab')₂ fragments induce autophagy similar to intact IVIG. This indicates that glycosylation pattern of Fc-fragments including sialylation have no major role in IVIG-induced autophagy. Indeed several F(ab')₂ fragments-mediated functions of IVIG on immune cells have been reported regulation of human DC functions, phagocytosis of immune complexes, expansion of human regulatory T cells and inhibition of Th17 responses (3, 4, 8). However, we found that IVIG may not induce autophagy by signaling via cell surface receptors as inhibition of endocytosis of IgG via cytochalasin D that inhibits phagocytosis and macropinocytosis, abrogated IVIG-induced autophagy. Several reports have demonstrated that both innate cells and adaptive immune cells internalize IVIG (38, 39). As equivalent amount of HSA failed to induce autophagy, rules out the possibility that IVIG-induced autophagy is due to stress following accumulation of IgG in the endosomes. As IVIG has been demonstrated to interact with various cytoplasmic antigens (74), suggests that IVIG might induce autophagy either by modifying the autophagy cargo or interacting with molecules that promote autophagy. Further work towards this intracellular pathway is required.

In summary, our study demonstrates that IVIG therapy induces autophagy in patients with inflammatory myopathies. Further investigations revealed that IVIG-mediated autophagy is restricted to inflammatory cells like monocytes, dendritic cells and M1 macrophages but not in cells associated with Th2 immune response like M2 macrophages. Mechanistically, IVIG induces autophagy by F(ab')₂-dependent process via activation of p38MAPK, AMPK and class III PI3K and requires endocytosis of IgG. Induction of autophagy by IVIG is of potential significance to its immunoregulatory action in autoimmune and inflammatory diseases.

Figure Legends

Figure 1. IVIG therapy in inflammatory myopathy patients induces autophagy. (A) Expression of LC3-II in PBMC of myopathy patients before (pre-IVIG) and post-IVIG therapy as analyzed by western-blot (5 patients, P1-P5). (B) The fold changes in LC3-II expression based on densitometry analysis of western blots (mean \pm SEM, n= 5). **, P<0.01 two-tailed Mann–Whitney test.

Figure 2. IVIG induces autophagy in PBMCs of healthy donors. (A) PBMC from healthy donors were cultured with IVIG (10 or 25 mg/ml) or HSA for 24 hours. Expression of LC3-II was analyzed by western blot. Representative blot of 4 different donors is shown. (B) The fold changes in LC3-II expression based on densitometry analysis of western blots (mean \pm SEM, n= 4). **, P<0.01; ***, P<0.001; ns, not significant; as determined by one-way ANOVA.

Figure 3: IVIG induces autophagy in monocytes, dendritic cells, CD4+ T cells and M1 macrophages.

Peripheral blood monocytes (A), monocyte-derived DC (B), M1 macrophages (C) and M2 macrophages (D) were cultured with IVIG (10 and 25 mg/ml) for 24 hours. (E) CD4+ T cells were stimulated and cultured with IVIG (10 and 25 mg/ml) for 5 days. Expression of LC3-II was analyzed by western blot. The the fold changes in LC3-II expression based on densitometry analysis of western blots were also presented (mean \pm SEM, n= 4). Starvation condition (Stv) was used as positive control for autophagy. (F) THP1 cells were treated with IVIG or HSA for 24 hours. The cells were then stained with LC3II-specific primary antibody followed by Alexa 488-conjugated secondary antibody. Nuclei were stained with DAPI. Representative fluorescent images and percent of LC3-II punctuated cells were shown. Results are from 5 experiments **, P<0.01; ***, P<0.001; ns, not significant; as determined by one-way ANOVA.

Figure 4: F(ab')₂ fragments of IVIG induce autophagy. (A) Monocytes were cultured with IVIG (25 mg/ml) and equimolar concentration of F(ab')₂ for 24 hours. Expression of LC3-II was analyzed by western blot. Representative blot of 4 different donors is shown. (B) The fold changes in LC3-II expression based on densitometry analysis of western blots (mean \pm SEM,

n= 4). **, P<0.01; ns, not significant; as determined by one-way ANOVA.

Figure 5: IVIG-mediated autophagy is associated with induction of class III PI3K, beclin-1, p-AMPK and p-p38MAPK and inhibition of p-mTORC1.

(A) Monocytes were cultured with IVIG (25 mg/ml) or HSA for 24 hours. Expression of p-mTORC1, mTORC1, beclin-1, p-p38MAPK, p38MAPK, AMPK, p-AMPK and PI3K class III was analyzed by western blot. Representative blots of 4 experiments by using monocytes from different donors are shown. (B-F) Densitometry analysis of fold changes (mean \pm SEM, n=4) in the expression of (B) p-mTORC1, (C) p-p38MAPK, (D) p-AMPK, (E) beclin-1, and (F) classIII PI3K. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant; as determined by one-way ANOVA.

Figure 6: Inhibition of Class III PI3K, p38MAPK and AMPK kinases abrogates IVIG-mediated autophagy.

(A-C) Peripheral blood monocytes were pretreated with (A) Class III PI3K inhibitor: 3MA, (B) p38MAPK inhibitor: SB202190 and (C) AMPK inhibitor: comp C followed by IVIG treatment for 24 hours. Expression level of LC3-II by western blot and densitometry analysis of fold changes (mean \pm SEM; n=4) are presented. (D-E) THP-1 cells were pretreated with respective inhibitors followed by IVIG treatment for 24 hours. Treated cells were stained with LC3-II primary antibody followed by Alexa 488-conjugated secondary. Nuclei were stained with DAPI. Representative fluorescent images and percent (mean \pm SEM; n=5) of LC3-II punctuated cells are shown. *, P<0.05; **, P<0.01; ***, P<0.00; as determined by one-way ANOVA.

Figure 7: Endocytosis of IgG is obligatory for the induction of autophagy by IVIG in innate immune cells.

Monocytes were pretreated with (A and B) Cyto D followed by IVIG treatment for 24 hours. Expression level of LC3-II by western blot and densitometry analysis (mean \pm SEM; n=4) of fold changes are shown. *, P<0.05; as determined by one-way ANOVA.

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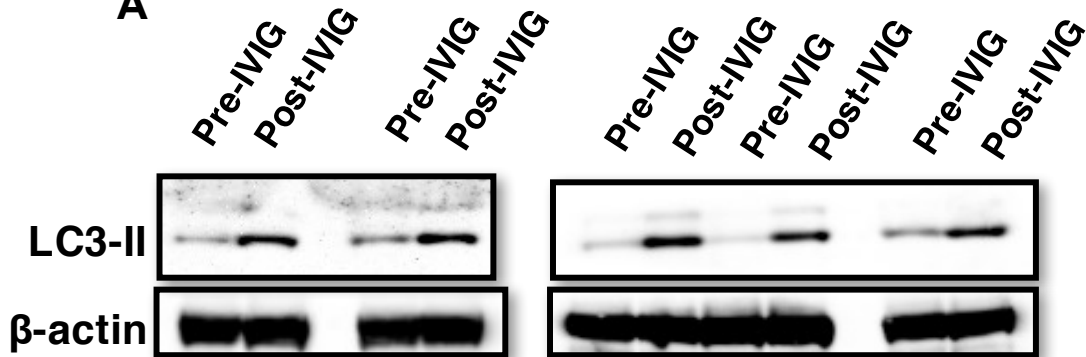
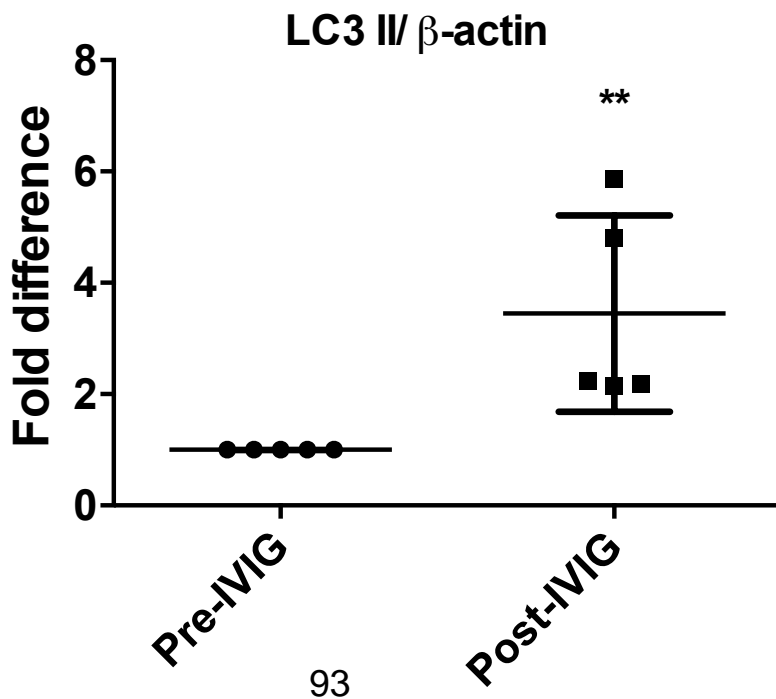
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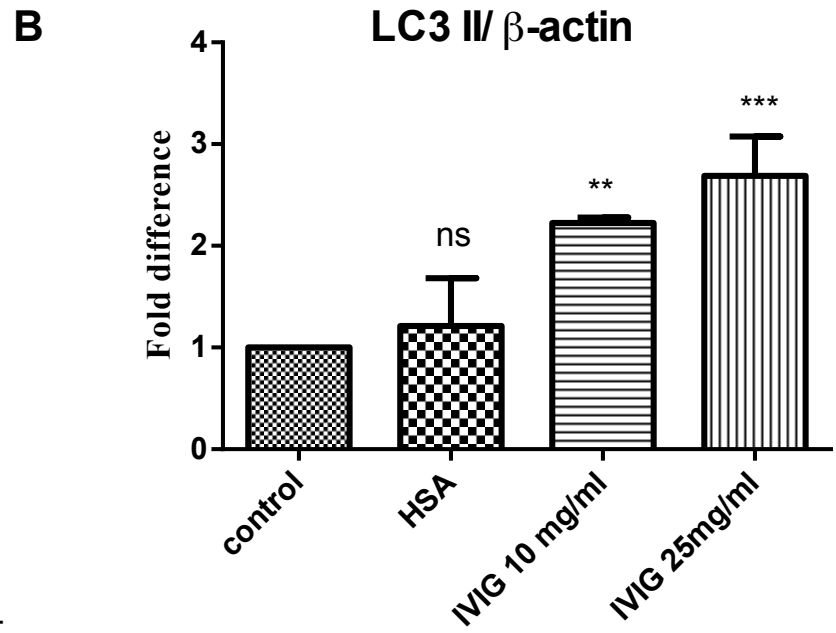
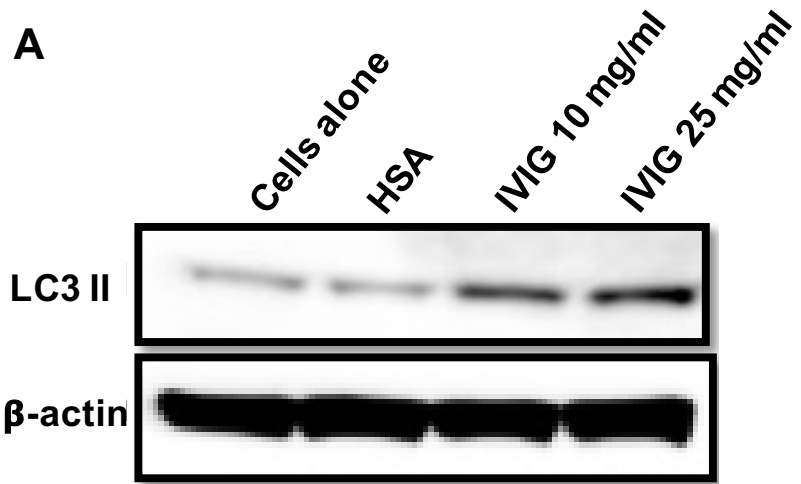
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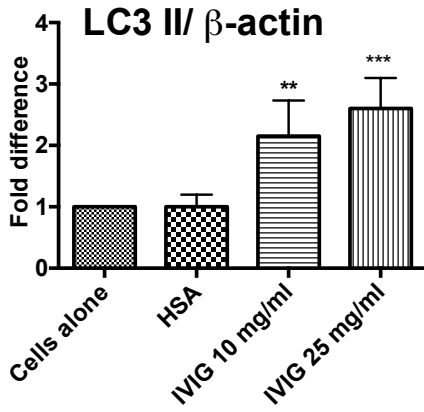
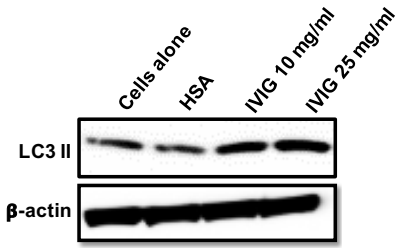
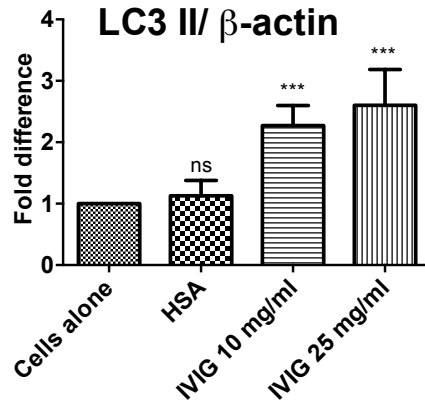
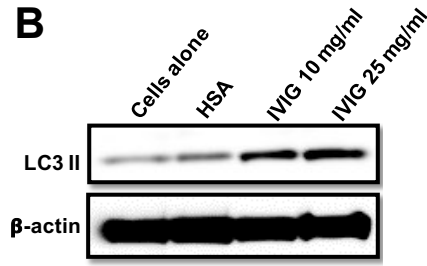
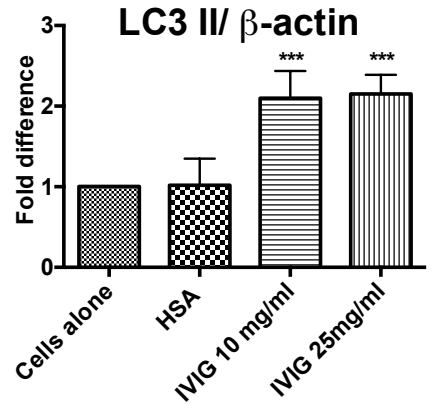
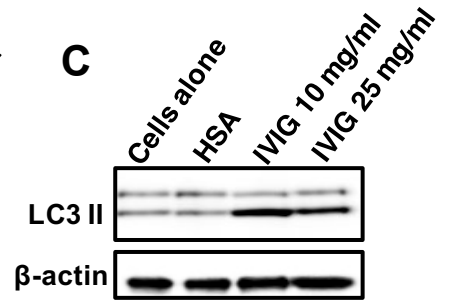
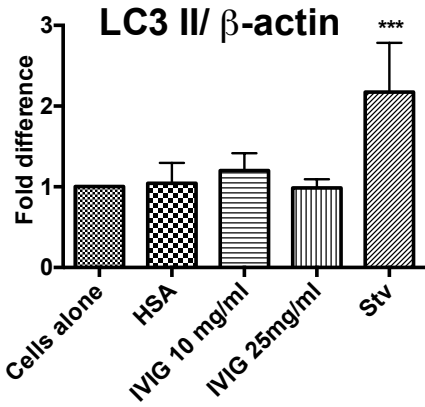
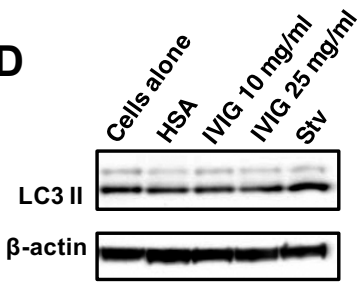
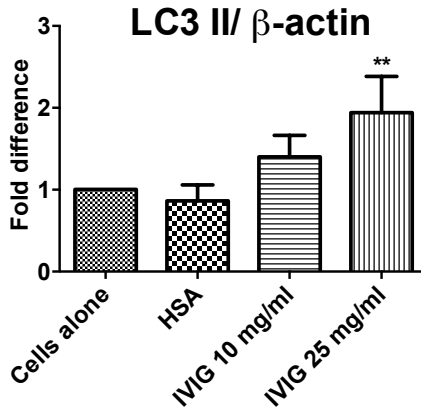
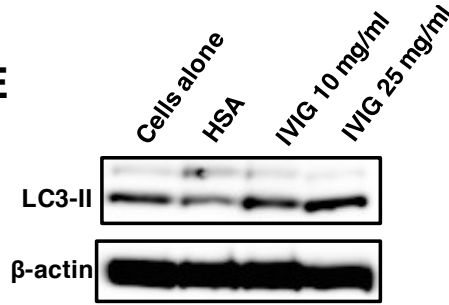
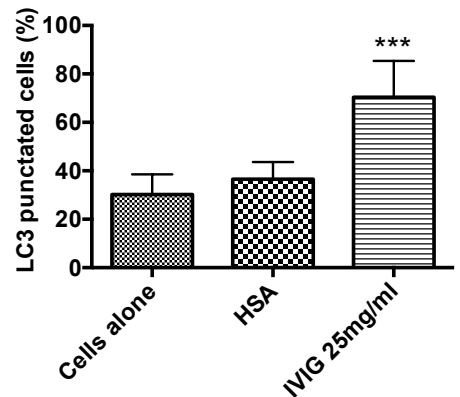
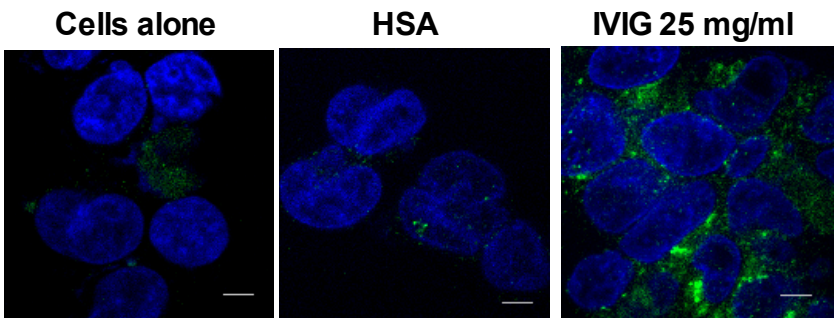
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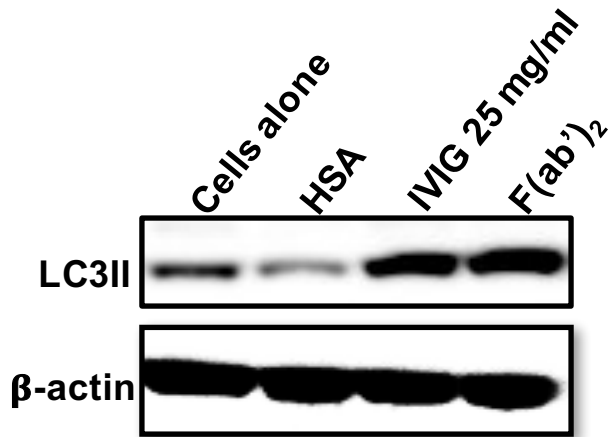
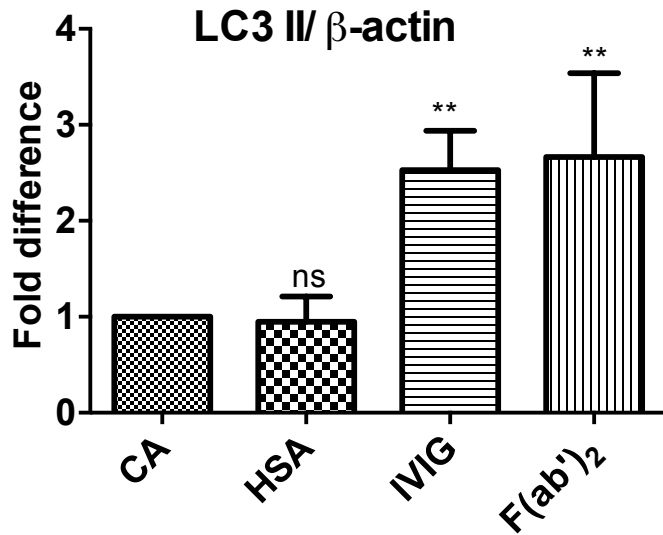
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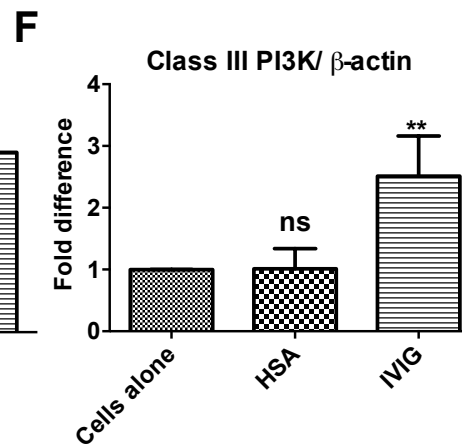
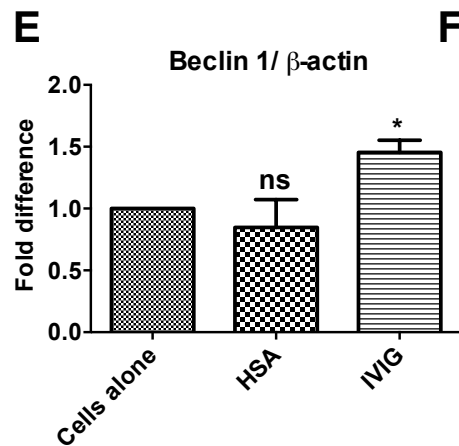
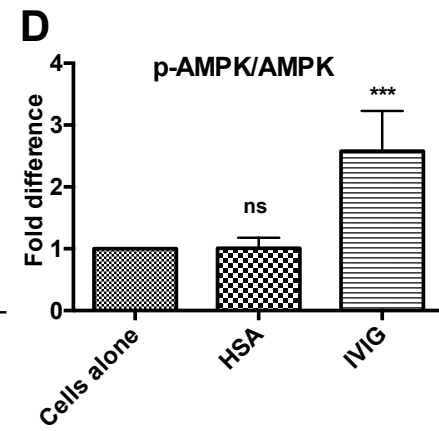
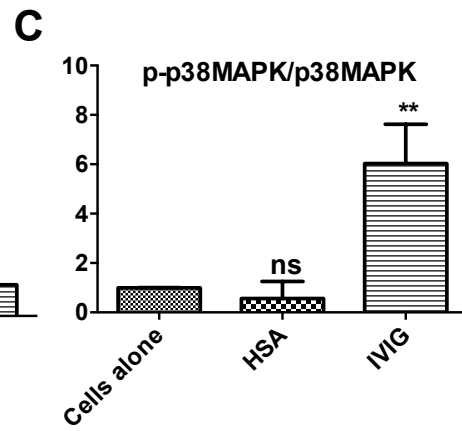
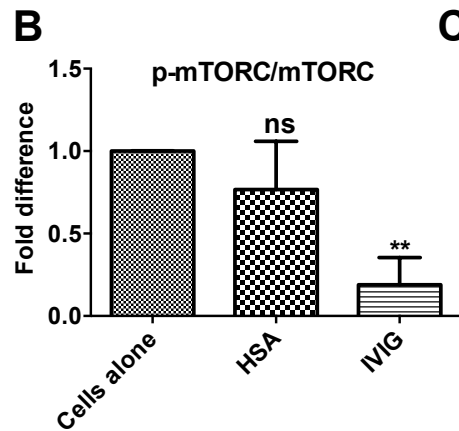
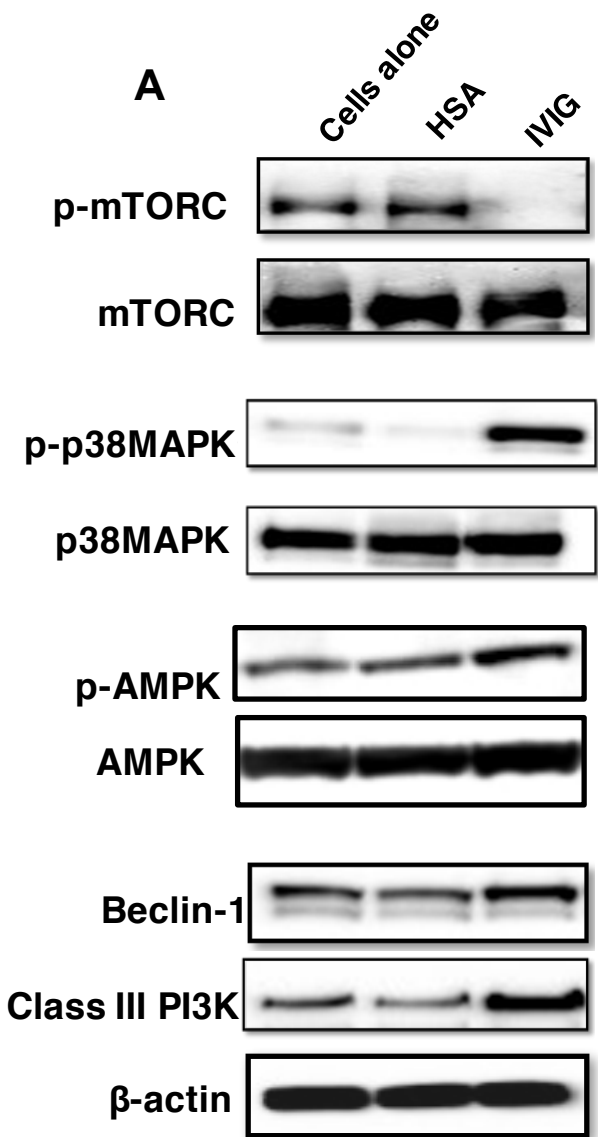
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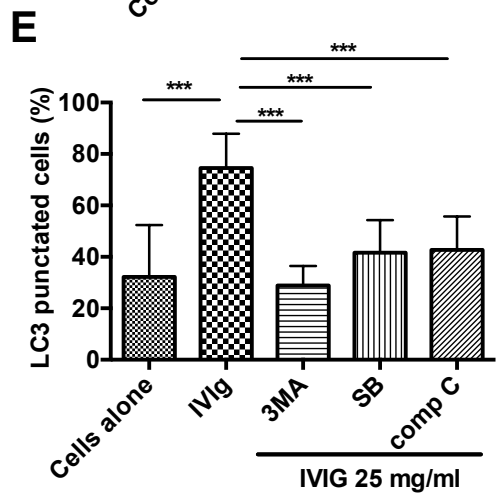
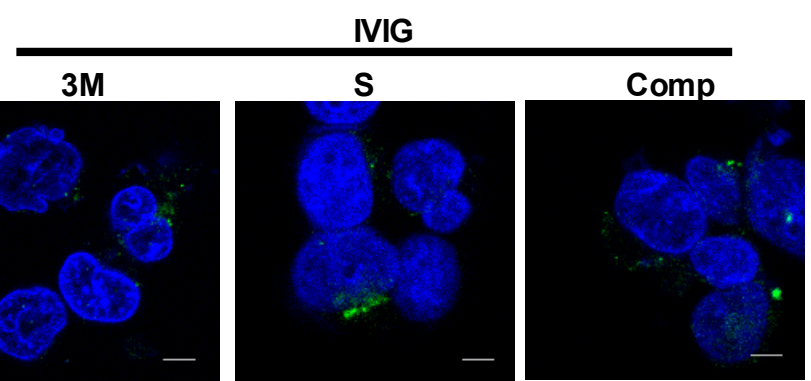
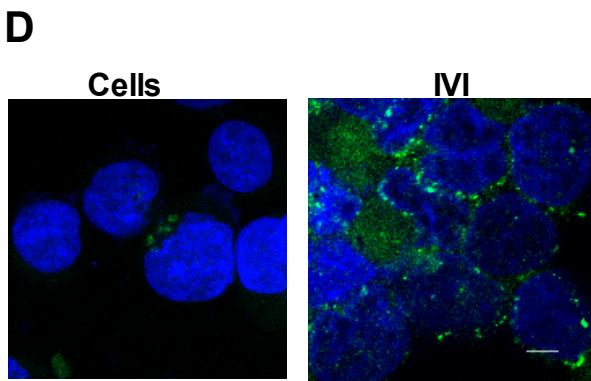
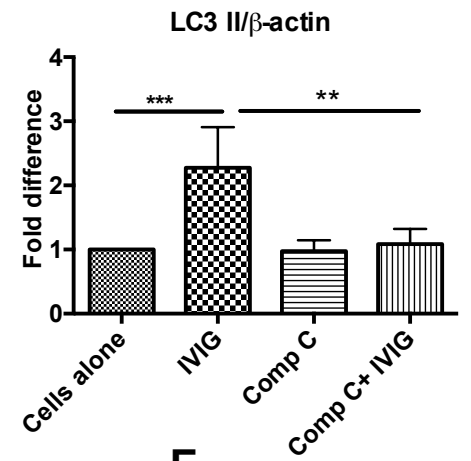
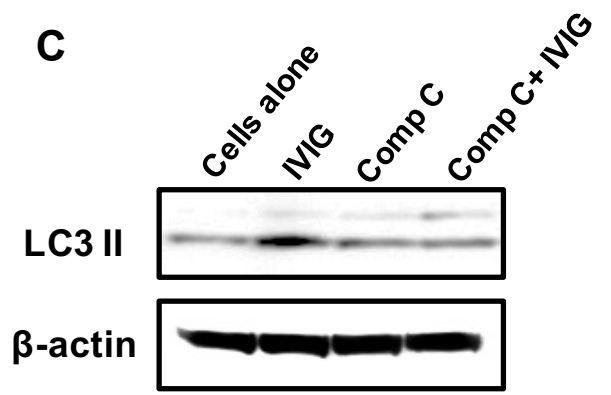
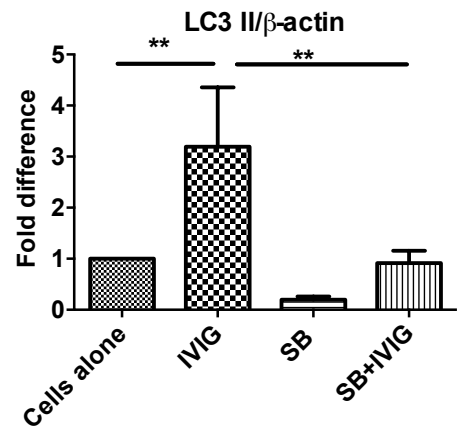
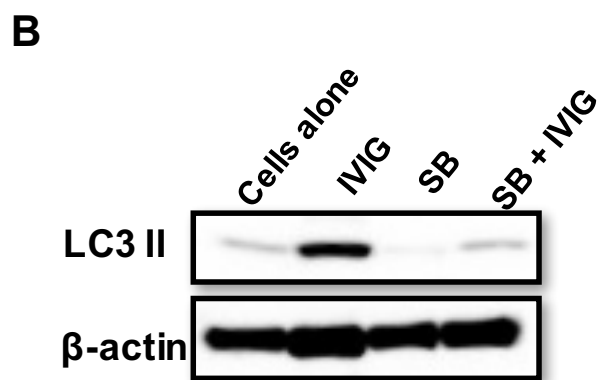
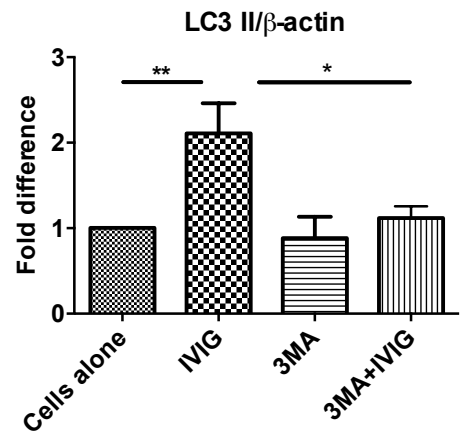
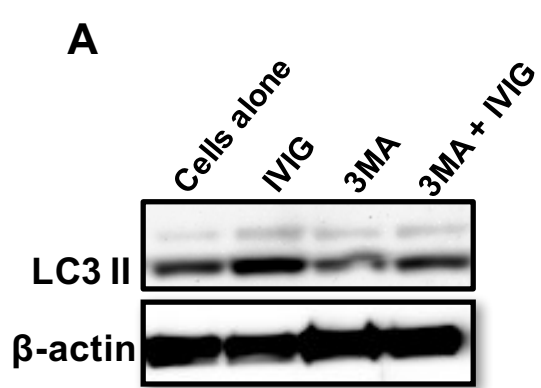
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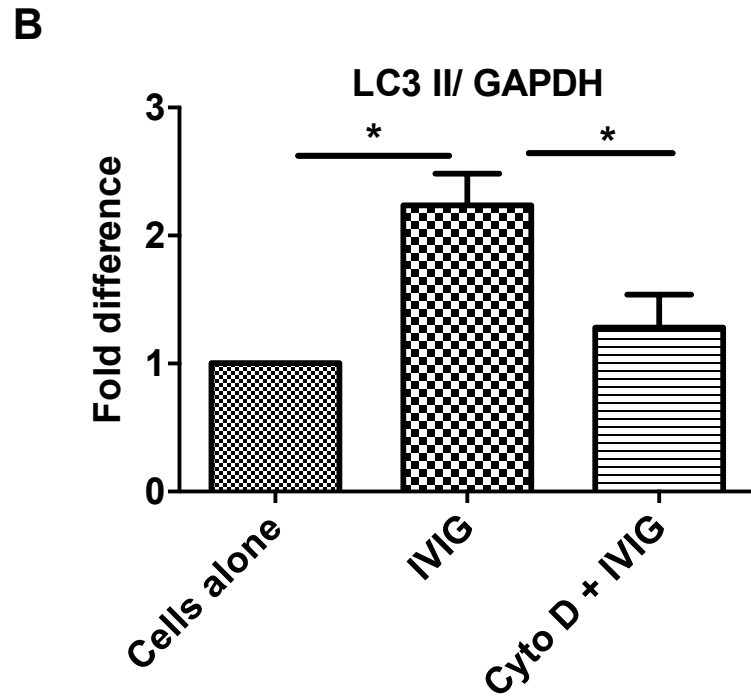
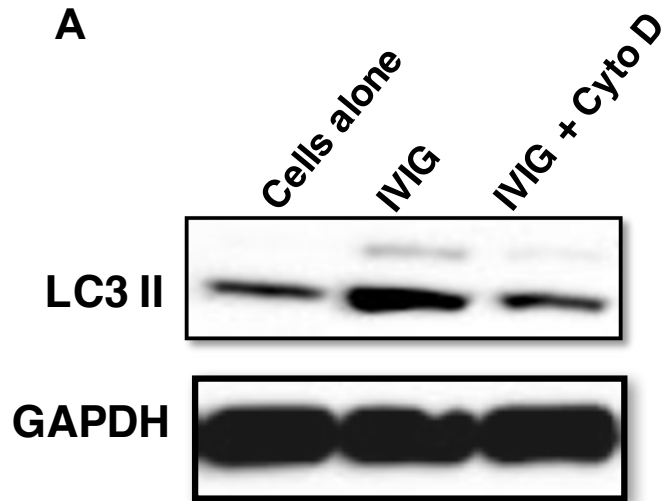


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Monomeric immunoglobulin A from plasma inhibits human Th17 responses *in vitro* independent of Fc α RI and DC-SIGN

(Article II)

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Monomeric Immunoglobulin A from Plasma Inhibits Human Th17 Responses *In Vitro* Independent of Fc α RI and DC-SIGN

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Circulating immunoglobulins including immunoglobulin G (IgG) and IgM play a critical role in the immune homeostasis by modulating functions of immune cells. These functions are mediated in part by natural antibodies. However, despite being second most abundant antibody in the circulation, the immunoregulatory function of IgA is relatively unexplored. As Th17 cells are the key mediators of a variety of autoimmune, inflammatory, and allergic diseases, we investigated the ability of monomeric IgA (mIgA) isolated from pooled plasma of healthy donors to modulate human Th17 cells. We show that mIgA inhibits differentiation and amplification of human Th17 cells and the production of their effector cytokine IL-17A. mIgA also suppresses IFN- γ responses under these experimental conditions. Suppressive effect of mIgA on Th17 responses is associated with reciprocal expansion of FoxP3-positive regulatory T cells. The effect of mIgA on Th17 cells is dependent on F(ab')₂ fragments and independent of Fc α RI (CD89) and DC-SIGN. Mechanistically, the modulatory effect of mIgA on Th17 cells implicates suppression of phosphorylation of signal transducer and activator of transcription 3. Furthermore, mIgA binds to CD4⁺ T cells and recognizes in a dose-dependent manner the receptors for cytokines (IL-6R α and IL-1RI) that mediate Th17 responses. Our findings thus reveal novel anti-inflammatory functions of IgA and suggest potential therapeutic utility of mIgA in autoimmune and inflammatory diseases that implicate Th17 cells.

Keywords: monomeric IgA, Th17, IL-17, natural antibodies, IVIG, Fc α RI, CD89, DC-SIGN, Treg

INTRODUCTION

CD4⁺ T-helper (Th) cells play an important role in the immune responses against both pathogens and self-antigens. Based on distinct cytokine and transcription factor profiles, several subsets of CD4⁺ Th cells have been elucidated. These include Th1, Th2, and CD4⁺CD25⁺ regulatory T cells (Tregs). In addition, Th17 cells that are characterized by lineage-specific transcription factor retinoic acid-related orphan receptor C (RORC) and the secretion of signature cytokine IL-17A are also identified (1). Phosphorylation of signal transducer and activator of transcription 3 (STAT3) is

essential for the differentiation and amplification of Th17 cells. Th17 cells have been shown to play an important role in the defense against extracellular pathogens and in the pathogenesis of a variety of autoimmune, inflammatory, and allergic diseases (1, 2). Therefore, modulation of Th17 responses represents one of the fast evolving therapeutic options for the treatment of autoimmune and systemic inflammatory diseases.

Immunoglobulin A (IgA) is the most prevalent antibody at mucosal sites and the second most abundant antibody in the circulation. Secretory IgA at mucosal sites is dimeric in nature. It has been proposed that high affinity secretory IgA prevents mucosal colonization of invading pathogens and low affinity secretory IgA regulates commensal bacteria (3, 4). In the circulation, IgA is mostly monomeric although nearly 10–20% of circulating IgA are presented as dimeric or polymeric in nature (5). Currently, three bona fide receptors have been identified for IgA. Fc α RI (CD89) expressed on various innate cells such as monocytes, macrophages, dendritic cells (DCs), and neutrophils is specific for IgA and mediates effector functions of IgA. The other two receptors, polymeric Ig receptor and Fc α / μ R recognize both IgA and IgM and are therefore not specific for IgA. In addition to the aforementioned receptors, two alternative IgA receptors have also been identified. They are the asialoglycoprotein receptor that is implicated in the catabolism of IgA, and the transferrin receptor that mediates deposition of IgA in tissues (6).

Although triggering of Fc α RI by IgA immune complexes or polymers induces activation of immune cells (7–9) *via* complete phosphorylation of tyrosine residues of immunoreceptor tyrosine-based activation motif (ITAM) within the associated Fc γ adaptors, naturally occurring monomeric IgA (mIgA) in the plasma was found to exert inhibitory effects on the activation of immune cells by triggering inhibitory ITAM (ITAMi) signaling through the associated Fc γ chain and recruitment of tyrosine phosphatase Src homology 2 domain-containing phosphatase-1 (SHP-1) (10–13). mIgA also induces death in activated neutrophils (14) and inhibits complement deposition mediated by anti-ganglioside antibodies (15). The anti-inflammatory effects of mIgA have been explored in various experimental models (10–13, 16).

Thus, so far anti-inflammatory effects of mIgA have been elucidated mainly in the context of innate immune cells and Fc α RI. It is not known whether anti-inflammatory effects of mIgA observed in various experimental models are solely due to the modulation of innate cells or also due to anti-inflammatory effects on the cells of adaptive immune compartment and particularly CD4⁺ T cells that are critical players in the pathogenesis of autoimmune and inflammatory diseases. Therefore, in view of emerging roles of Th17 cells in the pathogenesis of autoimmune, allergy, and inflammatory diseases, we explored the immunomodulatory role of mIgA isolated from the pooled plasma of healthy donors on the human Th17 cell differentiation, amplification, and secretion of effector cytokine IL-17A. Our data indicate that mIgA binds to CD4⁺ T cells independent of Fc α RI (CD89), and reciprocally regulates human Th17 and FoxP3-positive Treg cells. The effect of mIgA on Th17 cells is dependent on F(ab')₂ fragments and implicates suppression of phosphorylation of STAT3. Our data

thus reveal Fc α RI-independent immunomodulatory functions of naturally occurring mIgA and potential therapeutic utility of mIgA in autoimmune and inflammatory diseases that implicate Th17 cells.

MATERIALS AND METHODS

Cell-Culture Reagents and Antibodies

Anti-CD3 (clone UCHT1), anti-CD28 mAbs (clone 37407), and TGF- β 1 were procured from R&D Systems (Lille, France). IL-1 β , IL-6, and IL-21 were purchased from Immuno Tools (Friesoythe, Germany). Plasma-derived human serum albumin (HSA) was from Laboratoire Française de Biotechnologies (Les Ulis, France).

Immunoglobulins

Monomeric IgA and F(ab')₂ fragments of mIgA and IVIG (Privigen[®]) were provided by CSL Behring AG (Bern, Switzerland).

Monomeric IgA was derived from the AIEIX chromatographic step of the IVIG manufacture process of CSL Behring AG. Fraction F4 was obtained after a post-wash of the Macro-Prep High Q (BioRad, Hercules, CA, USA) column with 10 mM phosphate/30 mM acetate at pH 6.5 by elution with 55 mM tartrate/5 mM acetate at pH 7.6. Fraction F4 was then brought to approximately 1 mg/ml in PBS by ultra-/diafiltration and then depleted of IgG by affinity chromatography using an IgSelect resin (GE Healthcare, Glattbrugg, Switzerland). mIgA was directly harvested from the flow through fraction of the IgSelect chromatography and brought to its final formulation *via* ultra-/diafiltration of 48.5 g/l in PBS.

F(ab')₂ fragments from IgA were generated by solid phase pepsin digestion using pepsin-coupled beads (Thermo Fisher Scientific, Allschwil, Switzerland). The F(ab')₂ fragments were recovered by centrifugation. The supernatant was sterile filtered (0.45 μ m) and formulated in PBS using ultrafiltration centrifugal devices (30,000 Da MWCO; Sartorius, Tagelswangen, Switzerland). Purity and integrity were controlled by SDS-PAGE and SE chromatography.

The labeling of mIgA and IVIG was done with the Lightning-Link[®] Rapid DyLight[®] 650 kit (Innova Biosciences, Cambridge, UK) according to manufacturer's instructions.

Cell Purification

Buffy coats from the healthy donors were processed to purify peripheral blood mononuclear cells (PBMCs). Ethics committee approval for the use of such material (Institut National de la Santé et de la Recherche-EFS ethical committee convention 15/EFS/012) was obtained and experiments were performed in accordance with the approved guidelines of INSERM. The CD4⁺ T cell isolation kit-II (Miltenyi Biotec, Paris, France) was used to isolate untouched total CD4⁺ T cells by negative selection. Subsequently, CD45RA⁺ and CD45RO⁺ CD4⁺ T cells were separated by using CD45RO microbeads (Miltenyi Biotec). Furthermore, CD25⁺ cells were depleted from the CD45RA⁺ fraction by using CD25 microbeads (Miltenyi Biotec) to obtain CD4⁺CD25⁻CD45RO⁻ naïve T cells. The purity of all subpopulations was more than 96%.

Monocytes were isolated from PBMC by using CD14 microbeads (Miltenyi Biotec) and were cultured for 5 days

with GM-CSF (1,000 IU/million cells) and IL-4 (500 IU/million cells) (both from Miltenyi Biotec) for the differentiation into DCs (17).

T-Cell Stimulation and Culture

Forty-eight well flat bottom plates were coated with 1.5 µg/ml anti-CD3 mAb for at least 5 h at 37°C. At the end of incubation, the wells were rinsed once with RPMI-1640 medium. A total of 5×10^4 CD4⁺ T cells/well/500 µl were stimulated in serum-free X-VIVO 15 medium with soluble anti-CD28 mAb (1.0 µg/ml) in presence of cytokines including acid-treated TGF-β1 (5 ng/ml), IL-21 (25 ng/ml) for naïve T cells, and IL-1β (12.5 ng/ml) and IL-6 (25 ng/ml) for memory T cells (18). Indicated concentrations of IgA, F(ab')₂ fragments of IgA, IVIG, or HSA were added to the cells 12 h after the initiation of culture. The cells were cultured for 6 days at 37°C in 5% CO₂. The supernatants were collected at the end of experiments for cytokine analysis and the cells were used for intracellular staining.

Intracellular Staining for CD4⁺ T Cells and Cytokine Assays

Cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml, Sigma-Aldrich, Saint Quentin Fallavier, France) and ionomycin (500 ng/ml, Sigma-Aldrich) at 37°C for 6 h with GolgiStop (BD Biosciences Le Pont de Claix, France) for the last 3 h. Cells were surface stained with BV421-conjugated CD4 mAb (clone RPA-T4, BD Biosciences). Cells were fixed, permeabilized (Fix/Perm, eBioscience, Paris, France), and incubated with APC-conjugated anti-human Foxp3 (clone 236A/E7, eBioscience), PE-conjugated anti-human IL-17A (clone eBio64CAP17, eBioscience), and FITC-conjugated anti-human IFN-γ (Clone 4S.B3, BD Biosciences) at 4°C. Ten thousand cells were acquired for each sample and data were analyzed by using FACS DIVA and Flowjo softwares.

Intracellular staining for the phosphorylated STAT3 (pSTAT3) was carried out on stimulated CD4⁺ T cells at indicated time points. Cells were harvested and fixed in pre-warmed BD Cytotfix buffer by incubation for 10 min at 37°C. The cells were washed twice with staining buffer (1% fetal calf serum/PBS), permeabilized with chilled BD Phosflow Perm Buffer III on ice for 30 min. At the end of incubation, the cells were washed twice with staining buffer, incubated with PE-conjugated anti-STAT3 pY705 (clone 4/P-STAT3, BD Biosciences) at room temperature for 45–60 min. Cells were washed and resuspended in staining buffer before acquisition.

Amounts of IL-17A in the cell-free supernatants were quantified by ELISA (DuoSet ELISA kit, R&D Systems).

Analysis of Expression of CD89 and DC-SIGN

CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 mAbs for 24 h. Cells were surface stained with BV421-conjugated CD4 mAb and APC-conjugated CD89 (clone A59, BioLegend, London, UK) or FITC-conjugated DC-SIGN (clone DCN46, BD Biosciences) mAbs for 30 min at 4°C. Monocytes were stained with FITC-conjugated HLA-DR (clone TU36, BD Biosciences) and APC-conjugated CD89, while DCs were stained with

APC-conjugated HLA-DR (clone G46-6, BD Biosciences) and FITC-conjugated DC-SIGN mAbs. The expression of markers was analyzed by flow cytometry.

IgA- and IgG (IVIG)-Binding Assay

Total CD4⁺ T cells were stimulated with anti-CD3/CD28 mAbs for 24 hrs. To analyze the binding of IgA or IgG (IVIG) to CD4⁺ T cells, either resting or anti-CD3, anti-CD28-stimulated T cells were incubated with BV421-conjugated anti-human CD4, FITC-conjugated CD45RA (clone HI100, BD Biosciences), and BV510-conjugated CD45RO (clone UCHL1, BD Biosciences) antibodies and DyLight 650-conjugated-IgA or IgG (IVIG) (3 µg/10⁶ cells) for 30 min. Cells were analyzed by flow cytometry.

Immunoglobulin A and IgG binding to IL-1RI and IL-6Rα was measured by ELISA. ELISA plates were coated with recombinant IL-1RI (human IL-1Ra, expressed in 293E cells) and IL-6Rα (human IL-6Rα, expressed in insect cells) (1 µg/ml) (BioLegend) overnight at 4°C. After blocking with 5% BSA in PBS, 0.02% Tween 20 for 1 h at 37°C, wells were incubated with different concentrations of IgA, or IVIG for 2 h at 37°C. Following washings, the plates were incubated with horseradish peroxidase-labeled anti-human IgA or IgG for 2 h, followed by development with 3,3',5,5'-tetramethylbenzidine. The absorbance was measured at 450 and 570 nm. The data were analyzed by subtracting the absorbance of 570 nm from those of 450 nm.

Statistical Analysis

The data were analyzed by one-way ANOVA (repeated measures with Tukey's multiple comparison test) or two-tailed Student's *t*-test using GraphPad Prism software.

RESULTS

mIgA Inhibits Differentiation of Human Th17 Cells

CD45RA⁺CD25⁻ naïve CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the presence TGF-β and IL-21 for 6 days to differentiate Th17 cells. TGF-β is required to induce both FoxP3 and RORC in naïve T cells and to inhibit IFN-γ while IL-21 relieves RORC from the FoxP3 by inducing STAT3 activation (2, 18). Twelve hours after the initiation of culture, mIgA was added to the cells at various concentrations (5, 15, and 25 mg/ml). We observed that mIgA significantly inhibits the differentiation of human Th17 cells (**Figures 1A,B**) as analyzed by intracellular staining for IL-17A. Significant inhibitory effect was observed even at low concentrations (5 mg/ml) of mIgA. In addition, mIgA also inhibited the production of IL-17A, the signature cytokine of Th17 cells (**Figure 1C**). The suppressive effect of mIgA was similar to that of therapeutic intravenous immunoglobulin IgG (IVIG) (**Figures 1A–C**) that is used in the therapy of various autoimmune, inflammatory, and infectious diseases (19–23) and was previously shown to inhibit Th17 responses both in experimental models and in patients with autoimmune diseases (24–29).

The effect of mIgA on the inhibition of Th17 differentiation was specific, as equimolar concentration of HSA (10 mg,

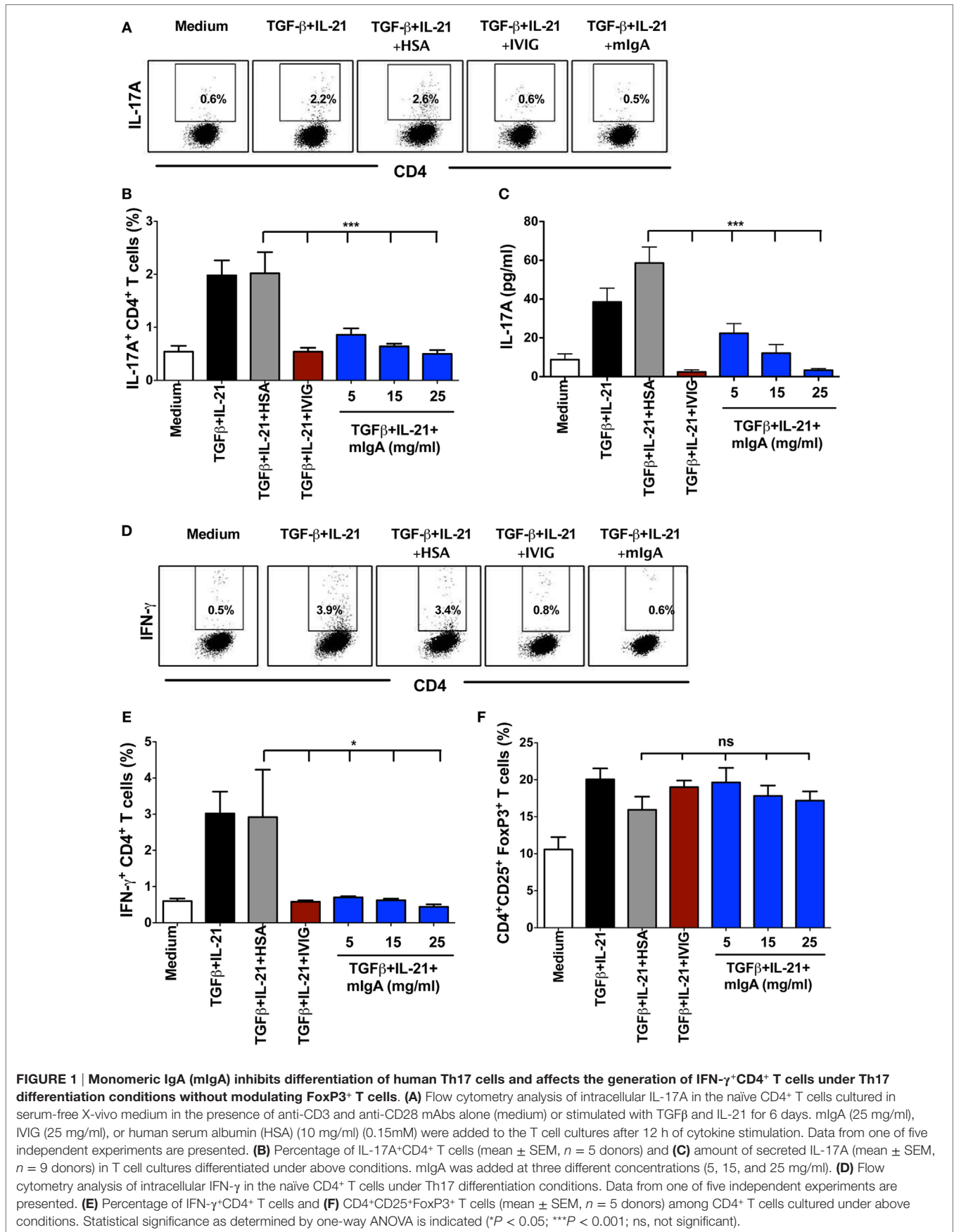


FIGURE 1 | Monomeric IgA (mIgA) inhibits differentiation of human Th17 cells and affects the generation of IFN-γ+CD4+ T cells under Th17 differentiation conditions without modulating FoxP3+ T cells. (A) Flow cytometry analysis of intracellular IL-17A in the naïve CD4+ T cells cultured in serum-free X-vivo medium in the presence of anti-CD3 and anti-CD28 mAbs alone (medium) or stimulated with TGFβ and IL-21 for 6 days. mIgA (25 mg/ml), IVIG (25 mg/ml), or human serum albumin (HSA) (10 mg/ml) (0.15mM) were added to the T cell cultures after 12 h of cytokine stimulation. Data from one of five independent experiments are presented. **(B)** Percentage of IL-17A+CD4+ T cells (mean ± SEM, n = 5 donors) and **(C)** amount of secreted IL-17A (mean ± SEM, n = 9 donors) in T cell cultures differentiated under above conditions. mIgA was added at three different concentrations (5, 15, and 25 mg/ml). **(D)** Flow cytometry analysis of intracellular IFN-γ in the naïve CD4+ T cells under Th17 differentiation conditions. Data from one of five independent experiments are presented. **(E)** Percentage of IFN-γ+CD4+ T cells and **(F)** CD4+CD25+FoxP3+ T cells (mean ± SEM, n = 5 donors) among CD4+ T cells cultured under above conditions. Statistical significance as determined by one-way ANOVA is indicated (*P < 0.05; ***P < 0.001; ns, not significant).

0.15 mM), used as protein control, did not alter Th17 differentiation (Figures 1A–C). Also, the inhibitory effect of mIgA on Th17 responses was not due to toxic effects of the immunoglobulins as we did not observe differences in the yield of cells in mIgA-treated conditions as compared to cytokine-treated control cells or HSA-treated cells.

We also analyzed the effect of mIgA on frequency of IFN- γ -secreting CD4⁺ T cells in the culture. We found that mIgA was equally effective to inhibit IFN- γ responses under Th17 differentiation conditions (Figures 1D,E). Further, similar to our previous report on IVIG (24), inhibition of Th17 differentiation by mIgA was not associated with the reciprocal enhancement of CD4⁺CD25^{high} FoxP3⁺ Tregs (Figure 1F). Thus, these results demonstrate that mIgA exhibits inhibitory effects on the differentiation of human Th17 cells.

mIgA Suppresses Amplification of Human Th17 Cells

For the amplification of human Th17 cells, CD45RO⁺ memory CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of IL-1 β and IL-6 for 6 days. By signaling through corresponding cytokine receptors, IL-1 β and IL-6 induce phosphorylation of STAT3 and amplify Th17 cells in the memory CD4⁺ T cell pool (2, 18). Similar to Th17 cell differentiation conditions, various concentrations of mIgA (5, 15, and 25 mg/ml) were added to the cells 12 h post initiation of culture. The pro-inflammatory cytokines IL-1 β and IL-6 significantly enhanced the frequency of IL-17A-producing cells as compared to memory CD4⁺ T cells stimulated with anti-CD3 and anti-CD28 mAbs alone (Figures 2A,B). Remarkably, mIgA significantly suppressed the amplification of Th17 cells (Figures 2A,B) and the amount of IL-17A (Figure 2C) produced by these cells. Its inhibitory effect was similar to that of high-dose IVIG (Figures 2A–C). Of note, the inhibitory effect of mIgA on IFN- γ responses under Th17 amplification conditions was observed only at the highest immunoglobulin concentration (Figure 2D).

Under Th17 amplification conditions, the inhibitory effect of mIgA on Th17 cells was associated with a reciprocal enhancement of FoxP3⁺ Tregs (Figure 2E). The effect of mIgA on Tregs was prominent at the highest concentration (25 mg/ml). These results thus suggest that both mIgA and IVIG exert similar modulatory effects on Th17 amplification.

Fc α RI (CD89) and DC-SIGN Are Dispensable for the Inhibition of Th17 Response by mIgA

Recent reports have indicated that Fc α RI (CD89) plays a major role in mediating anti-inflammatory effects of mIgA on innate immune cells (10–13, 16). In addition, SIGN-R1 on DCs was also implicated in the immunoregulatory functions of secretory IgA (30). To explore if Fc α RI and DC-SIGN (human counterpart of SIGN-R1) receptors are implicated in the inhibitory effect of mIgA, we investigated the expression of these two receptors on activated CD4⁺ T cells. We found that human CD4⁺ T cells were negative for both, Fc α RI and DC-SIGN (Figures 3A,B), thus

ruling out their implication in the inhibition of Th17 responses by mIgA. Importantly, monocytes and DCs that were used as positive controls stained with specific anti-Fc α RI and anti-DC-SIGN fluorescent antibodies, respectively (Figures 3A,B).

Inhibition of Th17 Response by mIgA Implicates F(ab')₂ Fragments

Lack of expression of Fc α RI and DC-SIGN on CD4⁺ T cells raised an important prospect that suppressive effects of mIgA on Th17 cell responses might be mediated *via* F(ab')₂ fragments. Indeed, we found that F(ab')₂ fragments of mIgA significantly inhibited the frequency of IL-17A-positive T cells under Th17 differentiation conditions (Figure 3C). This effect was also associated with significant downregulation of IFN- γ -secreting CD4⁺T cells in the culture (Figure 3D).

mIgA Binds to CD4⁺ T Cells

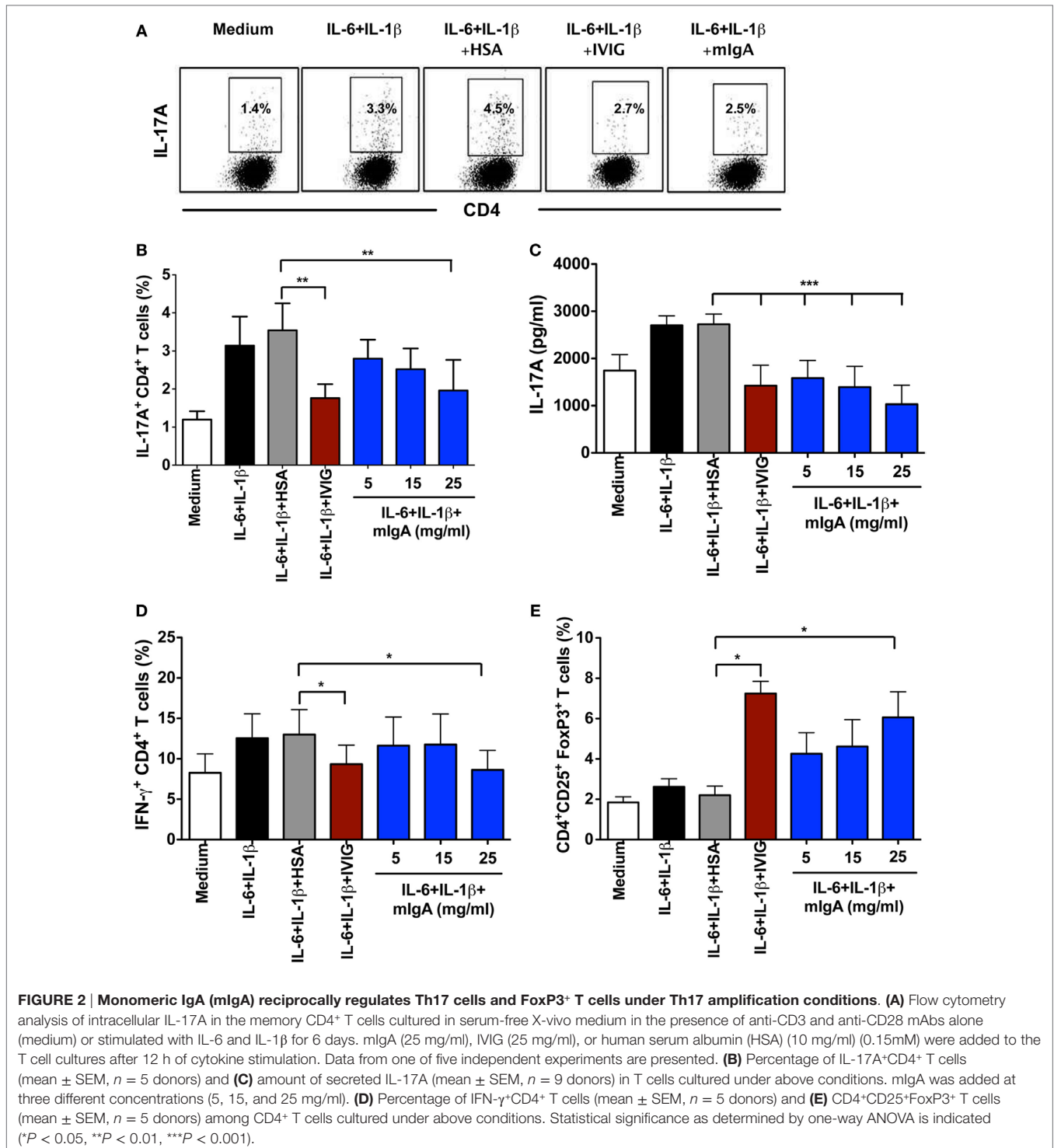
Natural antibodies that recognize various self-motifs have been identified (31–34). Therefore, to further understand the mechanisms underlying the inhibitory effect of IgA on Th17 responses, we analyzed the binding of mIgA to CD4⁺ T cells by flow cytometry. We found that nearly 45% of the stimulated CD4⁺ T cells ($n = 9$ donors) were positive for mIgA binding (Figures 4A,B). mIgA however did not bind to resting CD4⁺ T cells ($n = 3$ donors) suggesting that activation signals license T cells for immunoglobulin binding. Further, the extent of binding of mIgA was similar for CD45RA⁺ naive and CD45RO⁺ memory CD4⁺ T cells (Figures 4C,D). This finding indicates that naturally occurring mIgA exerts its immunomodulatory effects by binding to CD4⁺ T cells.

mIgA and IVIG Recognize CD4⁺ T Cells to a Similar Extent

As both mIgA and IVIG reciprocally regulated Th17 and Tregs at equivalent concentration, rises the possibility that they recognize CD4⁺ T cells to a similar extent. Confirming our proposition, we observed that both immunoglobulin fractions bind CD4⁺ T cells to a similar magnitude (Figures 5A–C). To further substantiate these results, we investigated the surface molecules on CD4⁺ T cells that could be recognized by mIgA and IVIG. As mIgA and IVIG inhibited cytokine-mediated Th17 differentiation and amplification, we hypothesized that the corresponding cytokine receptors on CD4⁺ T cells are the targets for these immunoglobulin fractions. Therefore, we analyzed binding of mIgA and IVIG to IL-6R α and IL-1RI, the receptors for IL-6 and IL-1 β that are implicated in the amplification of Th17 cells (1, 2, 18, 35). mIgA recognized both IL-6R α and IL-1RI in a dose-dependent manner (Figures 5D,E). The binding of mIgA was however stronger for IL-6R α . Importantly, IVIG and mIgA showed similar pattern of recognition of IL-6R α and IL-1RI (Figures 5D,E).

mIgA Interferes with STAT3 Activation

STAT3 has a key role in the Th17 cell programming by relieving RORC from FoxP3-mediated inhibition. Together with RORC, it facilitates the secretion of effector cytokines of



Th17 cells. STAT3 is activated by Th17-polarizing cytokines (1, 2). As we found that mIgA recognizes cytokine receptors implicated in Th17 responses, we aimed at exploring if this binding of mIgA on CD4⁺ T cells has a repercussion on STAT3 phosphorylation and hence interferes with early signaling events of Th17 cells. In line with our proposition, we uncovered that mIgA significantly suppresses the phosphorylation of

STAT3 at Y705 both in Th17 differentiation and amplification conditions (Figures 6A–E).

DISCUSSION

Patients with selective IgA deficiency not only exhibit enhanced predisposition to mucosal infections but also to

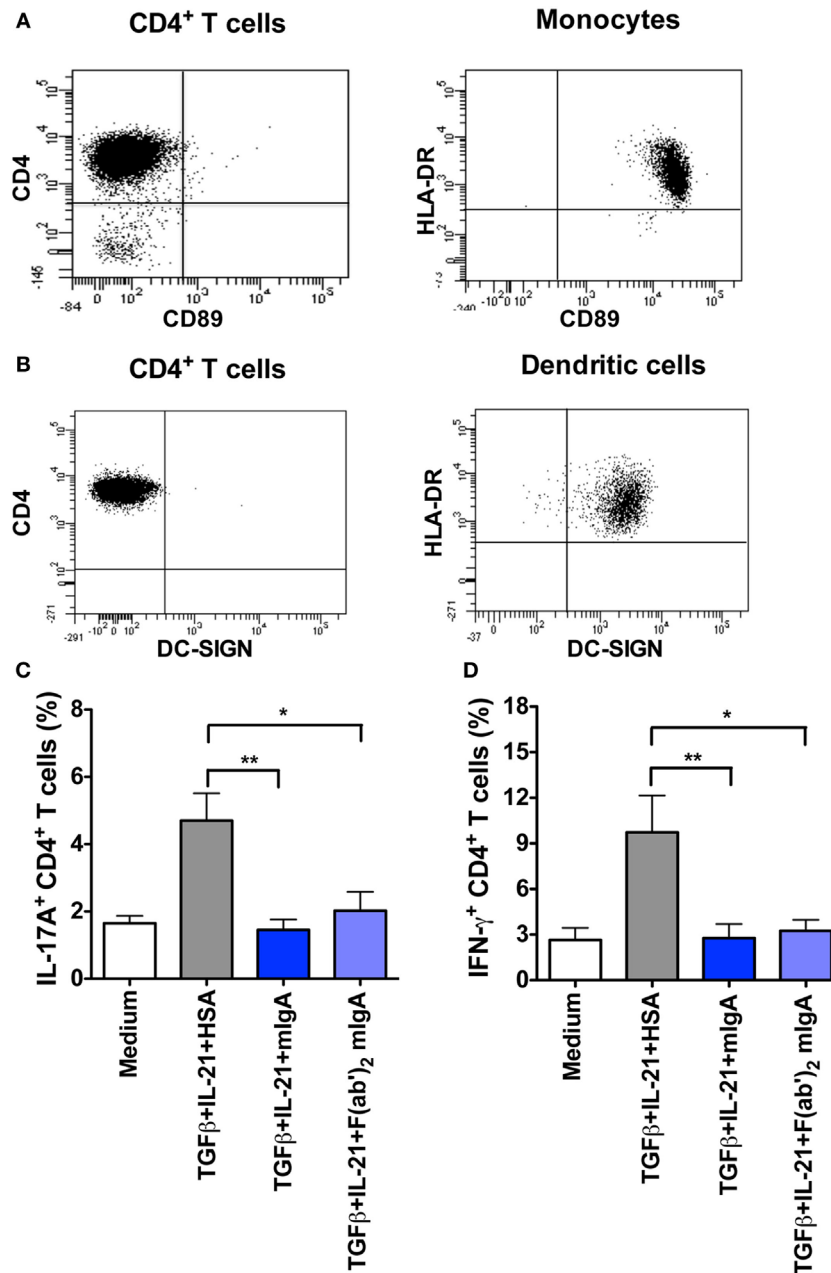


FIGURE 3 | Inhibition of Th17 response by monomeric IgA (mIgA) implicates F(ab')₂ fragments while CD89 and DC-SIGN are dispensable. (A,B) Flow cytometric analysis human CD4⁺ T cells, monocytes, and dendritic cells for the expression of CD89 and DC-SIGN. **(C,D)** Percentage of **(C)** IL-17A⁺CD4⁺ T cells and **(D)** IFN- γ ⁺CD4⁺ T cells (mean \pm SEM, $n = 4$ donors) in T cells cultured in serum-free X-vivo medium in the presence of anti-CD3 and anti-CD28 mAbs alone (Medium) or stimulated with TGF β and IL-21 for 6 days. mIgA (25 mg/ml), F(ab')₂ fragments of mIgA (15 mg/ml), or human serum albumin (HSA) (10 mg/ml) (0.15mM) were added to the T cell cultures after 12 h of cytokine stimulation. Statistical significance as determined by one-way ANOVA is indicated (* $P < 0.05$; ** $P < 0.01$).

several autoimmune and allergic conditions including arthritis, autoimmune endocrinopathies, and intestinal inflammatory diseases such as ulcerative colitis and Crohn's disease (36). These observations support the role of IgA in the immune regulation and homeostasis. The immunoregulatory functions of IgA are mediated mainly *via* Fc α RI, expressed on various

innate immune cells. Whether such an interaction is inflammatory or anti-inflammatory is determined by nature of the IgA. Thus, IgA immune complexes and polymeric IgA were reported to induce activation of innate immune cells while mIgA was found to be anti-inflammatory. The anti-inflammatory effects of mIgA include its inhibitory effect on the chemotaxis of immune

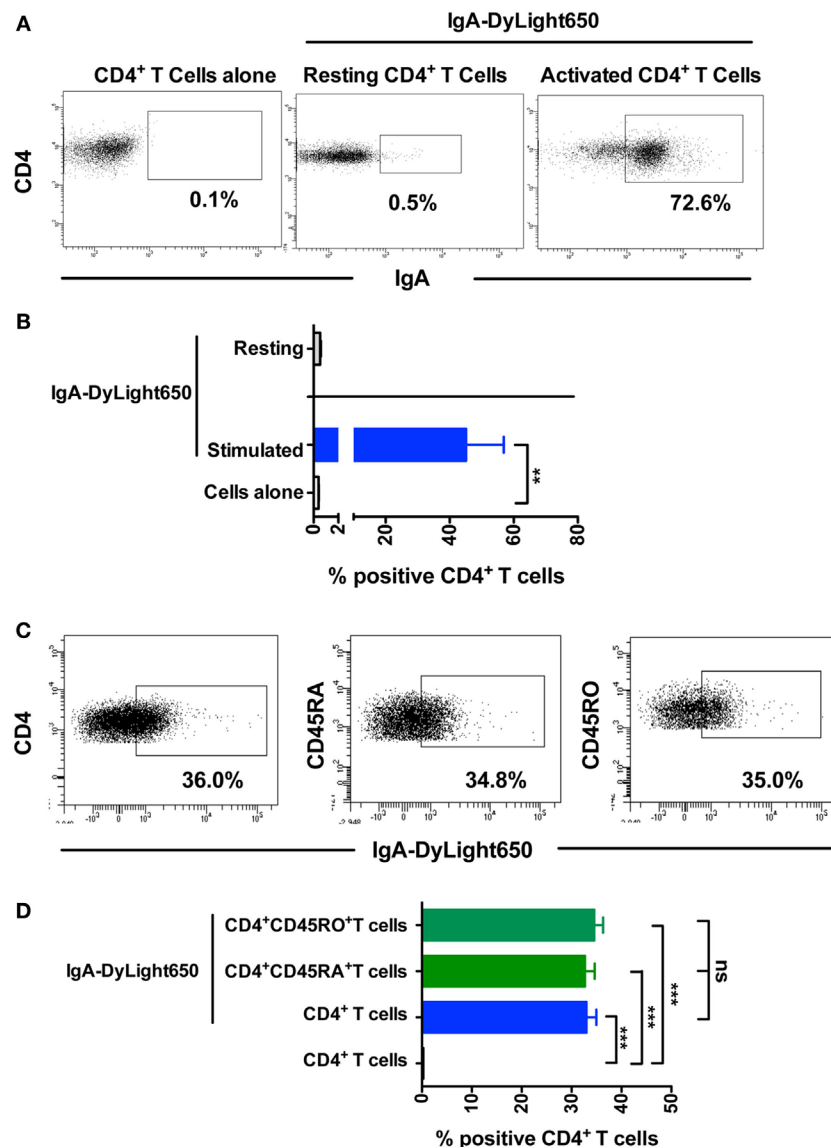
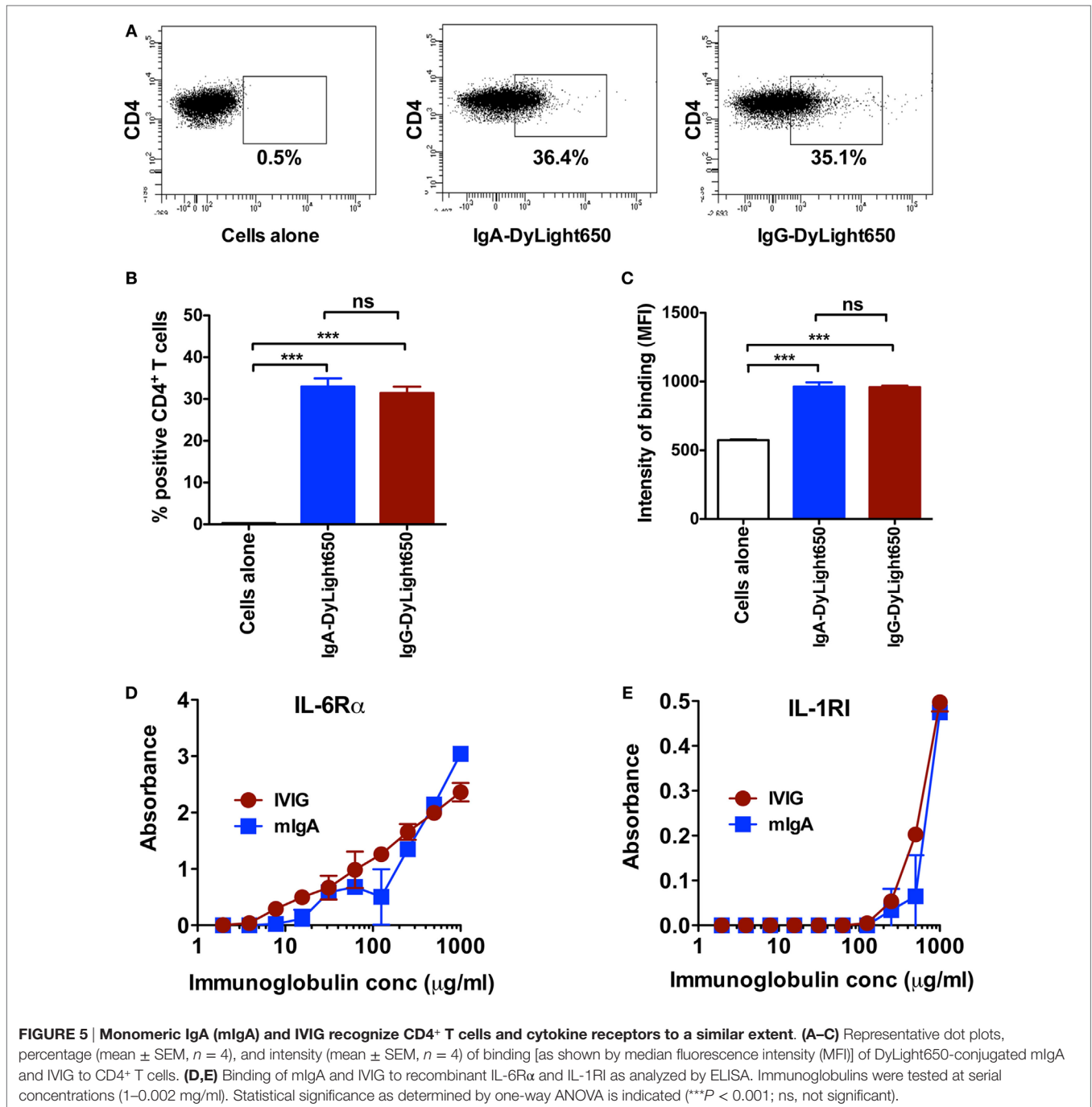


FIGURE 4 | Monomeric IgA (mIgA) binds to CD4⁺ T cells. (A,B) Representative dot plots and percentage (mean \pm SEM, $n = 3-9$ donors) of binding of DyLight650-conjugated mIgA to CD4⁺ T cells. Statistical significance as determined by two-tailed Student's *t*-test is indicated (** $P < 0.01$). **(C,D)** Representative dot plots and percentage (mean \pm SEM, $n = 4$) of binding of DyLight650-conjugated mIgA to CD4⁺ T cells, CD4⁺CD45RA⁺ naive T cells, and CD4⁺CD45RO⁺ memory T cells. Statistical significance as determined by one-way ANOVA is indicated (** $P < 0.01$; *** $P < 0.001$; ns, not significant).

cells, IgG-mediated phagocytosis and bactericidal activity of polymorphonuclear cells, and secretion of inflammatory cytokines such as TNF and IL-6 (37–43). Furthermore, mIgA also induces the production of IL-10 by human monocytes and monocyte-derived DCs (44). Of note, recognition of secretory IgA *via* carbohydrate-recognizing receptors on innate cells such as SIGN-R1 has also been suggested (30, 45, 46). This interaction rendered DC tolerogenic characterized by the secretion of IL-10 and gaining the ability to expand Tregs. Our current results show that mIgA exerts direct anti-inflammatory functions on effector T cells, independent of Fc α RI, DC-SIGN, and innate cells. These

data thus further expand the landscape of immunoregulatory functions of IgA and of natural immunoglobulins.

Aberrant activation of Th17 cells and their effector cytokines IL-17A and GM-CSF are implicated in the pathogenesis of various autoimmune and inflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis, psoriasis, dermatomyositis, allergy, asthma, and others (2). Importantly, targeting Th17 responses have given promising results in experimental models of autoimmune diseases and in patients (47). Recent data from ours and others show that beneficial effects of therapeutic IVIG containing IgG from pooled plasma of thousands of healthy



donors is associated with inhibition of Th17 responses (24–29, 48–50), indicating that immunoglobulins have regulatory functions on Th17 cells. The data from current report show that in addition to IgG, mIgA also exerts modulatory effects on Th17 responses. In fact, mIgA was recently demonstrated to attenuate experimental arthritis in human CD89 transgenic mice (11), a disease where Th17 cells have a key role in the pathogenesis. It should be noted that the serum levels of IgA ranges from 2 to 3 mg/ml, but we observed consistent inhibitory effect of mIgA both on differentiation and amplification of Th17 cells at higher

doses (25 mg) and was analogous to what is observed with IVIG (24). The lower concentration of mIgA (5 mg) although shown inhibitory effects on Th17 differentiation, significant effects were not observed on all parameters of Th17 amplification. Immunoglobulins exert their anti-inflammatory effects *via* several mutually non-exclusive mechanisms and it might explain requisite of higher concentrations of immunoglobulins for the therapeutic purposes to inhibit inflammation.

Recent reports show that Th17 cells are required for the production of high affinity secretory IgA at intestinal mucosal

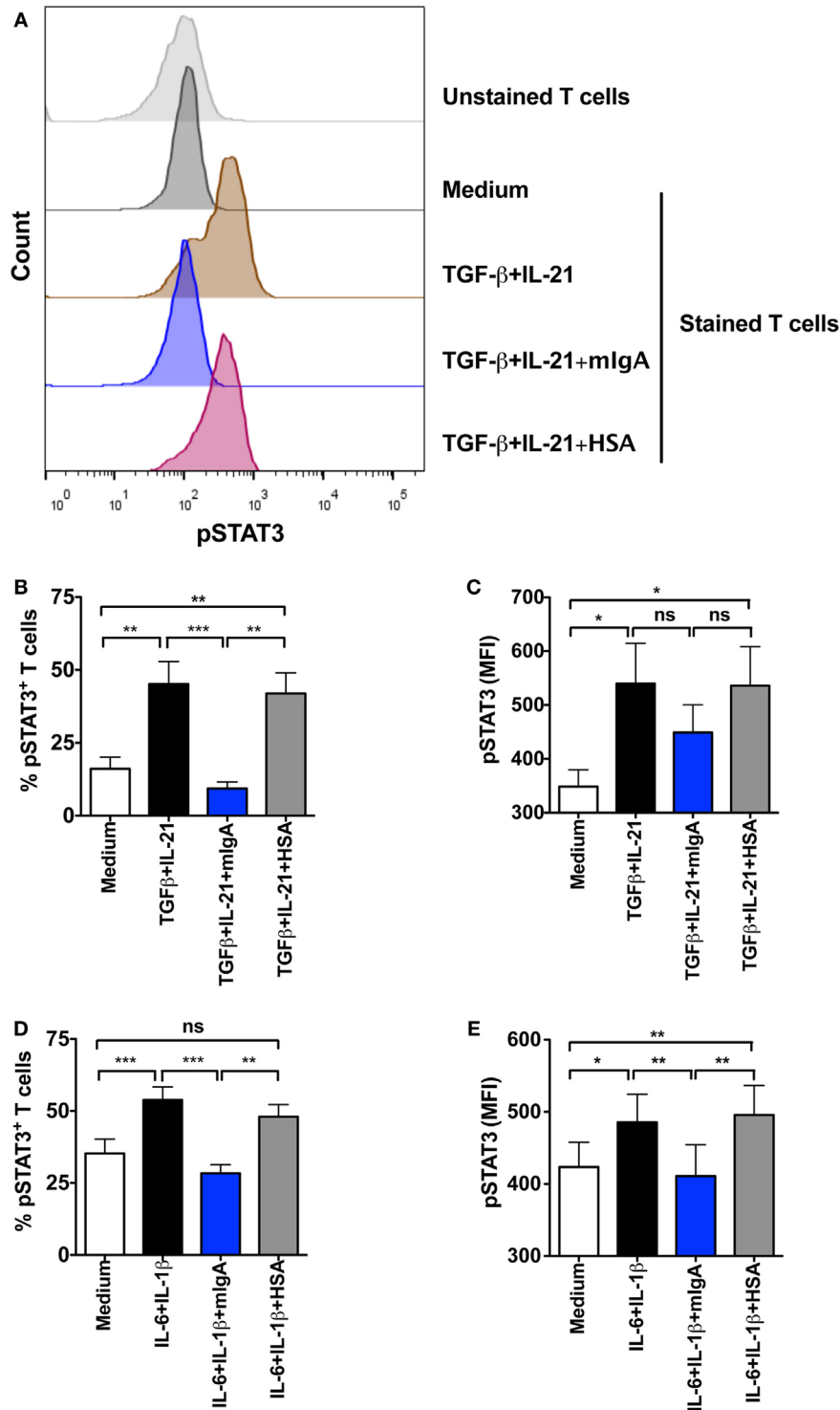


FIGURE 6 | Monomeric IgA (mIgA) interferes with signal transducer and activator of transcription 3 (STAT3) activation. CD4⁺ T cells were cultured in serum-free X-vivo medium in the presence of anti-CD3 and anti-CD28 mAbs alone (medium) or stimulated with cytokines for the differentiation of Th17 cells [TGFβ and IL-21, panels (A–C)] or for the amplification of Th17 cells [IL-1β and IL-6, panels (D,E)]. mIgA (25 mg/ml) or human serum albumin (HSA) (10 mg/ml) (0.15 mM) were added to the T cell cultures. Phosphorylation of STAT3 was analyzed after 72 h. (A) Representative histograms showing pSTAT3 in different experimental conditions for differentiating Th17 cells. (B–E) Percentage of pSTAT3⁺CD4⁺ T cells (B,D) and median fluorescence intensity (MFI) of pSTAT3 (C,E) under Th17 differentiation (B,C) (mean ± SEM, n = 5 donors) and amplification (D,E) (mean ± SEM, n = 5 donors) conditions. Statistical significance as determined by one-way ANOVA is indicated (*P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant).

surfaces (51, 52). Payer patch-homing Th17 cells induce IgA-producing germinal center B cells by acquiring the phenotype of follicular T cells and producing IL-21. A similar mechanism was also reported for the promotion of local IgA responses in lungs by vaccine-induced Th17 cells (53). Thus, Th17 cells and secretory IgA work in cooperation to protect mucosal surfaces against microbial invasion and regulating the microbiota. Although Th17 cells were reported to provide help for B cells to produce systemic IgG response *via* IL-21 and IL-17 (54), their role for circulating IgA response is not known. As we uncovered that mIgA inhibits Th17 responses, these data together suggest that under the conditions where Th17 cells are hyperactivated, IgA has the ability to control its own helpers to keep the immune response at check.

Natural antibodies (immunoglobulins) that are produced in the absence of deliberate immunization and independent of external antigens constitute an integral part of immunoglobulin repertoire (55–64). A major fraction of these natural antibodies recognize self-motifs and are termed as natural autoantibodies. These natural autoantibodies have important role in the therapeutic benefits of IVIG in autoimmune and inflammatory conditions (23, 31, 65). Our results indicate that inhibitory effect of mIgA on Th17 cells is mediated in part *via* natural IgA autoantibodies that recognize cytokine receptors on CD4⁺ T cells and interfere with Th17 programming. In fact, we found that both mIgA and IVIG recognize CD4⁺ T cells as well as IL-6R α and IL-1RI to a similar extent. Also, significant downregulation of STAT3 phosphorylation by mIgA support our proposition.

Although we found that mIgA directly inhibits Th17 responses independent of Fc α RI and DC-SIGN, we believe that the effect of mIgA on Th17 cells *in vivo* also implicates innate cells such as DCs, monocytes, and macrophages, which are known to provide signals for Th17 responses (2). In fact, several reports have now shown that mIgA exerts Fc α RI-mediated anti-inflammatory effects on innate cells by prompting ITAMi configuration (10–13, 16). It should be noted that IVIG (IgG) and IgA display distinct differences in their glycosylation pattern. IgG is glycosylated at Asn297 of the Fc-fragment and about 15–25% of IgG are glycosylated at the Fab region (66). IgA on the other hand is the most glycosylated form of immunoglobulin and over 6% of IgA content are represented by sugars (67). In addition to *N*-linked glycans at asparagine 263 and asparagine 459 of Fc region, up to five *O*-linked glycan chains containing of *N*-acetylgalactosamine with β 1,3-linked galactose and sialic acids can be found at the hinge region serine and threonine residues of IgA1, the predominant IgA subclass in the circulation (68, 69). Similar to

IgG, about 30% of Fab fragments of IgA1 also contain *N*-linked glycans (69). Further, IgA1 and IgG display significant differences in the sialylation content of *N*-glycans. In contrast to IgG that contains sialic acid in less than 10% *N*-glycans, nearly 90% of the *N*-glycans in IgA1 are sialylated mainly with α 2,6-configuration (69). Whether differences in the glycosylation patterns of IgA and IgG impact modulation of innate cells and innate cell-mediated Th17 responses remains to be investigated. Due to heterogeneous composition of glycan chains, further work is also necessary with mIgA preparations containing defined glycosylation patterns to finely dissect the role of IgA in modulating Th17 responses. Since F(ab)₂ fragments of mIgA could inhibit Th17 responses similar to intact mIgA and that F(ab)₂ fragments of IVIG were previously reported to inhibit Th17 responses both *in vitro* and *in vivo* (24, 70) imply that *N*-linked glycans (and hence sialylation) at Fc region of mIgA might not have a role in regulating Th17 responses.

In conclusion, our data highlight the promise of plasma-derived mIgA as therapeutic molecule for autoimmune and inflammatory diseases and hence represents an innovative plasma-derived therapeutic product (71). Although shorter half-life of IgA as compared to IgG is the major drawback, the efficacy of mIgA on Th17 cells should instigate the therapeutic development of monomeric plasma IgA as an analog to IVIG.

AUTHOR CONTRIBUTIONS

SK and JB designed the research. CS, MD, VP, ES-V, and MS performed the research. SW, MJ, and CV provided research tools. CS, MD, VP, SW, MJ, CV, SK, and JB contributed to data analyses and data interpretation. JB wrote the manuscript. CS, MD, VP, ES-V, MS, SW, MJ, CV, SK, and JB revised the manuscript critically for important intellectual content and approved the final version.

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**Discussion
and
perspectives**

Discussion

The immune system with its innate and adaptive components has developed to defend the body. Its principal functions are recognition and subsequent elimination of foreign antigens, induction of immunologic memory, and development of tolerance to self-antigens. Autoimmunity is defined as the development of immune system reactivity in the form of auto-antibodies and T-cell responses to self-structures. There are various symptoms and disorders which are encompassed in autoimmune diseases. They vary from organ specific to systemic, and include, insulin-dependent diabetes mellitus, RA, SLE, scleroderma, thyroiditis and MS to name but a few. The most common areas in the body which are targeted by autoimmune diseases are the thyroid gland, stomach, adrenal glands and pancreas. Systemic autoimmune diseases most commonly include the skin, joints and muscle tissue.

Immunosuppressants have been used to treat autoimmune diseases for decades, so their benefits are well-established. However, all immunosuppressant drugs carry the serious risk of adverse reactions and infection and many have been linked to a higher risk for cancer. For example, chronic use of corticosteroids increases your risk for atherosclerosis (hardening of the arteries), diabetes, and osteoporosis. Methotrexate—a drug commonly used to treat rheumatoid and psoriatic arthritis—can suppress bone marrow and, in rare instances, trigger a fatal condition known as “methotrexate lung.”

Natural antibodies (immunoglobulins) that are produced in the absence of deliberate immunization and independent of external antigens constitute an integral part of immunoglobulin repertoire (576–580). A major fraction of these natural antibodies recognize self-motifs and are termed as natural autoantibodies. These natural autoantibodies have important role in the therapeutic benefits of IVIg in autoimmune and inflammatory conditions (441, 580, 581). Several mutually non-exclusive mechanisms have been proposed to explain the immune- regulatory role of IVIg in autoimmunity and inflammation. IVIg acts on different cells of immune system, including DC, monocytes/macrophages, granulocytes, natural killer cells, B-cells and various subset of T-cells. In addition, IVIg also modulates complement and cytokine network (491, 497, 524, 515, 517, 520). Although the mechanisms underlying these effects are broad and may differ in each disease state. Together, IVIg exerts immune-regulatory effects in autoimmune and inflammatory conditions without suppressing the immune system, which provides a strong immunological rationale for its therapeutic use.

Autophagy is required for normal cell functioning and survival. Autophagy regulates energy and nutrient homeostasis and has an essential role in tissue development (582). A number of immunological processes are highly dependent on autophagy, including pathogen recognition and destruction, antigen presentation, lymphocyte development and effector functions of immune cells (414, 416, 583, 584). A number of pro-inflammatory cytokines, including IFN- α , TNF- α , IL-1 and IL-23 induce autophagy (585, 586), while Th2 and regulatory cytokines, including IL-4, IL-13 and IL-10 are inhibitory (587, 588). Although autophagy is regulated by cytokines, autophagy itself can directly influence the transcription, processing and secretion of a number of cytokines and chemokines (589). For example, autophagy can modulate the secretion of members of the IL-1 cytokine family, IL-23 and, as a consequence, IL-17 (585). The regulation of IL-1 β and IL-23 secretion by autophagy is critical for the prevention of autoimmune and auto-inflammatory diseases (181, 589). Defective autophagy process is linked to severe pathologies, including metabolic disorders, neurodegenerative diseases and cancer (591, 592). Autophagy also play a fundamental role in the regulation of innate and adaptive immune system (583), lymphocyte survival, lymphocyte homeostasis (583, 584) and differentiation (414). There are several evidences that support the involvement of autophagy in autoimmune and auto-inflammatory disorders including the discovery of polymorphisms in autophagy-related genes. Disorders such as SLE, IBD, RA, psoriasis, MS and myositis (593-596) were suggested to be associated with defects in autophagy process.

In the present study, I have demonstrated that IVIg induces autophagy in PBMCs. Furthermore, our results indicate that IVIg-mediated autophagy induction is restricted to inflammatory cells like monocytes, monocyte-derived DCs, M1 m ϕ but not in immunosuppressive cells, for example M2 m ϕ . Furthermore, I have observed that IVIG-mediated autophagy induction is F(ab')₂-dependent. Interestingly, endocytosis of IgG is required in IVIg-mediated autophagy induction.

Autophagy process is controlled by products of numerous autophagy-specific genes (Atg) and several macromolecular signaling complexes including mTOR complex-1 (mTORC1). Inhibition of mTORC1 is essential for autophagy to initiate, and allows the translocation of a complex containing Atg1/unc-51-like kinase (ULK)1/2, Atg13, FIP200 and Atg101 from cytosol to the endoplasmic reticulum (ER) (597). This leads to the recruitment of the type III

PI3K, VPS34, in a complex with other proteins, including beclin-1, to the developing autophagosome (598). Rapamycin that inhibits mTOR and enhances autophagy has been successfully used in the treatment of autoimmune and auto-inflammatory diseases (599, 600). In addition, rapamycin has been described to increase Tregs (601), which play a crucial role in the prevention of autoimmunity (31).

In conditions of low cellular energy and essential amino acid deprivation, the induction of autophagy is driven by AMPK, which is antagonized by mTOR when nutrients are sufficient (326). There is emerging evidence that AMPK is a key modulator of immune response. AMPK activation is a critical point at which anti-inflammatory signals converge to elicit suppressive responses. AMPK can reduce the severity of inflammation and tissue damage in colitis and EAE (602). Also, AMPK suppresses LPS-induced IL-6 and TNF- α in neutrophils (603). In addition, report has shown that AMPK can drive the induction of Tregs. Several studies suggest that p38MAPK also regulates autophagy both positively and negatively (604, 605). In the present study I have shown that IVIg induces AMPK, beclin-1, class III PI3K and p38MAPK and inhibits mTORC1. Inhibition of PI3K, p38MAPK and AMPK by 3MA, SB202190 and compound C respectively, abrogated IVIg-mediated autophagy induction.

IVIg is used for the treatment of large number of autoimmune and system inflammatory diseases. Several studies revealed the beneficial effects of IVIg in inflammatory myopathies (442, 518). Inflammatory myopathies are a group of disease that involve chronic muscle inflammation, accompanied by muscle weakness. Inflammatory myopathies classified into four main categories: dermatomyositis, polymyositis, immune-mediated necrotizing myopathy and inclusion body myositis (606). Recent report showed that, rapamycin, which enhances autophagy, decreases the severity of myositis as curative and preventative treatment in murine model of experimental myositis. The improvement in the disease phenotype was associated with decrease in the frequency of effector T cells and a relative increase in Tregs (607). In the present study, I have shown IVIg therapy significantly induces autophagy *in vivo* in inflammatory myopathies. My observations suggest that the beneficial effects of IVIg in inflammatory myopathies may be in part due to modulation of autophagy as an analogue to rapamycin effect.

Taken together, my study demonstrated that IVIg induces autophagy particularly in inflammatory cell type. IVIG induces autophagy via p38MAPK, AMPK and class III PI3K. Furthermore, I have shown that IVIg therapy induces autophagy *in vivo* in inflammatory

myopathies. Thus our results uncover a novel mechanism of action IVIg in achieving a therapeutic effect in inflammatory and autoimmune diseases via autophagy.

CD4⁺ T cells after being activated and differentiated into distinct effector subsets play a major role in mediating immune response through the secretion of subset-specific cytokines. Any defect in the T cell populations may result in diseases and autoimmune diseases are result from failure to sustain tolerance to self-and/or cross-reactive molecules. Depending on the cytokines they produce, these T cell subsets have very different properties. Th cells include the well-defined effector Th1 and Th2 subsets as well as the more recently described Th17 cells, but also regulatory subsets like Treg. The Th17 and Treg developmental pathways are reciprocally interconnected. This implicates a balance between both cell types which influences the outcome of the immune responses. Research on the immunopathogenesis of autoimmune and inflammatory diseases has made significant progress in the past few years and Th17 and Tregs have emerged as major players in autoimmunity. Th17 cells represent a pro-inflammatory subset, which when in excess contributes to autoimmunity and tissue damage whereas Tregs have an antagonistic effect. The Th17/Treg balance provides a basis for understanding the immunological mechanisms that induce and regulate autoimmunity and chronic inflammation.

Aberrant activation of Th17 cells and their effector cytokines IL-17A and GM-CSF are implicated in the pathogenesis of various autoimmune and inflammatory (139). Importantly, targeting Th17 responses have given promising results in experimental models of autoimmune diseases and in patients. Recent data from ours and others show that beneficial effects of therapeutic IVIG is associated with inhibition of Th17 responses (525-529), indicating that immunoglobulins have regulatory functions on Th17 cells.

Patients with selective IgA deficiency not only exhibit enhanced predisposition to mucosal infections but also to several autoimmune and allergic conditions including arthritis, autoimmune endocrinopathies, and intestinal inflammatory diseases such as ulcerative colitis and Crohn's disease (608). These observations support the role of IgA in the immune regulation and homeostasis. The immunoregulatory functions of IgA are mediated mainly via Fc α RI, expressed on various innate immune cells. Whether such an interaction is inflammatory or anti-inflammatory is determined by nature of the IgA. Thus, IgA immune complexes and polymeric IgA were reported to induce activation of innate immune cells while mIgA was found to be

anti-inflammatory. The anti-inflammatory effects of mIgA include its inhibitory effect on the chemotaxis of immune cells, IgG-mediated phagocytosis and bactericidal activity of polymorphonuclear cells, and secretion of inflammatory cytokines such as TNF and IL-6 (566-568). Furthermore, mIgA also induces the production of IL-10 by human monocytes and monocyte-derived DCs (609). Of note, recognition of secretory IgA via carbohydrate-recognizing receptors on innate cells such as SIGN-R1 has also been suggested (575, 610). This interaction rendered DC tolerogenic characterized by the secretion of IL-10 and gaining the ability to expand Tregs. My current results show that mIgA exerts direct anti-inflammatory functions on effector T cells, independent of Fc α RI, DC-SIGN, and innate cells. These data thus further expand the landscape of immunoregulatory functions of IgA and of natural immunoglobulins.

The data from current report show that in addition to IgG, mIgA also exerts modulatory effects on Th17 responses. In fact, mIgA was recently demonstrated to attenuate experimental arthritis in human CD89 transgenic mice (611), a disease where Th17 cells have a key role in the pathogenesis. It should be noted that the serum levels of IgA ranges from 2 to 3 mg/ml, but we observed consistent inhibitory effect of mIgA both on differentiation and amplification of Th17 cells at higher doses (25 mg) and was analogous to what is observed with IVIg (524). The lower concentration of mIgA (5 mg) although shown inhibitory effects on Th17 differentiation, significant effects were not observed on all parameters of Th17 amplification. Immunoglobulins exert their anti-inflammatory effects via several mutually non-exclusive mechanisms and it might explain requisite of higher concentrations of immunoglobulins for the therapeutic purposes to inhibit inflammation.

Recent reports show that Th17 cells are required for the production of high affinity secretory IgA at intestinal mucosal surfaces (612, 613). Payer patch-homing Th17 cells induce IgA-producing germinal center B cells by acquiring the phenotype of follicular T cells and producing IL-21. A similar mechanism was also reported for the promotion of local IgA responses in lungs by vaccine-induced Th17 cells (614). Thus, Th17 cells and secretory IgA work in cooperation to protect mucosal surfaces against microbial invasion and regulating the microbiota. Although Th17 cells were reported to provide help for B cells to produce systemic IgG response via IL-21 and IL-17 (615), their role for circulating IgA response is not known. As I uncovered that mIgA inhibits Th17 responses, these data together suggest that under the conditions where Th17 cells are hyperactivated, IgA has the ability to control its own helpers to keep the immune response at check.

My results indicate that inhibitory effect of mIgA on Th17 cells is mediated in part via natural IgA autoantibodies that recognize cytokine receptors on CD4⁺ T cells and interfere with Th17 programming. In fact, I found that both mIgA and IVIG recognize CD4⁺ T cells as well as IL-6R α and IL-1RI to a similar extent. Also, significant downregulation of STAT3 phosphorylation by mIgA support this proposition. Although I found that mIgA directly inhibits Th17 responses independent of Fc α RI and DC-SIGN, I believe that the effect of mIgA on Th17 cells *in vivo* also implicates innate cells such as DCs, monocytes, and macrophages, which are known to provide signals for Th17 responses. In fact, several reports have now shown that mIgA exerts Fc α RI-mediated anti-inflammatory effects on innate cells by prompting ITAMi configuration.

IgA is the most prevalent antibody at mucosal sites and the second most abundant antibody in the circulation. IVIg (IgG) and IgA display distinct differences in their glycosylation pattern. IgG is glycosylated at Asn297 of the Fc-fragment and about 15–25% of IgG are glycosylated at the Fab region. IgA on the other hand is the most glycosylated form of immunoglobulin and over 6% of IgA content are represented by sugars (608). In addition to N-linked glycans at asparagine 263 and asparagine 459 of Fc region, up to five O-linked glycan chains containing of N-acetylgalactosamine with β 1,3-linked galactose and sialic acids can be found at the hinge region serine and threonine residues of IgA1, the predominant IgA subclass in the circulation (534, 537). Similar to IgG, about 30% of Fab fragments of IgA1 also contain N-linked glycans (534). Further, IgA1 and IgG display significant differences in the sialylation content of N-glycans. In contrast to IgG that contains sialic acid in less than 10% N-glycans, nearly 90% of the N-glycans in IgA1 are sialylated mainly with α 2,6-configuration (69). Due to heterogeneous composition of glycan chains, further work is necessary with mIgA preparations containing defined glycosylation patterns to finely dissect the role of IgA in modulating Th17 responses. Since F(ab')₂ fragments of mIgA could inhibit Th17 responses similar to intact mIgA and that F(ab')₂ fragments of IVIG were previously reported to inhibit Th17 responses both *in vitro* and *in vivo* (515, 524) imply that N-linked glycans (and hence sialylation) at Fc region of mIgA might not have a role in regulating Th17 responses.

In conclusion, my data highlight the promise of plasmaderived mIgA as therapeutic molecule for autoimmune and inflammatory diseases and hence represents an innovative plasma-derived therapeutic product (71). Although shorter half-life of IgA as compared to IgG is the major

drawback, the efficacy of mIgA on Th17 cells should instigate the therapeutic development of monomeric plasma IgA as an analog to IVIg.

Perspectives

Currently, IVIG is used in the therapy of a large number of autoimmune and inflammatory diseases. Despite considerable progress has been made in understanding the cellular and molecular basis of immunomodulatory effects of IVIG, the precise mechanisms responsible for sustained therapeutic benefits of IVIG in diverse immunological disorders remain an enigma. Several mutually non-exclusive mechanisms have been proposed to explain the immune-regulatory role of IVIG. Thus, IVIG acts on different cells of immune system, including DCs, monocytes/macrophages, granulocytes, natural killer cells, B-cells and various subsets of T-cells. In the present study, I have shown that IVIG therapy in autoimmune patients induces autophagy in immune cells and dissected the molecular mechanisms. Thus my results uncover a novel mechanism of action IVIG in achieving a therapeutic effect in inflammatory and autoimmune diseases via autophagy.

In current study, IVIG therapy induced autophagy in all tested inflammatory myopathy patients. It would be interesting to examine whether IVIG therapy induces autophagy in all treated patients irrespective of clinical outcome or autophagy induction is restricted to only those patients who respond to IVIG therapy so that autophagy can be used a one of the biomarkers of IVIG response. The influence of polymorphism(s) in genes implicated in autophagy pathway towards clinical response to IVIG therapy is another domain to investigate. The mechanisms of IVIG-induced autophagy can be further explored in animal models of autoimmune diseases such as EAE and CIA by using various knock-out mouse strains.

In my present study I have also shown that mIgA inhibit human Th17 response. Thus, my results demonstrate immunomodulatory functions of naturally-occurring mIgA and potential therapeutic utility of mIgA in autoimmune and inflammatory diseases that implicate Th17 cells. Further, the immunomodulatory effects of mIgA could be investigated *in vivo* using animal models of autoimmune disease such as EAE where Th17 cells have prime role in the pathogenesis. It would be also interesting to investigate the regulation of autophagy by mIgA and underlying molecular mechanism.

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Annexes

Publications

- Article 1:** Stephen-Victor E*, Das M*, Sharma M*, Galeotti C, Fohrer-Ting H, Sendid B, Darnige L, Terris B, Badoual C, Bruneval P, Kaveri SV, Bayry J. Demystification of enigma on antigen presenting cell features of human basophils: data from secondary lymphoid organs. (2017). *Haematologica*. (*co-first authors)
- Article 2:** Maddur MS*, Stephen-Victor E*, Das M*, Prakhar P, Sharma VK, Singh V, Rabin M, Trinath J, Balaji KN, Bolgert F, Vallat JM, Magy L, Kaveri SV, Bayry J. Regulatory T cell frequency, but not plasma IL-33 levels, represents potential immunological biomarker to predict clinical response to intravenous immunoglobulin therapy. (2017). *J Neuroinflammation*. (*co-first authors)
- Article 3:** Das M, Galeotti C, Stephen-Victor E, Karnam A, Kaveri SV, Bayry J. Human basophils may not undergo modulation by DC-SIGN and mannose receptor-targeting immunotherapies due to absence of receptors. (2017). *J Allergy Clin Immunol*. (Correspondence)
- Article 4:** Stephen-Victor E, Sharma VK, Das M, Karnam A, Saha C, Lecerf M, Galeotti C, Kaveri SV, Bayry J. IL-1 β , But Not Programed Death-1 and Programed Death Ligand Pathway, Is Critical for the Human Th17 Response to *Mycobacterium tuberculosis*. (2016). *Front Immunol*.
- Article 5:** Das M, Kaveri SV, Bayry J. Cross-presentation of antigens by dendritic cells: role of autophagy. (2015). *Oncotarget*. (Editorial)
- Article 6:** Galeotti C, Hegde P, Das M, Stephen-Victor E, Canale F, Muñoz M, Sharma VK, Dimitrov JD, Kaveri SV, Bayry J. Heme oxygenase-1 is dispensable for the anti-inflammatory activity of intravenous immunoglobulin. (2016). *Sci Rep*
- Article 7:** Saha C, Das M, Stephen-Victor E, Friboulet A, Bayry J, Kaveri SV. Differential Effects of Viscum album Preparations on the Maturation and Activation of Human Dendritic Cells and CD4⁺ T Cell Responses. (2016). *Molecules*.

Demystification of enigma on antigen-presenting cell features of human basophils: data from secondary lymphoid organs

Antigen presenting cells (APCs) play a key role in the uptake of antigens, and processing and presentation of antigen-derived peptides in the context of MHC molecules to T cells. The co-stimulatory molecules CD80 and CD86 provide signals for T-cell activation and proliferation. Dendritic cells (DCs), macrophages/monocytes, and memory B cells are classically known for their role as APCs. In contrast to this dogma, recent reports from mice show that basophils can also function as APCs. These reports were revolutionary as basophils were previously known as accessory cells that produce IL-4 required for the Th2 responses mediated by classical APCs.^{1,2} In fact, it was shown that murine basophils express MHC class II, and co-stimulatory molecules CD80 and CD86

required for the T-cell stimulation.³⁻⁵ Additionally, these basophils were also reported to process and present antigens that are associated with classical Th2 responses such as allergens and proteases, and mediate Th2 differentiation *in vitro* and *in vivo*. Although *in vivo* data were later contradicted,^{6,7} the *ex vivo* and *in vitro* data clearly demonstrated functioning of murine basophils as APCs.

In contrast to mice, several data from humans have shown that basophils, either from healthy donors or from allergic and lupus patients, lack the features of APCs.⁸⁻¹² These basophils were negative for the expression of antigen presenting molecule HLA-DR, and co-stimulatory molecules CD80 and CD86, and failed to polarize Th2 as well as Th17 responses when co-cultured with CD4⁺ T cells. However, all these reports were based on basophils from the circulation, while APC functions of murine basophils were demonstrated from the secondary lymphoid organs, like spleen and lymph nodes. One of the hypotheses is that basophils, upon migration to the secondary lymphoid organs, might gain characteristics of

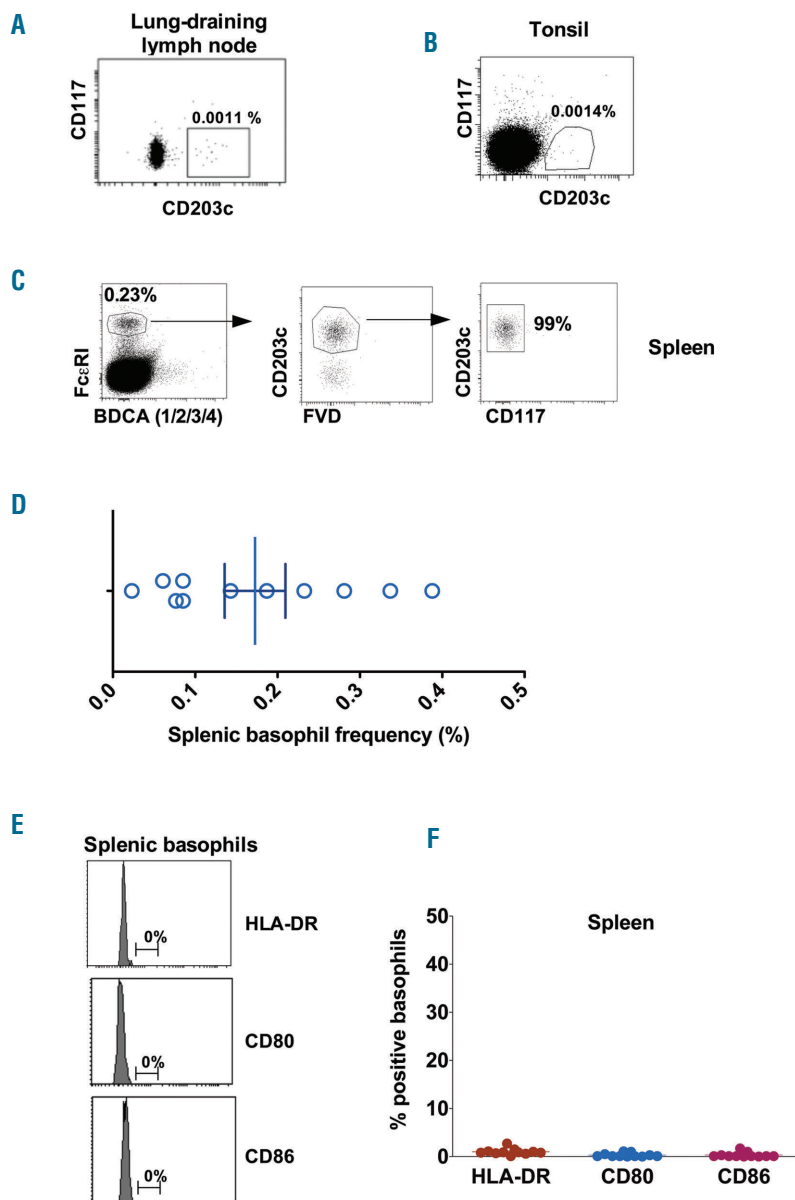


Figure 1. Human basophils from secondary lymphoid organs lack the phenotypic features of APCs. (A, B) The frequency of basophils in lung-draining lymph nodes and tonsils. Representative data of three or four subjects. (C) CD203c⁺FcεRI⁺BDCA-(1/2/3/4)⁻CD117⁻ splenic basophils negative for fixable viable dye (FVD). (D) The frequency of splenic basophils from 11 subjects. (E, F) Representative histograms and mean±SEM (n=11 subjects) of HLA-DR, CD80 and CD86 expressions on steady-state splenic basophils.

APCs. Therefore, the debate on whether human basophils display the features of APCs or not remains an unresolved issue unless and until their features are probed in secondary lymphoid tissues of humans.

In order to unequivocally address whether human

basophils, like their murine counterparts, display features of APCs or not, we studied basophils from human secondary lymphoid tissues such as spleen, lung-draining lymph nodes and tonsils (See *Online Supplementary Methods*). In general, c-kit (CD117) and BDCA-4 (CD304)

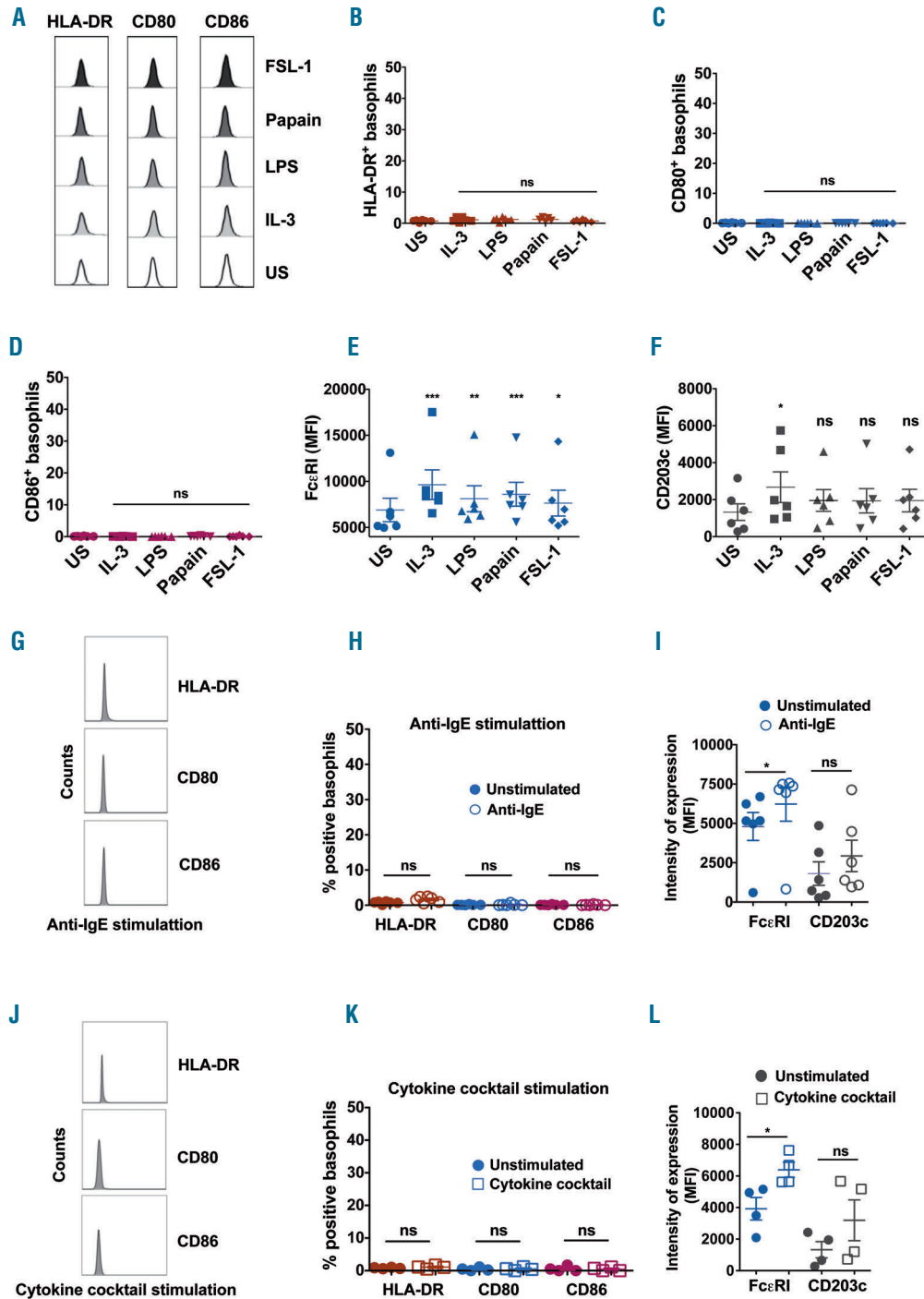


Figure 2. Expression of HLA-DR, B7 co-stimulatory molecules and activation-associated markers on human splenic basophils under various stimulatory conditions. (A-L) Spleenocytes were stimulated with IL-3, LPS, papain, FSL-1 (A-F), or anti-IgE (G-I), or cytokine cocktail (IL-3, GM-CSF and IFN- γ) (J-L) for 4 to 24 hours. Representative histograms showing the expression of HLA-DR, CD80 and CD86 on splenic basophils (A, G, J); mean \pm SEM values of % of splenic basophils positive for HLA-DR, CD80 and CD86 (B, C, D, H, K); and mean \pm SEM values of intensity of expression (MFI) of Fc ϵ RI and CD203c (E, F, I, L) on stimulated splenic basophils are shown. Data are from four to six subjects. US, unstimulated cells; * P <0.05; ** P <0.01; *** P <0.001; ns, not significant; by One-way analysis of variance (B-F) or two-tailed Mann-Whitney test (H, I, K, L).

markers were used to distinguish basophils from mast cells and plasmacytoid DCs (pDCs), while CD203c distinguished basophils from other immune cells. Basophils were identified as CD203c⁺FcεRI⁺BDCA-4⁻c-kit⁻ cells. As myeloid DC population was also reported to express

FcεRI and that pDCs also express BDCA-2 (CD303), BDCA-1 (CD1c), BDCA-3 (CD141) and BDCA-2 markers were additionally used to distinguish splenic basophils from different DC subsets. The phenotype of basophils was analyzed in steady state and under various stimula-

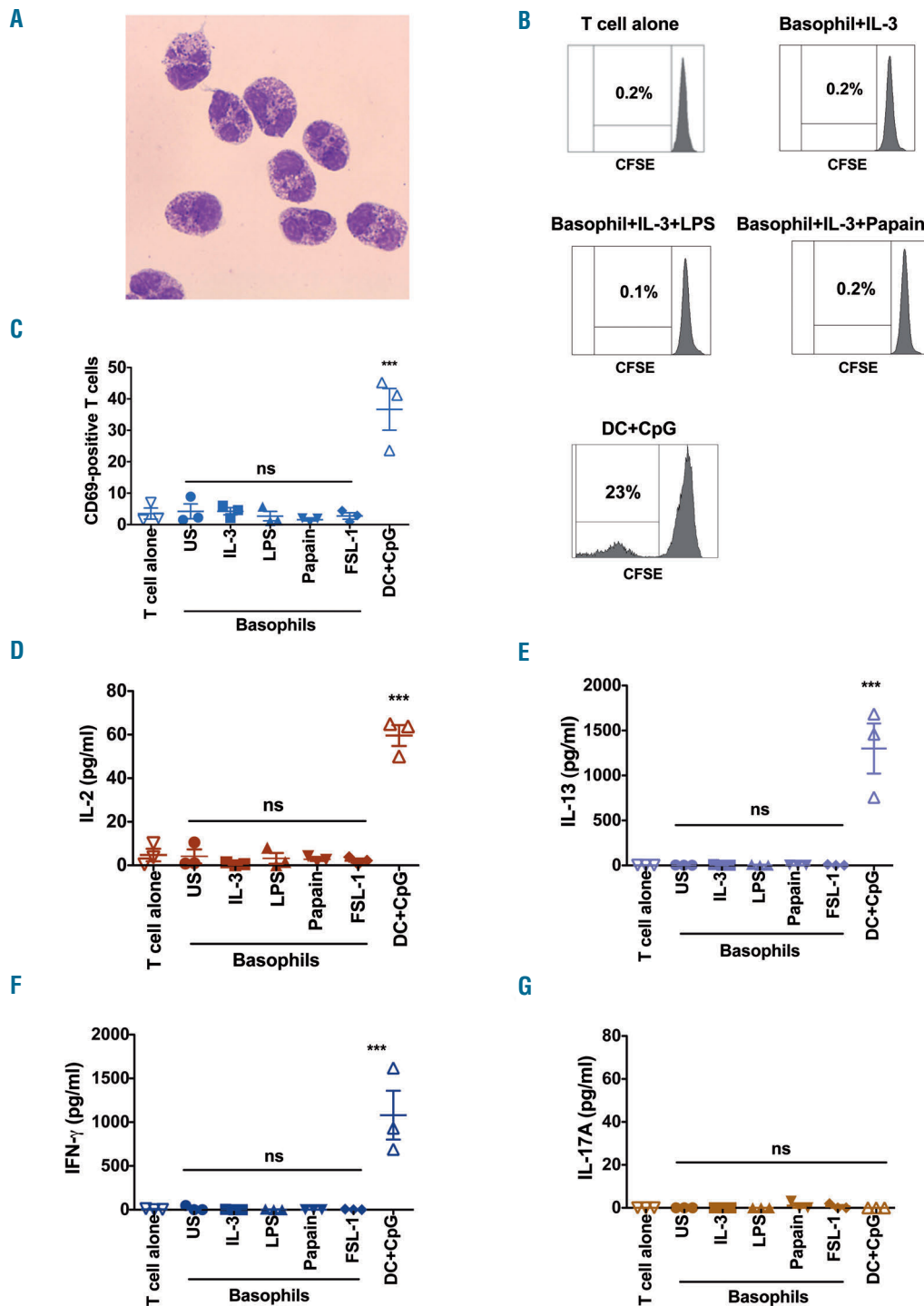


Figure 3. Human splenic basophils lack the functional features of APCs. (A) May-Grünwald Giemsa staining of cytospin preparations of sorted splenic basophils. (B) Ability of splenic basophils to stimulate CD4⁺ T-cell proliferation as analysed by CFSE staining. Representative of three experiments. (C) CD69 expression on CD4⁺ T cells co-cultured with sorted splenic basophils. (D-G) Induction of IL-2, and Th2 (IL-13), Th1 (IFN-γ) and Th17 (IL-17A) cytokines in sorted splenic basophil-CD4⁺ T-cell co-cultures (n=3). US: unstimulated cells; ***P<0.001; ns: not significant; by One-way analysis of variance.

tory conditions. Basophils were also sorted from the spleen, and the morphology of cytospin preparations of sorted basophils was analyzed by May-Grünwald Giemsa staining. The APC features of sorted splenic basophils were investigated in the functional assays by probing their ability to stimulate CD4⁺ T-cell activation, proliferation and cytokine production (See *Online Supplementary Methods*).

Basophils are rare leukocytes and represent <1% blood leukocytes in the blood.¹ However, basophils were sparsely present in the human lung-draining lymph nodes (0.0013% (± 0.0002 SD), n=3) and tonsils (0.004 \pm 0.0024%, n=4) (Figure 1 A-B). Therefore, we could not analyze APC features of basophils from these secondary lymphoid organs.

Basophils in the spleen (0.172 \pm 0.122%, n=11) (Figure 1 C-D) were nearly 130 times higher than lung-draining lymph nodes. We found that splenic basophils under steady state condition did not express HLA-DR, CD80 and CD86 (Figure 1 E-F). On the other hand, splenic DCs expressed high levels of HLA-DR and varying degrees of CD80 and CD86 (*Online Supplementary Figure S1*). These results thus indicate that under steady state, human basophils residing at secondary lymphoid tissues do not display phenotypic characteristics of APCs.

Basophils receive signals from various sources such as cytokines, toll-like receptor (TLR) agonists and IgE-bound antigens. Therefore, we explored if basophils express phenotypic markers of APCs upon activation by these different categories of stimulation. IL-3, in addition to supporting differentiation and survival of basophils, also primes their activation.² Previous reports have shown that murine basophils cultured in IL-3-conditioned medium express MHC class II and co-stimulatory molecules.³⁻⁵ On the other hand, GM-CSF and IFN- γ were previously reported to induce HLA-DR and co-stimulatory molecules on human innate cells. Therefore, IL-3 and cytokine cocktail containing IL-3, GM-CSF and IFN- γ were used for the stimulation of basophils (See *Online Supplementary Methods*).

Human basophils express various TLRs, including TLR4 and 2, and undergo activation upon TLR signaling.¹³ Therefore, we stimulated basophils with lipopolysaccharide from *E. coli* (LPS, TLR4 agonist), FSL-1 (Pam2CGDPKHPKSF, TLR2/6 agonist), or CpG ODN (TLR9 agonist). Furthermore, we also used papain as a model allergen in our experiments, and this cysteine protease allergen can directly activate basophils and promote murine basophil-mediated Th2 responses.⁴ Since, IgE-bound antigens/allergens provide the most-potent activation signal for basophils,^{13,14} this stimulation condition was mimicked by crosslinking of surface Fc ϵ RI-bound IgE by using anti-IgE antibodies (See *Online Supplementary Methods*).

Splenocytes were stimulated for 4 to 24 hours and analyzed for the expression of HLA-DR and B7 molecules on the basophils. Notably, expression of HLA-DR, CD80 and CD86 on basophils did not change significantly despite stimulation with IL-3, LPS, papain or FSL-1. The expression of these markers on stimulated basophils was on par with steady state cells (Figure 2 A-D). We confirm that basophils were indeed activated by these stimuli as analyzed by the expression levels of Fc ϵ RI and CD203c (Figure 2 E-F). The expression of Fc ϵ RI was significantly increased on splenic basophils irrespective of stimulatory conditions. Although expression of CD203c was also enhanced upon stimulation, due to variations among the donors, significance was not reached in all stimulatory conditions. Similar results were also obtained when

splenocytes were stimulated with anti-IgE antibodies or cytokine cocktail (Figure 2 G-L). These results thus provide a pointer that human splenic basophils lack the phenotypic characteristics of APCs irrespective of activation signals they receive.

One of the hallmarks of APCs is their ability to stimulate CD4⁺ T-cell proliferation and cytokine production. Therefore, to prove indisputably that human basophils lack the functions of APCs, we sorted splenic basophils for co-culture with CD4⁺ T cells (See *Online Supplementary Methods*). Basophils from splenocytes were first enriched using basophil isolation kit II (Miltenyi Biotec). Enriched cells were labelled, and live basophils were sorted on FACS Aria III flow cytometer (BD Biosciences) as cells positive for Fc ϵ RI and CD203c, and negative for BDCA-4 and c-kit. The purity of sorted basophils was 98-99%. May-Grünwald Giemsa staining of cytospin preparations confirmed morphology of basophils with basophilic granules in the cytoplasm. Nucleus was partly covered by the basophilic granules. Cells presented relatively narrow cytoplasm that was loosely permeated by intensely basophilic granules. The basic color of the cytoplasm was pale blue to pale pink (Figure 3 A). Isolated splenic basophils were functionally viable. Upon IL-3 stimulation, isolated cells presented with enhanced CD69 expression and released histamine in response to degranulation stimuli.

We co-cultured sorted splenic basophils with allogeneic CD4⁺ T cells at a ratio of 1:10 under various stimulatory conditions, such as IL-3, LPS, papain or FSL-1. CD4⁺ T cells were isolated from peripheral blood mononuclear cells of healthy donors using CD4 MicroBeads (Miltenyi Biotec). Consistent with the phenotypic characteristics, stimulated basophils failed to induce CD4⁺ T-cell proliferation (Figure 3 B), T-cell activation marker CD69 (Figure 3 C) and secretion of IL-2 (Figure 3 D). In addition, basophils did not induce Th2 (IL-13), Th17 (IL-17A) or, as expected, Th1 (IFN- γ) cytokines (Figure 3 E-G). CpG or cytokine cocktail-stimulated basophils also presented similar results. Splenic DCs used as control, induced CD4⁺ T-cell proliferation, expression of CD69, and secretion of IL-2, IFN- γ and IL-13 (Figure 3 B-F).

Several models of allergy or parasitic infection have demonstrated induction of Th2 response *in vivo* in the spleen⁷ suggesting that the spleen has an important role in Th2 responses. Furthermore, previous studies in helminth-infected or papain-immunized mice have used splenic basophils to demonstrate their APC features³⁻⁵ and capacity to induce Th2 responses.^{3,5} These lines of evidence thus validate the use of human splenic basophils for the analysis of APC characteristics. Altogether, our results indicate that in humans, the role of basophils in Th2 responses is restricted to provision of IL-4 in the microenvironment,¹⁵ but not as APCs.

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SHORT REPORT

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Regulatory T cell frequency, but not plasma IL-33 levels, represents potential immunological biomarker to predict clinical response to intravenous immunoglobulin therapy

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Abstract

Background: Intravenous immunoglobulin (IVIg) is a polyspecific pooled immunoglobulin G preparation and one of the commonly used therapeutics for autoimmune diseases including those of neurological origin. A recent report in murine model proposed that IVIg expands regulatory T (T_{reg}) cells via induction of interleukin 33 (IL-33). However, translational insight on these observations is lacking.

Methods: Ten newly diagnosed Guillain-Barré syndrome (GBS) patients were treated with IVIg at the rate of 0.4 g/kg for three to five consecutive days. Clinical evaluation for muscular weakness was performed by Medical Research Council (MRC) and modified Rankin scoring (MRS) system. Heparinized blood samples were collected before and 1, 2, and 4–5 weeks post-IVIg therapy. Peripheral blood mononuclear cells were stained for surface CD4 and intracellular Foxp3, IFN- γ , and tumor necrosis factor alpha (TNF- α) and were analyzed by flow cytometry. IL-33 and prostaglandin E2 in the plasma were measured by ELISA.

Results: The fold changes in plasma IL-33 at week 1 showed no correlation with the MRC and MRS scores at weeks 1, 2, and ≥ 4 post-IVIg therapy. Clinical recovery following IVIg therapy appears to be associated with T_{reg} cell response. Contrary to murine study, there was no association between the fold changes in IL-33 at week 1 and T_{reg} cell frequency at weeks 1, 2, and ≥ 4 post-IVIg therapy. T_{reg} cell-mediated clinical response to IVIg therapy in GBS patients was associated with reciprocal regulation of effector T cells-expressing TNF- α .

Conclusion: T_{reg} cell expansion by IVIg in patients with autoimmune diseases lack correlation with IL-33. T_{reg} cell frequency, but not plasma IL-33 levels, represents potential immunological biomarker to predict clinical response to IVIg therapy.

Keywords: IVIg, Guillain-Barré syndrome, T_{reg} cells, IL-33, TNF- α , Clinical response, Regulatory T cells

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Findings

Introduction

Intravenous immunoglobulin (IVIG) is a polyspecific pooled immunoglobulin G preparation and one of the commonly used immunotherapeutics for the treatment of autoimmune diseases including those of neurological origin [1–4]. Various reports demonstrate that IVIG exerts beneficial effects through several mutually non-exclusive mechanisms including expansion of CD4⁺Foxp3⁺ regulatory T cells (T_{reg} cells) [5–10]. Some of these mechanisms are also being explored as biomarkers of IVIG response [11, 12]. T_{reg} cells play an indispensable role in the maintenance of immune tolerance and suppress unnecessary deleterious immune responses, such as autoimmunity [13]. IVIG is shown to expand T_{reg} cells and enhance their functions in experimental animal models and patients with autoimmune diseases [9, 14–17]. Recent studies suggested that IVIG-induced expansion of T_{reg} cells requires the prime role of dendritic cells (DCs), and involves interaction of IVIG with C-type lectin receptors, such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and dendritic cell immunoreceptor (DCIR), and is mediated through prostaglandin (PG) E₂ secreted by DCs [15, 18]. Interestingly, based on the murine models of autoimmune diseases, i.e., antibody-mediated K/BxN arthritis and T cell-mediated experimental autoimmune encephalitis, a recent report proposed that induction of interleukin 33 (IL-33) in macrophages by IVIG through interaction of α 2,6-sialylated crystallizable fraction (Fc) with SIGN-R1 or human DC-SIGN is essential for the expansion of T_{reg} cells [19, 20]. However, translational insight on these observations in patients with autoimmune diseases is lacking.

Guillain-Barré syndrome (GBS) is an autoimmune disease of neurological origin affecting the peripheral nerves [21]. GBS is believed to be caused by effector T cells and autoantibodies to myelin components [22, 23]. Furthermore, GBS patients display reduced frequency of T_{reg} cells that are required for the prevention of autoimmunity [24]. Amelioration of experimental autoimmune neuritis, an experimental model of GBS, was associated with up-regulation of T_{reg} cells [25]. Currently IVIG is used as a first-line therapy for GBS [26]. Of note, sialylated IVIG has been shown to suppress anti-ganglioside antibody-mediated nerve injury in experimental GBS model and was associated with increased expression of IL-33 mRNA [27]. Sialylated IVIG also inhibited anti-ganglioside antibody-mediated complement deposition *in vitro* [28]. In view of these reports, we investigated the role of IL-33 towards clinical response to IVIG treatment and T_{reg} cell expansion in GBS patients. We found that kinetics of peripheral T_{reg}

cell expansion and improvement of clinical symptoms in GBS patients following IVIG therapy lack correlation with the level of induction of IL-33 in the blood.

Materials and methods

Clinical evaluation and sample collection

A total of ten treatment-naïve patients (mean age of 68 years) exhibiting neurological signs of GBS were enrolled based on the diagnostic criteria (Table 1). The study was approved by relevant ethical committee (84-2012-08, CHU Limoges) and consent was obtained from the enrolled patients. The patients received IVIG at the rate of 0.4 g/kg for three (three patients) or five (seven patients) consecutive days. Clinical evaluation for the muscular weakness using Medical Research Council (MRC) and modified Rankin score (MRS) grading systems and collection of heparinized blood samples were done before and 1, 2, and 4–5 weeks after the initiation of IVIG treatment (post-IVIG) [29]. For all the patients, the MRC is a sum score of ten muscle groups tested bilaterally (min = 0, max = 100).

Flow cytometry and ELISA

Plasma and peripheral blood mononuclear cells (PBMCs) were separated from the heparinized blood samples. PBMCs were stained for surface CD4 and intracellular Foxp3, and also interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) following stimulation with phorbol myristate acetate (50 ng/mL) and ionomycin (500 ng/mL, Sigma-Aldrich, France), along with GolgiStop (BD Biosciences, France), for 4 h. The intracellular staining was performed using Foxp3 staining kit (eBioscience, France) as per the manufacturer's instructions. Stained cells were acquired on LSR II (BD Biosciences) flow cytometer and data was analyzed using BD FACS DIVA (BD Biosciences) and FlowJo (FlowJo LLC, USA) softwares. IL-33 was measured in the plasma by ELISA (R&D systems, France). The amount of PGE₂ in the

Table 1 Demographics and clinical information of GBS patients

Sl no.	Age (years)	Sex	MRC score (pre-IVIG)	MRS (pre-IVIG)
P1	61	Male	30	5
P2	68	Female	80	4
P3	57	Male	85	1
P4	70	Female	68	4
P5	82	Male	70	3
P6	66	Female	78	2
P7	85	Male	54	4
P8	60	Female	70	4
P9	68	Male	54	5
P10	59	Female	13	4

plasma samples was estimated by ELISA as described previously [15].

Statistical analysis

One-way ANOVA “Kruskal-Wallis test” was used for the analysis of IL-33 in the plasma. Pearson correlation (*r*) was used to determine the association between MRC or MRS scores and plasma levels of IL-33 and PGE₂ and the frequency of T_{reg} cells in the blood of GBS patients at different time points following IVIG treatment. Same assay was also used for determining the association

between frequency of T_{reg} cells and CD4⁺IFN-γ⁺ and CD4⁺TNF-α⁺ T cells.

Results and discussion

The overall rate of new incidence of GBS is 0.6–4.0/year/100,000 people. We limited our investigation only to newly diagnosed and treatment-naïve GBS patients to avoid possible modulatory effects of past IVIG therapy or other therapeutic agents on the T_{reg} cells and other immune parameters. This explicates the difficulties in enrolling treatment-naïve patients for the investigation.

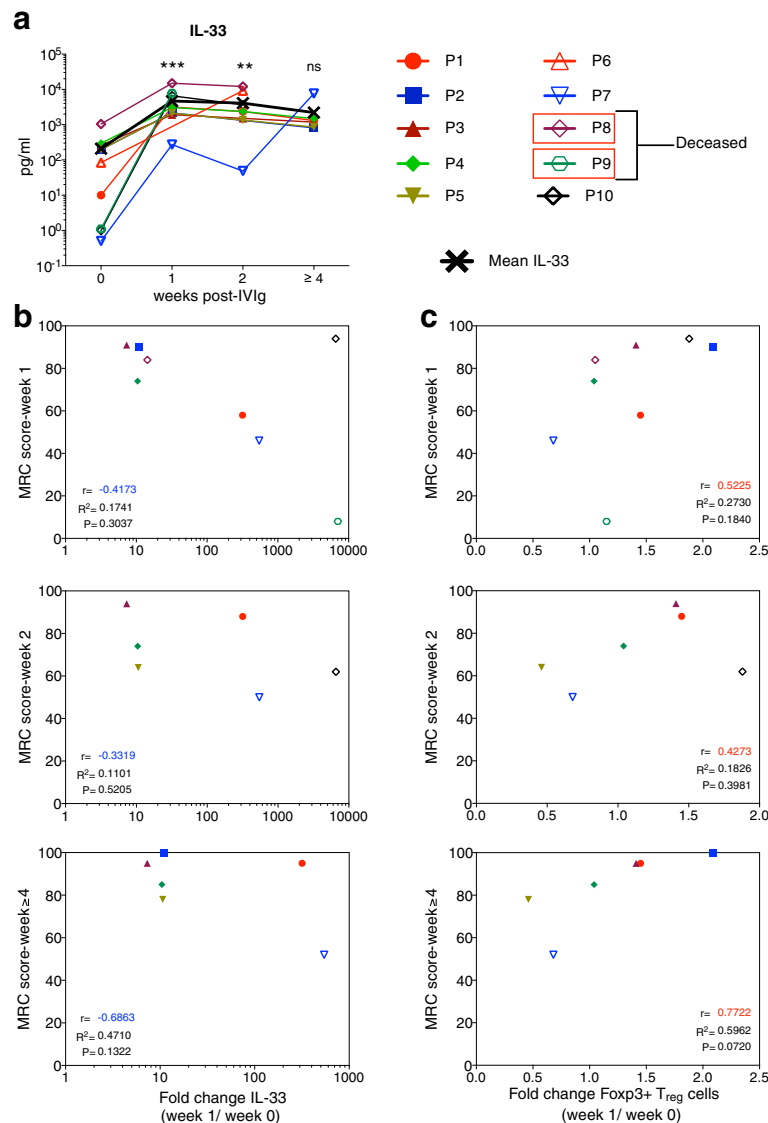


Fig. 1 Correlation between changes in the plasma IL-33 and regulatory T (T_{reg}) cell frequencies and clinical response to IVIG therapy in GBS patients. **a** Temporal changes in the amount of IL-33 (pg/ml) in the plasma of ten Guillain-Barré syndrome patients before (week 0) and weeks 1, 2, and ≥4 following initiation of IVIG therapy. Statistical significance as determined by one-way ANOVA. ****P* < 0.001, ***P* < 0.01, ns = not significant. **b** Correlation between the clinical response (MRC score) at different time points (weeks 1, 2, and ≥4) after initiation of IVIG therapy and the fold changes in plasma IL-33 at week 1. **c** Correlation between the clinical response (MRC score) at different time points (weeks 1, 2, and ≥4) after initiation of IVIG therapy and the fold changes in circulating T_{reg} cells at week 1. Each symbol represents individual patient. *r* = Pearson correlation. *p* = statistical significance

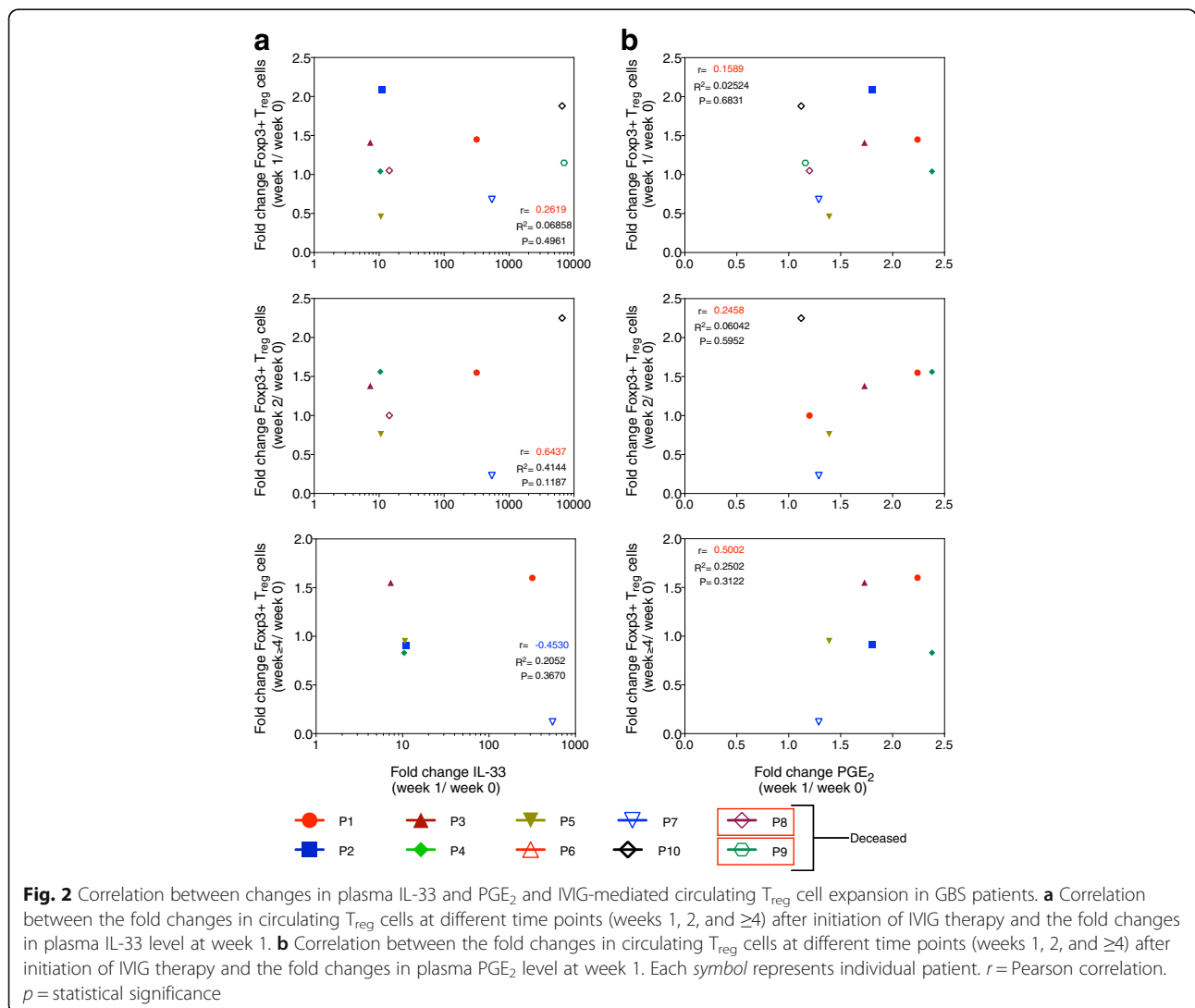
Table 2 Correlation between MRS scores at different time points (weeks 1, 2, and ≥ 4) after initiation of IVIG therapy and the immunological parameters such as IL-33, T_{reg} cells, and PGE₂ at week 1. The values in the parenthesis denote statistical significance

<i>r</i> values (<i>p</i> values)	MRS (week post-IVIG)			
	Week	1	2	≥ 4
IL-33	1	0.57 (0.14)	-0.60 (0.20)	0.71 (0.42)
T _{reg} cell	1	-0.37 (0.3601)	-0.61 (0.1067)	-0.47 (0.0684)
PGE ₂	1	-0.16 (0.6954)	-0.29 (0.4361)	-0.07 (0.8936)

As kinetics of IL-33 induction in the plasma of patients with autoimmune diseases following IVIG therapy was not explored earlier, we first analyzed plasma levels of IL-33 in ten GBS patients at various time points (pre-IVIG and weeks 1, 2, and ≥ 4 after initiation of IVIG therapy). Patients had variable pre-IVIG plasma IL-33 levels that significantly increased at week 1 in all the

patients. This data thus confirms our recent observation in IVIG-treated inflammatory myopathy and anti-neutrophil cytoplasmic antibody-associated vasculitis patients [30]. At subsequent time points, IL-33 levels in the plasma of IVIG-treated GBS patients declined progressively but remain significantly higher than the pre-IVIG levels (Fig. 1a) indicating sustained effect of IVIG beyond its half-life on plasma IL-33 levels.

In order to explore the role of IL-33 in IVIG-mediated therapeutic effects, we analyzed whether IL-33 induction correlates with clinical response to IVIG therapy and T_{reg} cell expansion [29]. Of note, the fold changes in plasma IL-33 level at week 1 showed no correlation with the MRC scores at weeks 1, 2, and ≥ 4 post-IVIG therapy (Fig. 1b). Also, the *r* values are consistently negative at all the time points. On the other hand, T_{reg} cell response following IVIG therapy appears to be linked with clinical recovery from GBS and is consistent with the previous observations on essential



role of T_{reg} cells in IVIG-induced protection in mouse models [18, 31]. Yet, we observed no correlation between the fold-changes in T_{reg} cells at week 1 and clinical score at weeks 1, 2, and ≥ 4 (Fig. 1c). These results suggest that T_{reg} cells, but not IL-33, might predict clinical response to IVIG therapy.

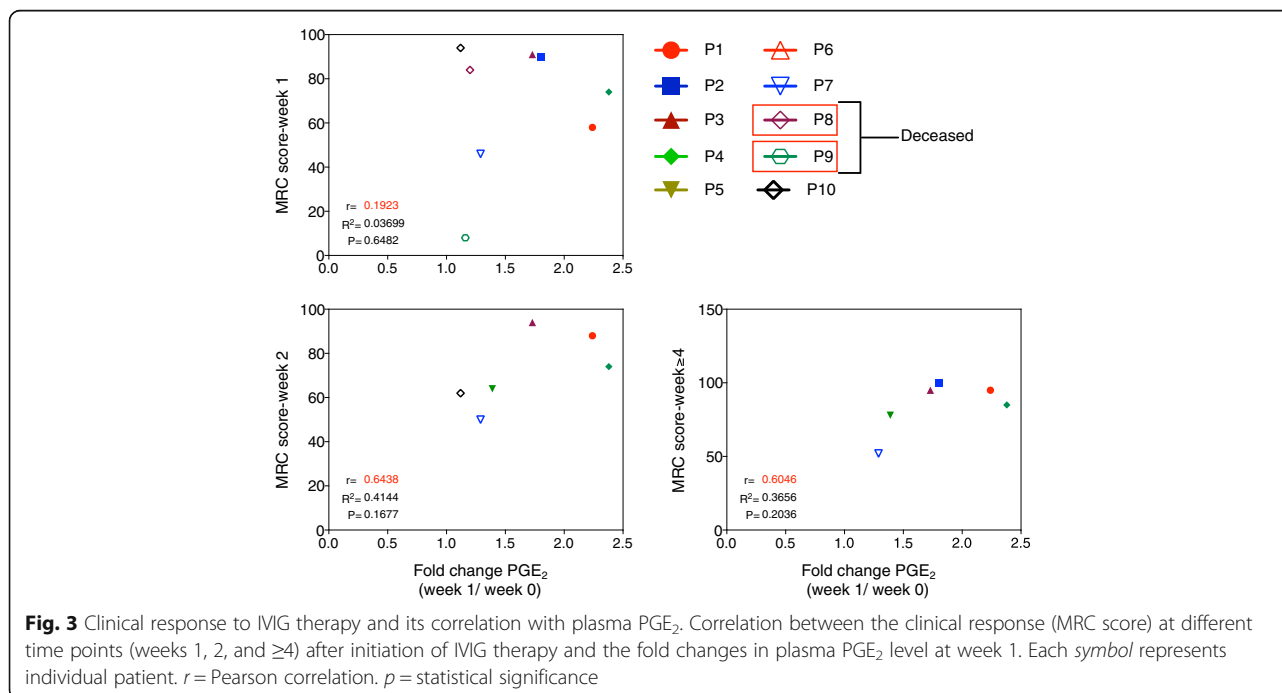
Similar observations are also made with MRS parameters. We found that the fold changes in IL-33 level at week 1 displayed no correlation with MRS scores at weeks 1, 2, and ≥ 4 following initiation of IVIG therapy indicating that clinical improvement as analyzed by MRS is also not associated with changes in IL-33 levels in the blood. As in the case of MRC scores, changes in T_{reg} cells were suggestive of clinical response to IVIG therapy by MRS parameters as well. However, the fold changes in T_{reg} cells at week 1 and MRS scores at weeks 1, 2, and ≥ 4 post-IVIG therapy did not correlate (Table 2).

As data from the recent mouse study suggested that T_{reg} cell expansion by IVIG is dependent on IL-33 [20], we analyzed correlation between IL-33 and T_{reg} cells in these patients at various time points. Contrary to Fiebiger et al., we found no consistent association between the fold changes in IL-33 level at week 1 and T_{reg} cell frequency at weeks 1, 2, and ≥ 4 after initiation of IVIG therapy (Fig. 2a), implying that T_{reg} cell expansion by IVIG in patients with autoimmune diseases is not related to levels of IL-33 in the plasma.

Expansion of T_{reg} cells in the periphery is mediated by the signals derived from professional antigen presenting cells such as DCs. Unlike other cytokines, IL-33 can be released into the microenvironment only upon injury to

the cells and acts as alarmin to signal tissue damage to the immune system [32]. Our recent report shows that DC-SIGN-positive human innate cells derived either from the peripheral blood or from the spleen do not release IL-33 upon IVIG exposure [30]. Therefore, unlike murine models [19, 20], it is likely that damaged non-immune cells like endothelial cells or epithelial cells might have contributed to increased levels of IL-33 observed in the plasma of patients following IVIG therapy. Despite the lack of IL-33 induction, "IVIG-educated" DC-SIGN-positive human DCs induced T_{reg} cell expansion, a process mediated via cyclooxygenase-2-dependent PGE_2 and independent of Fc-fragments of IVIG [15]. Notably, there was a significant increase in the plasma levels of PGE_2 in IVIG-treated GBS patients [33]. In the current study, although plasma PGE_2 levels are enhanced in IVIG-treated patients, we found no correlation between the fold changes in PGE_2 level at week 1 and T_{reg} cell frequencies at week 1, 2, and ≥ 4 post-IVIG therapy (Fig. 2b). Similarly, fold changes in PGE_2 level at week 1 did not correlate with MRC score at weeks 1, 2, and ≥ 4 post-IVIG therapy (Fig. 3). The reason for the non-significant correlation of values might rests in the low number of patients. A recent report shows that plasma PGE_2 levels correlate with the prevention of IVIG resistance in Kawasaki disease [34]. Taken together, these results are suggestive of a role for PGE_2 -mediated expansion of T_{reg} cells in the clinical recovery of patients following IVIG therapy.

In the previous report [30], we analyzed IL-33 in the plasma of patients with rheumatic disorders as early as 48–72 h following initiation of IVIG therapy. With the



exception of two, the remaining 14 patients had IL-33 levels below 500 pg/ml of plasma following IVIG therapy. Although the underlying pathologies in that report and current study are different, we found that at 1 week following initiation of IVIG therapy, IL-33 level in the plasma was more than 1000 pg/ml in nine GBS patients. Furthermore, in majority of the patients (seven), IVIG was given daily for five consecutive days. Therefore, lack of consistent association between the fold changes in IL-33 level and T_{reg} cell frequency following IVIG therapy might not be due to loss of IL-33 subsequent to proteolytic cleavage and binding to ST2 receptor. Also, IL-33 levels observed at first week are maintained in majority of the patients even after 4 weeks post-IVIG. Whether polymorphisms in either ST2 receptor, IL-1RL1, and/or IL-33 itself, skew the plasma kinetics and/or downstream effects of IL-33 remains to be determined [35, 36].

Current literature suggests that IVIG can induce T_{reg} cell expansion by several mutually non-exclusive mechanisms

[9, 14, 16, 17, 31] that may vary among the pathologies and even among the patients with same underlying pathology. This might explain the lack of significant correlation between PGE_2 and T_{reg} cells. As we restricted our study only to treatment-naïve GBS patients, difficulty to get large number of patients provides an alternative explanation for the lack of significant positive correlation between changes in the IL-33 and PGE_2 , and T_{reg} cells. Role of IL-33 in the microenvironment towards DC-mediated human Treg expansion requires further exploration.

In addition to expansion of T_{reg} cells, IVIG is also known to enhance their functions and suppress effector T cell proliferation and production of cytokines such as $IFN-\gamma$ and $TNF-\alpha$ [16, 31]. Interestingly, in IVIG-treated GBS patients, there was a significant negative correlation between the fold changes in T_{reg} cells at week 1 and T cells expressing $TNF-\alpha$ at week 1 post-IVIG therapy. However, such consistent association was not observed with T cells expressing $IFN-\gamma$

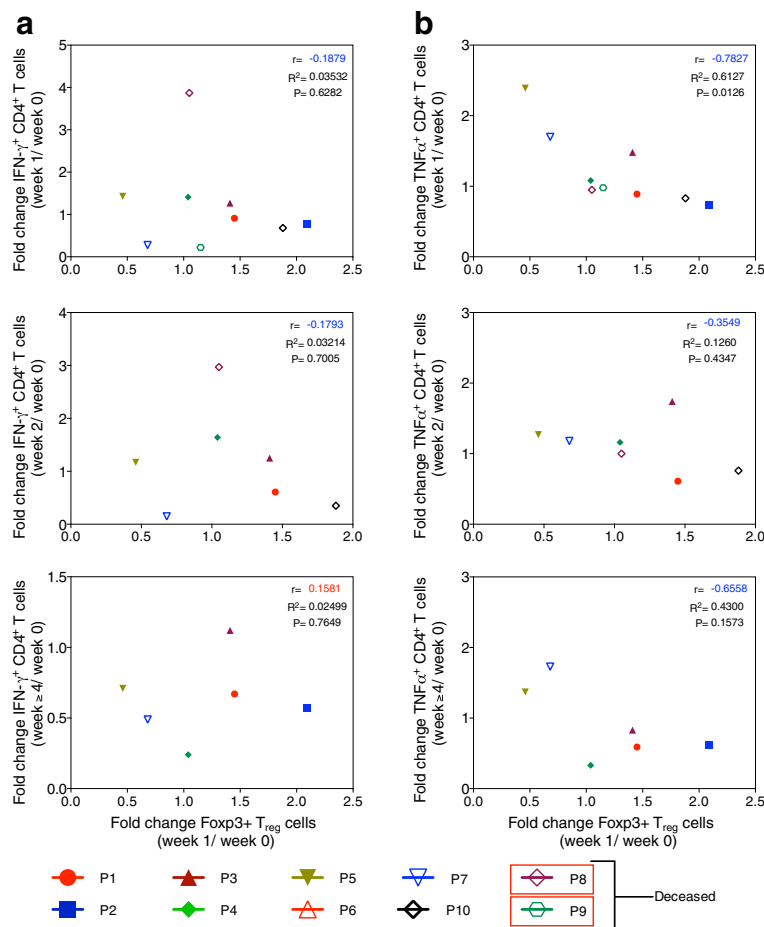


Fig. 4 T_{reg} cell response following IVIG therapy in GBS patients correlates negatively with effector T cells. **a** Correlation between the fold changes in circulating T_{reg} cells at week 1 after initiation of IVIG therapy and the fold changes in $IFN-\gamma^+$ $CD4^+$ T cells at weeks 1, 2, and ≥ 4 . **b** Correlation between the fold changes in circulating T_{reg} cells at week 1 following initiation of IVIG therapy and the fold changes in $TNF-\alpha^+$ $CD4^+$ T cells at weeks 1, 2, and ≥ 4 . Each symbol represents individual patient. r = Pearson correlation. p = statistical significance

(Fig. 4a, b). Thus, T_{reg} cell-mediated clinical response to IVIG therapy is associated with reciprocal regulation of effector T cells in GBS patients.

Conclusions

Because of its role as promoter of type 2 immune responses and regulator of innate and adaptive immune responses, IL-33 has been explored as therapeutic option in pre-clinical models of Alzheimer's disease [37], stroke [38], cerebral malaria [39], and transplantation [40]. In addition, IL-33 was also reported to be essential to attenuate viral-induced encephalitis [41]. In line with these reports, enhanced expression of IL-33 in mice was suggested to mediate T_{reg} cell expansion and protection by IVIG [19, 20, 27]. Translation of these results to human is complicated and requires thorough investigation. Indeed, we found that up-regulation of plasma IL-33 does not correlate with the clinical response to IVIG therapy and expansion of T_{reg} cells in GBS patients. T_{reg} cells on the other hand are negatively correlated to effector T cells expressing TNF- α . Thus, the mechanism of IL-33 induction by IVIG and its role in T_{reg} cell expansion observed in mouse models might not be applicable to human. Small size of the patients' cohort represents limitation of this study. Nevertheless, based on these findings, T_{reg} cell frequency, but not plasma IL-33 levels, represents potential immunological biomarker to predict clinical response to IVIG therapy.

Abbreviations

DCIR: Dendritic cell immunoreceptor; DCs: Dendritic cells; DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; GBS: Guillain-Barré syndrome; IFN- γ : Interferon gamma; IL-33: Interleukin 33; IVIG: Intravenous immunoglobulin; MRC score: Medical research council score; MRS: Modified Rankin score; PGE₂: Prostaglandin E₂; TNF- α : Tumor necrosis factor alpha; T_{reg} cells: Foxp3⁺ regulatory T cells

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Availability of data and materials

All data generated or analyzed during this study are included in this published article as figures.

Authors' contributions

MSM, ES-V, MD, PP, VKS, VS, MR, and JT performed research. FB, J-MV, and LM contributed analytical tools and evaluated the clinical scores. MSM, KNB, SVK, and JB analyzed the data. MSM and JB designed the research and wrote the paper. All authors read and approved the final manuscript.

Competing interests

This work is partially supported by financial support from CSL Behring, France.

Consent for publication

Not applicable

Ethics approval and consent to participate

The study was approved by relevant ethical committee (84-2012-08, CHU Limoges), and the consent was obtained from the enrolled patients.

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Human basophils may not undergo modulation by DC-SIGN and mannose receptor–targeting immunotherapies due to absence of receptors



To the Editor:

Sirvent et al¹ recently showed that novel vaccines targeting dendritic cells (DCs) by coupling glutaraldehyde-polymerized grass pollen allergoids to nonoxidized mannan enhance allergen uptake and induce functional regulatory T cells through programmed death ligand 1. Mechanistically, they found that nonoxidized mannan-coupled glutaraldehyde-polymerized grass pollen allergoids are captured and internalized by 2 lectin receptors on DCs: mannose receptor (CD206) and DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN or CD209). These data thus indicated that DCs could be targeted by C-type lectin receptors for efficient allergen immunotherapy.

Basophils are one of the key players of allergic responses. They mediate allergic inflammation by secretion of T_H2-polarizing cytokines IL-4 and IL-13 and by the release of effector molecules such as histamines and leukotrienes upon FcεRI signaling by IgE-allergen complexes.² Basophils receive activation signals not only via allergen-IgE complexes³ but also via Toll-like receptors⁴ and possibly C-type lectin receptors. In fact, basophils express several lectin receptors such as C-type lectin domain family 12 member A and DC immunoreceptor.^{5,6} Thus, it is likely that in addition to DCs, nonoxidized mannan-coupled allergoids might modulate basophil functions to exert immunotherapeutic benefits. Therefore, we analyzed the expression of mannose receptor and DC-SIGN on steady-state circulating human basophils and on stimulated basophils.

We analyzed basophils in whole blood of healthy donors without their purification to avoid any loss of cells and consequently misinterpretation of data (see this article's Online Repository at www.jacionline.org). Furthermore, erythrocyte-lysed whole blood cells were stimulated with IL-3 (100 ng/10⁶ cells) for 24 hours. IL-3-stimulated basophils were also stimulated for degranulation with anti-IgE antibodies (100 ng/10⁶ cells) for 30 minutes. As controls for the expression of DC-SIGN and CD206, we used CD14⁺ PBMCs (negative control), and rhIL-4 (500 IU/10⁶ cells) and rhGM-CSF (1000 IU/10⁶ cells)-differentiated monocyte-derived DCs (positive control).⁷

Circulating basophils were identified as positive for FcεRI and CD123 and negative for BDCA-4. We found that human basophils at steady state are negative for DC-SIGN and CD206 (Fig 1, A and B). Because basophils display enhanced expression of various receptors on receiving activation stimuli, we explored whether they express these lectin receptors on activation. However, irrespective of stimulation (IL-3 or degranulation stimuli), basophils remained negative for DC-SIGN and CD206 (Fig 1, B and C). Absence of DC-SIGN was also confirmed on isolated basophils. Furthermore, the absence of DC-SIGN and CD206 on basophils in our report is not due to nonreactivity of antibodies used in flow cytometry

because monocyte-derived DCs, used as positive control, uniformly expressed CD206 and DC-SIGN (Fig 1, A and B). As expected, CD14⁺ circulating monocytes, used as negative control, did not stain for both the markers, thus confirming lack of nonspecific binding of antibodies (Fig 1, A and B).

Our results thus indicate that human basophils lack DC-SIGN and mannose receptors and hence unlike DCs, they may not directly respond and be modulated by DC-SIGN- and mannose receptor-binding nonoxidized mannan-coupled allergoids. In addition, our data suggest that basophils do not get activated by DC-SIGN- and mannose receptor-binding allergens unless they are IgE-bound. Thus, the expression pattern of DC-SIGN and mannose receptor among innate cells diversifies allergic as well as tolerogenic responses.

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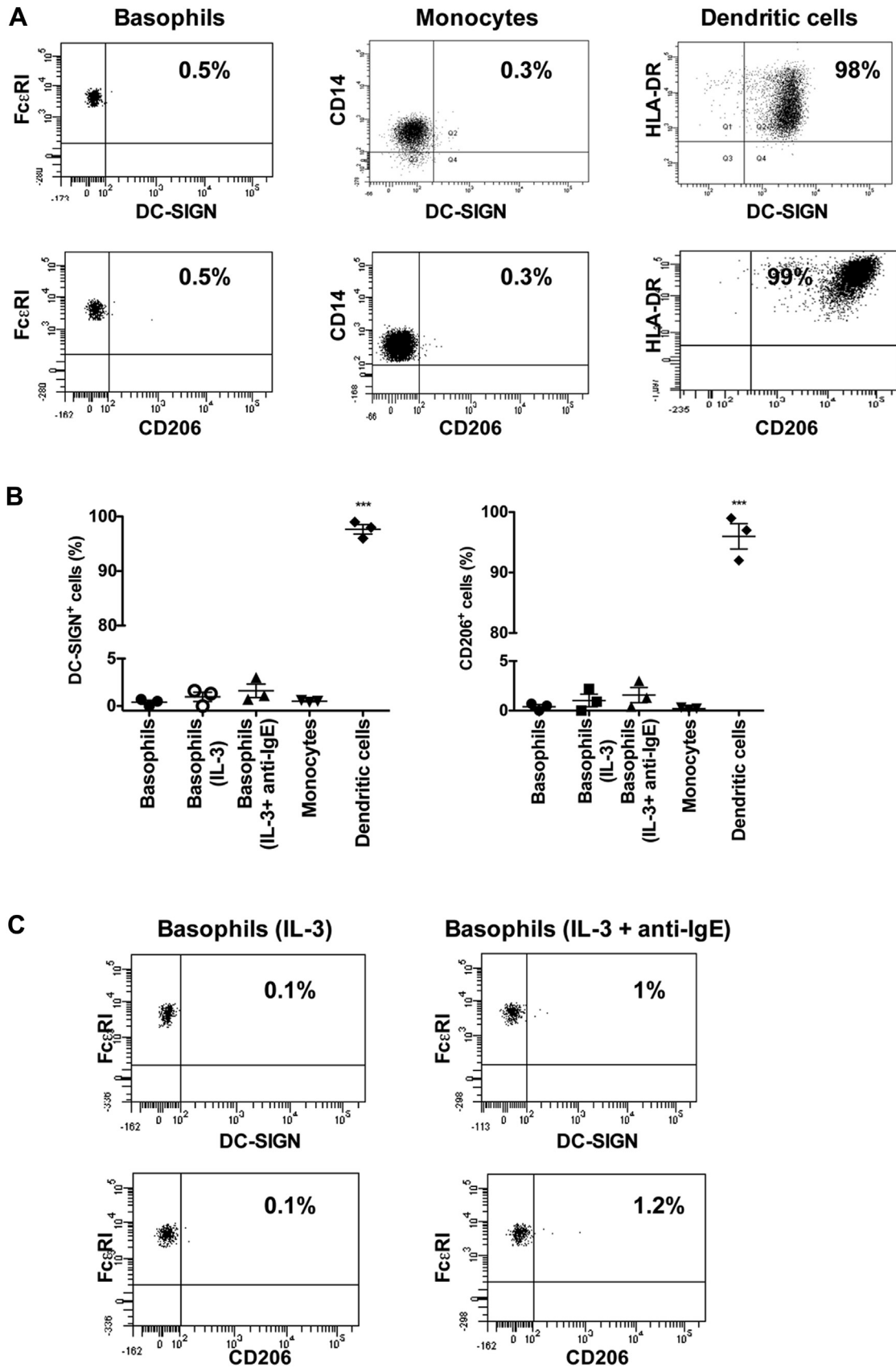


FIG 1. Human basophils are deficient for DC-SIGN and CD206. **A-C,** Flow cytometric analysis of DC-SIGN and CD206 on steady-state basophils, stimulated basophils (IL-3 or IL-3 and anti-IgE), and monocytes and monocyte-derived DCs. Representative dot-plots and percentage of cells (mean ± SEM, n = 3) positive for DC-SIGN and CD206. ****P* < .001.

METHODS

Cells and stimulation

Buffy bags of healthy donors were obtained from Centre Necker-Cabanel (EFS, Paris, France), and INSERM-EFS ethical committee approval (N°15/EFS/012) for the use of such material was obtained. Experiments were performed in accordance with the approved guidelines of INSERM.

Red blood cells were lysed using Ammonium-Chloride-Potassium Lysing Buffer (Lonza, Levallois, France). Briefly, blood was spun down and resuspended and incubated in Ammonium-Chloride-Potassium lysing buffer for 30 to 60 seconds. Cells were washed with medium and resuspended in serum-free X-VIVO medium. Cells were stimulated with IL-3 (100 ng/million cells; ImmunoTools, Friesoythe, Germany) for 24 hours. In addition, cells were cultured with IL-3 (100 ng/million cells) for up to 24 hours and during the last 30 minutes, cells were treated with anti-IgE antibodies (100 ng/million cells; Sigma-Aldrich, St Quentin Fallavier, France). Phenotype of basophils was analyzed in steady state and stimulated conditions by flow cytometry (LSR II, BD Biosciences, Le Pont De Claix, France) and the data were analyzed using FACSDiva software (BD Biosciences).

PBMCs were obtained from buffy bags of healthy donors by Ficoll density gradient centrifugation. Monocytes were isolated from PBMCs by using CD14 microbeads (Miltenyi Biotec, Paris, France) and were cultured for 5 days in rhIL-4 (500 IU/10⁶ cells) and rhGM-CSF (1000 IU/10⁶ cells) (both from Miltenyi Biotec) to obtain monocyte-derived DCs.

Antibodies for flow cytometry

The following antibodies were used for flow cytometry. FcεRIα-BV510 (Clone AER37 [CRA-1]) was from BioLegend (London, United Kingdom) and BDCA-4 (CD304)-APC (Clone AD5-17F6) was obtained from Miltenyi Biotec. CD123-BV421 (Clone 9F5), CD209-FITC (Clone DCN46), CD206-PE (Clone 19.2), HLA-DR-APC or PE (G46-6, BD Biosciences), and CD14-APC (Clone M5E2) antibodies were from BD Biosciences.

Statistical analysis

Levels of significance for comparison between samples were determined by 1-way ANOVA (repeated measures with Tukey's multiple comparison test). A *P* value of less than .05 was considered significant. Statistical analysis was performed by using Prism 5 GraphPad Software (La Jolla, Calif). Data are presented as mean ± SEM.



IL-1 β , But Not Programed Death-1 and Programed Death Ligand Pathway, Is Critical for the Human Th17 Response to *Mycobacterium tuberculosis*

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The programed death-1 (PD-1)–programed death ligand-1 (PD-L1) and PD-L2 co-inhibitory pathway has been implicated in the evasion strategies of *Mycobacterium tuberculosis*. Specifically, *M. tuberculosis*-induced PD-L1 orchestrates expansion of regulatory T cells and suppression of Th1 response. However, the role of PD pathway in regulating Th17 response to *M. tuberculosis* has not been investigated. In the present report, we demonstrate that *M. tuberculosis* and *M. tuberculosis*-derived antigen fractions have differential abilities to mediate human monocyte- and dendritic cell (DC)-mediated Th17 response and were independent of expression of PD-L1 or PD-L2 on aforementioned antigen-presenting cells. Importantly, we observed that blockade of PD-L1 or PD-1 did not significantly modify either the frequencies of Th17 cells or the production of IL-17 from CD4⁺ T cells though IFN- γ response was significantly enhanced. On the contrary, IL-1 β from monocytes and DCs were critical for the Th17 response to *M. tuberculosis*. Together, our results indicate that IL-1 β , but not members of the programed death pathway, is critical for human Th17 response to *M. tuberculosis*.

Keywords: PD-L1, PD-1, *Mycobacterium tuberculosis*, dendritic cells, monocytes, Th17, IL-23, IL-1 β

INTRODUCTION

Tuberculosis caused by *Mycobacterium tuberculosis* still remains a global threat with an estimated 1.5 million deaths annually. Cellular immunity plays a critical role in mediating the protection against tuberculosis. Indeed, IFN- γ -producing CD4⁺ T helper type 1 cells are critical for the control of *M. tuberculosis* in humans and murine models (1–4). Thus, general paradigm for tuberculosis vaccines has largely focused on enhancing the Th1/IFN- γ response (5, 6). However, despite enhancing IFN- γ response, the recombinant MVA85A vaccine failed to protect infants from tuberculosis (7). Therefore, it is pertinent to decipher the role played by other CD4⁺ T cell subsets and their cytokines in mediating immunity against *M. tuberculosis*.

Th17 cells that express transcription factor RORC and secrete archetype cytokine IL-17A represent a distinct subset of CD4⁺ T cells. Th17 cells also produce IL-17F, IL-21, IL-22, GM-CSF, and IL-26 and mediate pro-inflammatory responses (8–11). Th17 cells are associated with the pathogenesis of several autoimmune and inflammatory diseases (12–14). Evolutionarily, Th17 response is conserved to mediate protection at mucosal surfaces and against extracellular pathogens (10, 15). Recent

reports have also indicated that Th17 response may play a crucial role in mediating protection against intracellular pathogens, such as *Francisella tularensis* and *Chlamydia muridarum* (16–18). These data thus indicate the diverse role of Th17 cells in various physiopathologies.

Mycobacterium tuberculosis employs a plethora of mechanisms to suppress both innate and adaptive immune responses. The role of Th17 response to *M. tuberculosis* is largely pursued in mice, and it remains highly controversial (19–25). Recent reports in tuberculosis patients indicate that active disease and its severity are associated with low Th17 response (26, 27). Of note, anti-tuberculosis therapy is associated with enhanced Th17 response, suggesting that *M. tuberculosis* suppresses Th17 response as one of the immune evasion mechanisms (28).

Programed death-1 (PD-1)–programed death ligand-1 (PD-L1)/PD-L2 pathway occupies a unique place in the immune evasion strategies employed by *M. tuberculosis*. Recent data highlight the role of PD-1–PD-L1/PD-L2 axis in modulating regulatory T cell (Treg) and Th1 response to *M. tuberculosis* (29–33). Whether this pathway also regulates Th17 response to *M. tuberculosis* is not known. Therefore, in the present study, we have evaluated the role of PD pathway members (PD-L1, PD-L2, and PD-1) in mediating human monocyte- and dendritic cell (DC)-mediated Th17 response to *M. tuberculosis*.

Several reports have shown that DCs promote Th17 responses to either *M. tuberculosis* or its antigens (34–37). We found that monocytes and DCs have differential capacity to promote Th17 response to *M. tuberculosis* and *M. tuberculosis*-derived antigens. Notably, a prominent IL-17 response was mediated by DCs in comparison to monocytes. Although both monocytes and DCs did not express PD-L2, PD-L1 was significantly enhanced upon stimulation with *M. tuberculosis*. Similarly, *M. tuberculosis* stimulation of monocyte/DC–CD4⁺ cocultures also lead to significant increase in the frequency of PD-1⁺CD4⁺ T cells. Importantly, blocking PD-L1 or PD-1 neither significantly altered the frequencies of Th17 cells nor augmented IL-17 secretion from CD4⁺ T cells. Analysis of key Th17-polarizing cytokines indicated that the production of IL-1 β was crucial in the establishment of Th17 response to *M. tuberculosis*. These results thus reveal that the outcome of Th17 response to *M. tuberculosis* is dictated by the capacity of human innate cells to secrete key Th17-polarizing cytokine (IL-1 β) and not expression of members of the PD pathway.

MATERIALS AND METHODS

Antibodies

FITC-conjugated mAbs to CD86 [clone 2331 (FUN-1)], CD274 (clone MIH1), PE-conjugated mAbs to pSTAT3 (clone 4/P-STAT3), CD80 (clone L307.4), PD-L2 (clone 2D3/B7-H2), antigen-presenting cell (APC)-conjugated mAbs to HLA-DR (clone G46-6), PD-1 (clone MIH4), Alexa 700-conjugated mAb to CD4 (clone RPA-T4), and BV421-conjugated mAb to CD4 were from BD Biosciences (Le Pont de Claix, France). PE-conjugated mAbs to IL-17A (clone eBio64CAP17), human–mouse ROR γ t (AFKJS-9), APC-conjugated mAb to FoxP3 (clone 236A/E7), and Fixable Viability Dye eFluor[®] 506 were from eBioscience (Paris, France). PE-conjugated mAb to CD40 (clone MAB89) was from

Beckman Coulter (Villepinte, France). Blocking mAb to human PD-L1 (clone MIH1) and isotype control mAb were from eBioscience. Alexa-488 conjugated mAb to IL-10 (clone JES59D7) and blocking mAb to PD-1 (clone EH12.2H7) were from Biogen (London, UK).

M. tuberculosis Antigens

γ -irradiated *M. tuberculosis* (strain H37Rv) and *M. tuberculosis* cell wall, cell membrane cytoplasmic fractions were obtained from BEI resources NIAID, NIH.

Purification of Immune Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy bags of healthy donors by Ficoll density gradient centrifugation. Buffy bags of the healthy blood donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (Institut National de la Santé et de la Recherche-EFS ethical committee convention 15/EFS/012). Monocytes and autologous CD4⁺ T cells were isolated from PBMCs by positive selection using the human CD14 and the CD4 MicroBeads (Miltenyi Biotec, Paris, France), respectively. The cell purity was more than 97%.

Generation of DCs

Monocytes (0.5×10^6 cells/ml) were cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; 1,000 IU/ 10^6 cells) and IL-4 (500 IU/ 10^6 cells) (both cytokines from Miltenyi Biotec) for 5 days to obtain immature monocyte-derived DCs (38). The differentiation of DCs was confirmed by flow cytometry.

Stimulation of Monocytes and DCs with *M. tuberculosis* and Their Fractions

Monocytes or DCs (0.5×10^6 /ml) were cultured with (20 μ g/ml) γ -irradiated *M. tuberculosis* or *M. tuberculosis*-derived cell wall, cell membrane, or cytoplasmic fractions (10 μ g/ml) for 24 h. Activation of DCs and monocytes was assessed based on the expression of HLA-DR, CD40, CD80, and CD86 by using fluorescence-conjugated mAbs. In addition, induction of PD-L1 and PD-L2 on these cells was also analyzed.

Monocyte–CD4⁺ T Cell and DC–CD4⁺ T Cell Cocultures

Monocytes or DCs (10,000 cells/200 μ l/well) were cocultured with autologous CD4⁺ T cells at a ratio of 1:10 in U-bottom 96-well plates and stimulated with (20 μ g/ml) γ -irradiated *M. tuberculosis* or *M. tuberculosis*-derived cell wall, cell membrane, or cytoplasmic fractions (10 μ g/ml) for 5 days. After 5 days, cell-free supernatants were collected, and T cells were activated with phorbol myristate acetate (50 ng/ml) and ionomycin (500 ng/ml, Sigma-Aldrich, France), along with GolgiStop (BD Biosciences), for 4 h. For the analysis of IL-17⁺CD4⁺ T cells and IL-10⁺CD4⁺ T cells in the cocultures, surface staining was performed with fluorescence-conjugated mAbs to CD4. Then, cells were fixed, permeabilized using intracellular staining kit (eBioscience), and incubated at 4°C with fluorescence-conjugated mAbs to IL-17A,

IL-10, pSTAT3, and RORC. Samples were acquired by using LSR II (BD Biosciences) flow cytometry, and data were analyzed by BD FACS DIVA software (BD Biosciences).

For the analysis of FoxP3⁺CD4⁺ T in the cocultures, surface staining was performed with fluorescence-conjugated mAb to CD4. Then, cells were fixed, permeabilized using intracellular staining kit (eBioscience), and incubated at 4°C with fluorescence-conjugated mAb to FoxP3.

For the analysis of PD-1 on CD4⁺ T cells, surface staining was performed with fluorescence-conjugated mAbs to CD4 and PD-1.

PD-L1 and PD-1 Blocking Experiment

Autologous monocyte-CD4⁺ T cell and DC-CD4⁺ T cell cocultures were stimulated with *M. tuberculosis* for 18 h. Anti-PD-L1 (10 µg/ml), anti-PD-1 (10 µg/ml), or isotype control mAbs were then added to the coculture. After 5 days, frequency of IL-17A⁺CD4⁺ T cells and IL-17 secretion were analyzed.

Validation of Role for Innate Cytokines in *M. tuberculosis*-Mediated Th17 Responses

Dendritic cells (10,000 cells/200 µl/well) were cocultured with autologous CD4⁺ T cells at a ratio of 1:10 in U-bottom 96-well plates and stimulated with (20 µg/ml) γ -irradiated *M. tuberculosis* or *M. tuberculosis*-derived cytoplasmic fractions (10 µg/ml) either alone or in the presence of (10 ng/ml) rhIL-1 β (R&D systems, Lille, France) or rhIL-23 (PeproTech, Neuilly-Sur-Seine, France) for 5 days. After 5 days, cell-free supernatants were collected, and T cells were analyzed for Th17 responses by intracellular staining as described earlier.

Quantification of Cytokines

IL-17A, IFN- γ , IL-6, IL-1 β , and IL-23 in the cell-free supernatants of monocyte-CD4⁺ T cell and DC-CD4⁺ T cell cocultures were quantified by ELISA (Ready-SET-Go, eBioscience).

Statistical Analysis

Statistical analyses were performed by two-way non-parametric Mann-Whitney test or one-way ANOVA (Kruskal-Wallis test or Holm-Sidak's multiple comparisons test) as indicated using Prism 6 software. $P < 0.05$ was considered significant.

RESULTS

Human Monocytes Promote Th17 Response to *M. tuberculosis*

Tuberculosis is associated with an expansion of immunomodulatory CD16⁺ monocyte population (39). Monocytes have been implicated in the establishment of Th17 response in autoimmune diseases (40, 41) and infection (42). We first investigated the ability of human monocytes to promote Th17 response to *M. tuberculosis*. We found that *M. tuberculosis*-stimulated monocytes significantly enhanced both frequency of IL-17A⁺CD4⁺ T cells (Figures 1A,B) and the amount of secretion of IL-17A (Figure 1C). On further examination of downstream Th17 signaling events in CD4⁺ T cells, we found that *M. tuberculosis*-stimulated monocytes significantly enhanced the frequency of

pSTAT3 (Figure 1D) and RORC (Figure 1E). These results thus suggest that human monocytes have the ability to promote Th17 response to *M. tuberculosis*.

Different Antigen Fractions of *M. tuberculosis* Have Similar Capacity to Induce Monocyte-Mediated Th17 Response

Mycobacterium tuberculosis possesses a plethora of antigens to modulate immune response. Most of these antigens are either located in the cell wall, cell membrane, or cytosol. Hence, we investigated whether these different antigens of *M. tuberculosis* have similar or distinct ability to mount monocyte-mediated Th17 response. All these antigenic fractions induced similar level of activation of monocytes as shown by the significantly augmented expressions of CD80, CD86, and CD40 (Figure S1 in Supplementary Material). Consistent with the activation status of monocytes, all the antigen fractions, i.e., cell wall, cell membrane, and cytoplasmic fractions significantly enhanced the frequency of IL-17A⁺CD4⁺ T cells (Figures 2A,B) and the secretion of IL-17A (Figure 2C). However, we observed no significant differences in the extent of Th17 response mediated by different fractions of *M. tuberculosis*. Our data thus indicate that all the antigen fractions of *M. tuberculosis* have similar ability to promote monocyte-mediated Th17 responses.

Dendritic Cells Differentially Promote Th17 Response to *M. tuberculosis* and Its Antigen Fractions

Dendritic cells play a critical role in mediating protection to *M. tuberculosis* by priming T cell response. Hence, we investigated the capacity of DCs to promote Th17 response to *M. tuberculosis* and its antigen fractions. Our results indicate that DCs have the capacity to enhance Th17 response to *M. tuberculosis* (Figures 3A-C). However, in contrast to monocytes, human DCs displayed differential ability in stimulating Th17 response to different antigen fractions of *M. tuberculosis*. Thus, cell wall antigen fraction substantially enhanced Th17 response and was comparable to that induced by *M. tuberculosis* bacteria (Figures 3A-C). Although cell membrane fraction also enhanced Th17 response, it was lower than that observed with *M. tuberculosis* bacteria and cell wall fraction. Surprisingly, cytoplasmic fraction did not significantly enhance either frequencies of IL-17A⁺CD4⁺ T cells or the production of IL-17A (Figures 3A-C).

We confirm that low-level induction of Th17 response by cytoplasmic fraction was not because of lack of induction of DC maturation. In fact, DCs stimulated with cytoplasmic fraction significantly enhanced DC maturation markers HLA-DR, CD80, CD86, and CD40 and was similar to those observed with *M. tuberculosis* bacteria, cell wall, and cell membrane fractions (Figure S2 in Supplementary Material).

Further, the inability of cytoplasmic fraction to promote DC-mediated Th17 response was not due to increased frequency of suppressor T cells, such as IL-10⁺CD4⁺ T cells or FoxP3⁺CD4⁺ Treg cells. In fact, the frequency of IL-10⁺CD4⁺ T cells remain unaltered upon stimulation with *M. tuberculosis* or its antigen fractions (Figure 3D). Consistent with the previous findings,

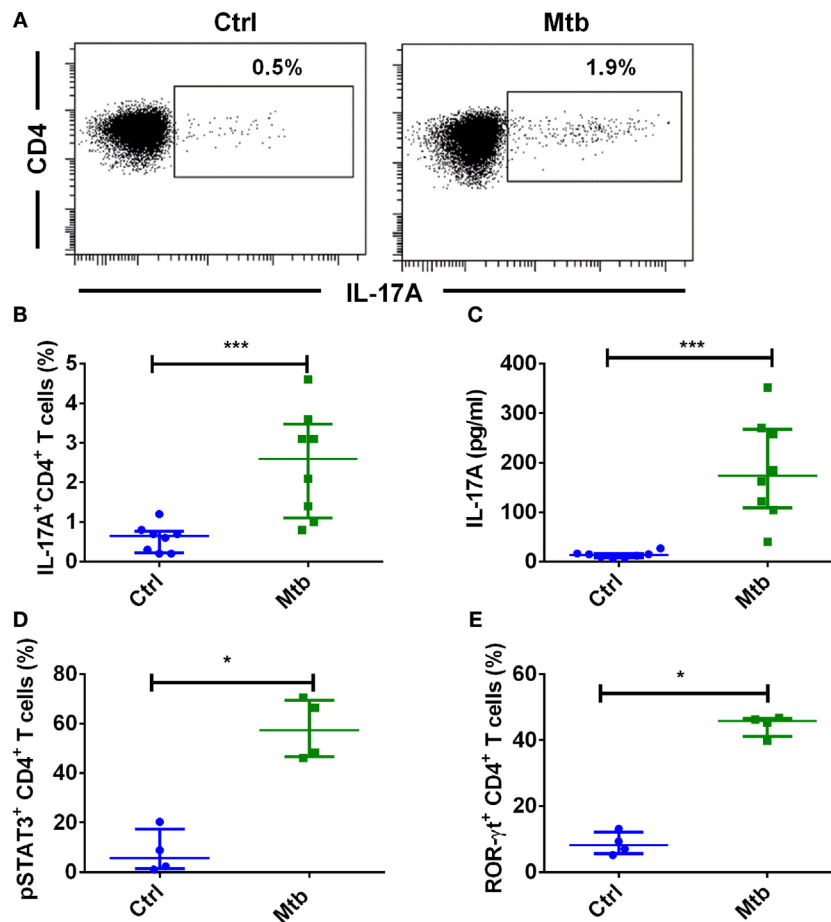


FIGURE 1 | Human monocytes stimulated with *M. tuberculosis* enhance Th17 response. Human peripheral blood monocytes were cocultured with autologous CD4⁺ T cells at a ratio of 1:10 in X-vivo medium for 5 days with or without γ -irradiated *M. tuberculosis* (Mtb). Th17 cells were analyzed by flow cytometry by combination of surface staining for CD4 and intracellular staining for IL-17A, pSTAT3, and ROR- γ t. IL-17A in the cell-free supernatants was quantified by ELISA. **(A,B)** Representative dot plots showing the frequencies of CD4⁺IL-17A⁺ T cells and **(B)** median \pm SEM data from eight donors. **(C)** The amount of secretion of IL-17A (median \pm SEM, $n = 8$). **(D)** Percentage of CD4⁺ T cells positive for pSTAT3 (median \pm SEM, $n = 4$). **(E)** Percentage of CD4⁺ T cells positive for ROR- γ t (median \pm SEM, $n = 4$). * $P < 0.05$; *** $P < 0.001$; as determined by Mann-Whitney test.

M. tuberculosis and its antigen fractions including cytoplasmic fraction significantly enhanced the frequency of FoxP3⁺CD4⁺ Tregs (Figure 3E). However, the extent of stimulation of Treg response was similar among all three antigen fractions of *M. tuberculosis*.

Differential Expression of PD-L1 on Monocytes and DCs upon Stimulation with *M. tuberculosis* and Its Antigen Fractions

M. tuberculosis employs several strategies to modulate effector CD4 T cell response (43). Several recent reports have indicated that PD-1–PD-L1/PD-L2 axis has a pivotal role in the regulation of T cell response to *M. tuberculosis* (29, 31, 33). Therefore, to decipher the role of PD-L1/PD-L2 in regulating Th17 response to *M. tuberculosis*, we first investigated the ability of *M. tuberculosis* and its different antigen fractions in modulating PD-L1 and PD-L2 expression on monocytes and DCs. Our results reveal that *M. tuberculosis* bacteria, cell wall fraction, and cell membrane

fractions significantly induce PD-L1 expression on both monocytes and DCs (Figures 4A,B). However, significant induction of PD-L1 by cytoplasmic fraction was observed only in monocytes but not in DCs (Figures 4A,B). Unstimulated monocytes and DCs did not express PD-L2 and was not altered upon stimulation with either *M. tuberculosis* or its different antigen fractions (Figures 4C,D).

PD-L1 on Human Innate Cells Regulates Th1, But Not Th17, Response to *M. tuberculosis*

PD-1–PD-L1 interaction has been reported to inhibit Th1 response to *M. tuberculosis*. To explore if PD-L1 also checks Th17 response to *M. tuberculosis*, we employed blocking antibodies to PD-L1. We found that blocking PD-L1 either in the monocyte–CD4⁺ T cell coculture or in the DC–CD4⁺ T cell coculture did not significantly modify either the frequencies of IL-17A⁺CD4⁺ T cells or the secretion of IL-17A (Figures 5A–F). These data are

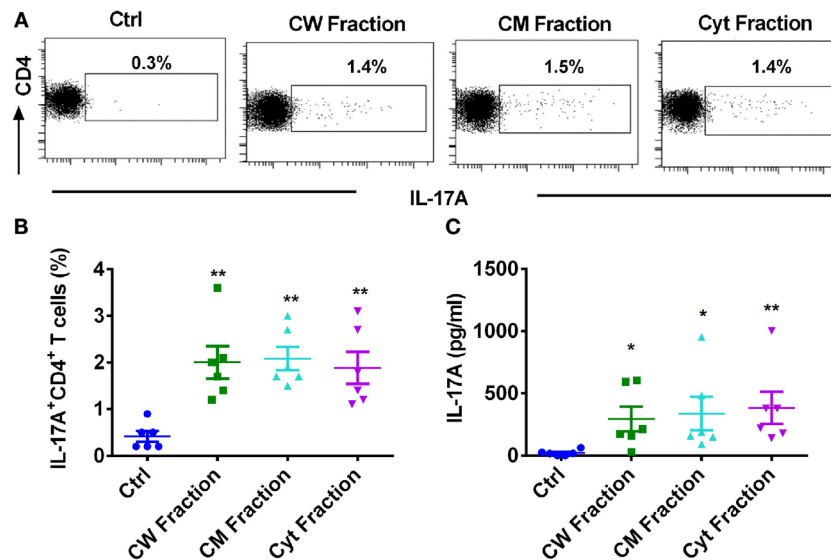


FIGURE 2 | Different antigen fractions of *M. tuberculosis* have similar capacity to amplify human monocyte-mediated Th17 response. Monocytes were cocultured with autologous CD4⁺ T cells either alone or were stimulated with *M. tuberculosis*-derived cell wall (CW), cell membrane (CM), or cytoplasmic (Cyt) fractions for 5 days. Th17 cells were analyzed by flow cytometry by combination of surface staining for CD4 and intracellular staining for IL-17A. IL-17A in the cell-free supernatants was quantified by ELISA. **(A,B)** Representative dot plots showing the frequencies of CD4⁺IL-17A⁺ T cells and **(B)** mean \pm SEM data from six independent donors. **(C)** The amount of secretion of IL-17A (mean \pm SEM, $n = 6$). * $P < 0.05$; ** $P < 0.01$; as determined by one-way ANOVA.

in line with the fact that lack of induction of PD-L1 on DCs by cytoplasmic fraction of *M. tuberculosis* was not associated with enhanced Th17 responses (Figures 3 and 4B). Whereas, same cytoplasmic fraction promoted Th17 response despite it induced significant expression of PD-L1 on monocytes (Figures 2 and 4A). Taken together, these results imply that PD-L1 does not function as negative regulator of Th17 response to *M. tuberculosis*.

It was important to demonstrate that observed phenomenon is not due to inefficient PD-L1 blocking. Previous results have demonstrated that PD-L1 negatively regulates IFN- γ responses. Therefore, to demonstrate the efficiency of PD-L1 blocking, we have assessed the secretion of IFN- γ in the monocyte-CD4⁺ T cell and DC-CD4⁺ T cell cocultures. Consistent with the previous data, blockade of PD-L1 significantly augmented the production of IFN- γ from CD4⁺ T cells (Figures 6A,B).

PD-1 Is Dispensable for the Regulation of Th17 Response to *M. tuberculosis*

Programmed death ligand-1 signals via PD-1 on CD4⁺ T cells, and recently it was reported that *Mycobacterium*-induced PD-1 coordinates suppression of Th17 response in tuberculosis patients (28). We confirm that *M. tuberculosis* significantly enhances the frequency of PD-1⁺ T cells in the DC/monocyte-CD4⁺ T cell cocultures (Figures 7A,D). However, blockade of PD-1 did not significantly alter either the frequencies of IL-17A⁺CD4⁺ T cells or the secretion of IL-17A (Figures 7B,C,E,F). Similar to PD-L1 experiments, blockade of PD-1 also lead to increased IFN- γ responses. Together, these data indicate that human Th17 response to *M. tuberculosis* is not under the control of PD pathway.

IL-1 β Is Critical for Mediating Th17 Response to *M. tuberculosis*

Upon encountering with pathogens, APCs secrete various cytokines that dictate the outcome of T cell response. Particularly, IL-6, IL-1 β , and IL-23 derived from innate immune cells play a crucial role in establishing and sustaining Th17 response. In addition, IL-21 and TGF- β also have important role in Th17 priming (8, 14). Thus, to decipher the mechanism of differential Th17 response to *M. tuberculosis* and its fractions by monocytes and DCs, we analyzed the Th17-polarizing cytokines secreted by these innate cells. IL-21 was not produced by monocytes and DCs upon stimulation with *M. tuberculosis* and its antigen fractions. Similarly, basal level of TGF- β was not significantly altered by *M. tuberculosis*. However, upon stimulation with *M. tuberculosis* and its antigen fractions, both innate cells secreted large quantities of IL-6 (Figures 8A,D) and moderate amounts of IL-1 β (Figures 8B,E). Of note, monocytes produced significantly lower amounts (~10-fold) of IL-23 as compared to DCs (Figures 8C,F). Interestingly, consistent with the lack of role in the modulation of Th17 response, none of these innate cytokines were significantly altered upon blockade of PD-L1 (Figure S3 in Supplementary Material).

Despite induction of large quantities of IL-6 in DCs similar to those induced by *M. tuberculosis* or its other antigen fractions, lack of Th17 response by cytoplasmic fraction provide a pointer toward dispensability of this cytokine in *M. tuberculosis*-mediated Th17 response. The similar amount of IL-1 β production by stimulated monocytes explains elicitation of Th17 response by all the antigen fractions of *M. tuberculosis*. On the contrary, cytoplasmic fraction was inferior to cell wall

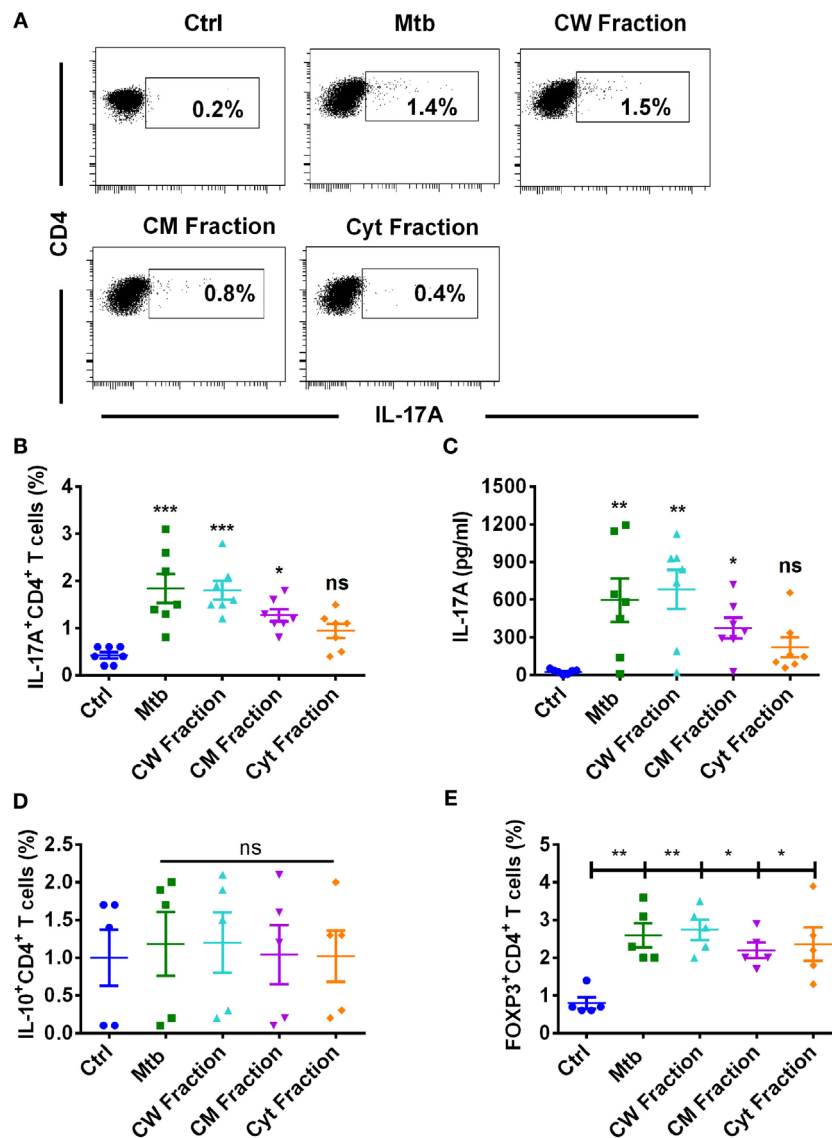


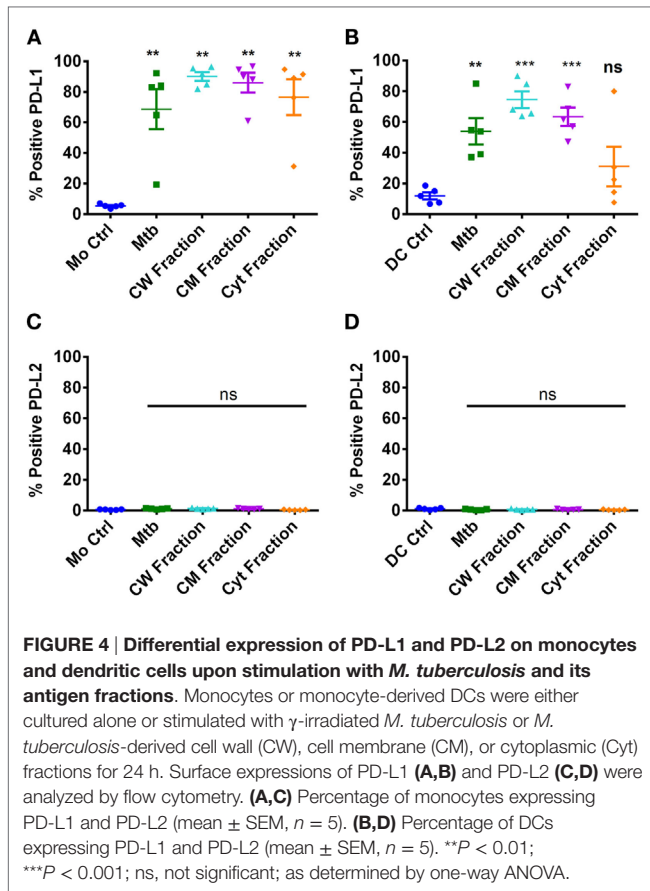
FIGURE 3 | Human dendritic cells differentially promote Th17 response to *M. tuberculosis* and its antigen fractions. Human monocyte-derived DCs were cocultured with autologous CD4⁺ T cells at a ratio of 1:10 in X-vivo medium alone or with γ -irradiated *M. tuberculosis* (Mtb) or *M. tuberculosis*-derived cell wall (CW), cell membrane (CM), or cytoplasmic (Cyt) fractions for 5 days. Th17 cells were analyzed by flow cytometry by combination of surface staining for CD4 and intracellular staining for IL-17A. IL-17A in the cell-free supernatants was quantified by ELISA. **(A,B)** Representative dot plots showing the frequencies of CD4⁺IL-17A⁺ T cells and **(B)** mean \pm SEM data from seven independent donors. **(C)** The amount of secretion of IL-17A (mean \pm SEM, $n = 7$). **(D,E)** Frequencies of IL-10⁺CD4⁺ T cells or FoxP3⁺CD4⁺ Treg cells in DC-T cell cocultures stimulated with various antigens of *M. tuberculosis* (mean \pm SEM, $n = 5$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant; as determined by one-way ANOVA.

and cell membrane antigen fractions in promoting IL-1 β and IL-23 by DCs, and this could explain the lack of Th17 responses observed in DCs upon stimulation with cytoplasmic fraction. Therefore, to establish a role for IL-1 β and IL-23 in *M. tuberculosis*-mediated Th17 responses, we supplemented IL-1 β or IL-23 to the DC-CD4⁺ T cell cocultures that are stimulated with cytoplasmic fraction of *M. tuberculosis*. We found that supplementation of IL-1 β enabled cytoplasmic fraction-stimulated DCs to mount strong Th17 response (**Figures 9A,B**), while IL-23 had no effect (**Figures 9C,D**). Our results thus reveal that innate

Th17-polarizing cytokine IL-1 β but not members of the PD pathway, dictates the outcome of human Th17 response to *M. tuberculosis* and its antigen fractions.

DISCUSSION

M. tuberculosis-loaded aerosols that enter the lungs interact with resident phagocytes, which include alveolar macrophages and DCs. Different subpopulations of DCs are known to coexist in the human lungs (44). Circulating monocytes form a major



reservoir for tissue macrophages and different subsets of DCs (45–47). Moreover, monocytes are known to give rise to DCs *in vivo* at mucosal surfaces, such as skin (48) and lungs (49, 50), thus suggesting an *in vivo* relevance of monocyte-derived DCs in mediating immune response to mucosal pathogens, such as *M. tuberculosis*. In comparison to other infections, there is a delay in the onset of adaptive immune response upon *M. tuberculosis* infection allowing the bacteria to form a niche in the lungs (51). While it is clear that Th1/IFN- γ response is indispensable for the protection against tuberculosis, the role of Th17/IL-17 in mediating protection is unclear.

Initial studies in mice suggested that the IL-23/Th17 axis is dispensable for the overall protection against *M. tuberculosis* challenge but is required for enhancing the formation of granuloma in the absence of an active IL-12/Th1 axis (52). Surprisingly, Th17 response mediated protection to a highly virulent *M. tuberculosis* strain HN878 by activating macrophages and curtailing bacterial burden in the lungs. However, it was dispensable to the less virulent strains of *M. tuberculosis* thus ascribing a protective role for Th17 to emerging virulent strains (53). Earlier reports in tuberculosis patients indicated that virulent strains, such as multi-drug resistant *M. tuberculosis*, strongly induce Th17 response. However, enhanced IL-17 was correlated to the severity of the disease and high bacterial burden in the lungs, suggesting a detrimental role for Th17 response in humans (54). More recent reports have demonstrated that tuberculosis patients

display lower antigen-specific Th17 response. Incidentally, anti-tuberculosis therapy not only enhanced Th1 response but also augmented Th17 response (28). Furthermore, individuals with biallelic RORC mutations with a defective Th17 response are associated with a compromised Th1 response and are susceptible to fungal and mycobacterial infections (55). Thus, it appears that the protective roles of Th17 response may vary depending on the stage of infection. Th17 response contributes to vaccine-mediated protection and during the earlier stages of infection by recruiting lymphocytes and promoting Th1 response. However, chronic exposure or during the later stages of infection it may be detrimental due to neutrophil recruitment that can mediate tissue damage leading to immunopathology.

Previous studies have shown that DCs can mediate Th17 response to *M. tuberculosis* by signaling through TLR-2, dectin-1, DC-SIGN, and mannose receptors (36, 53, 56). In the present study, we show that monocytes and DCs have differential capacity to modulate Th17 responses to *M. tuberculosis* and *M. tuberculosis*-derived antigen fractions. Notably, DCs evoked a much stronger IL-17A production from CD4⁺ T cells than monocytes. This might be due to the capacity of DCs to secrete large quantities of IL-23 than monocytes. IL-23 plays an important role in stabilizing and sustaining the ensuing Th17 response. Interestingly, unlike monocytes that promoted Th17 response to cell wall, cell membrane, and cytoplasmic fractions of *M. tuberculosis*, DCs displayed differential response to these antigen fractions. Thus, cell wall fraction triggered strong DC-mediated IL-17A production from CD4⁺ T cells; cell membrane fraction promoted intermediate IL-17A response, and the cytoplasmic fraction did not significantly modulate Th17 response. As immunosuppressor T cells have the ability to inhibit effector T cell responses, we surmised whether increased frequency of IL-10⁺CD4⁺ T cells or FoxP3⁺CD4⁺ Treg cells was responsible for low-level induction of DC-mediated Th17 responses by cytoplasmic fraction of *M. tuberculosis* (57–60). However, IL-10⁺CD4⁺ T cells were not induced under any stimulatory conditions. Although cytoplasmic fraction significantly enhanced the frequency of FoxP3⁺CD4⁺ Treg cells, this increase was similar to that observed with *M. tuberculosis* and other antigen fractions.

Suppression of cellular immunity reckons a major evasion strategy employed by *M. tuberculosis*. Of the numerous evasion strategies employed by *M. tuberculosis*, the exploitation of PD-L1/PD-L2–PD-1 axis occupies a central role due to its implication in the expansion of Treg response and suppression of effector Th1 response (61, 62). Considering that induced Tregs and Th17 responses are reciprocally regulated, the possible role of PD-L1/PD-L2 in regulating Th17 response has not been questioned. It is well established that *M. tuberculosis* infection induces PD-L1 on APCs and PD-1 on T cells. Interaction of PD-L1 with PD-1 promotes Treg differentiation and expansion by activating SHP1/2. Induced SHP1/2 suppresses STAT1 functions, abrogates IFN- γ production, and abolishes its inhibitory effect on FOXP3 leading to Treg expansion (63). Since PD-1 axis plays a critical role in mediating immune tolerance and loss of which can predispose to inflammatory conditions and autoimmune diseases (64, 65), it is deleterious to completely abrogate PD-L1–PD-1 signaling during *M. tuberculosis* infection. This is

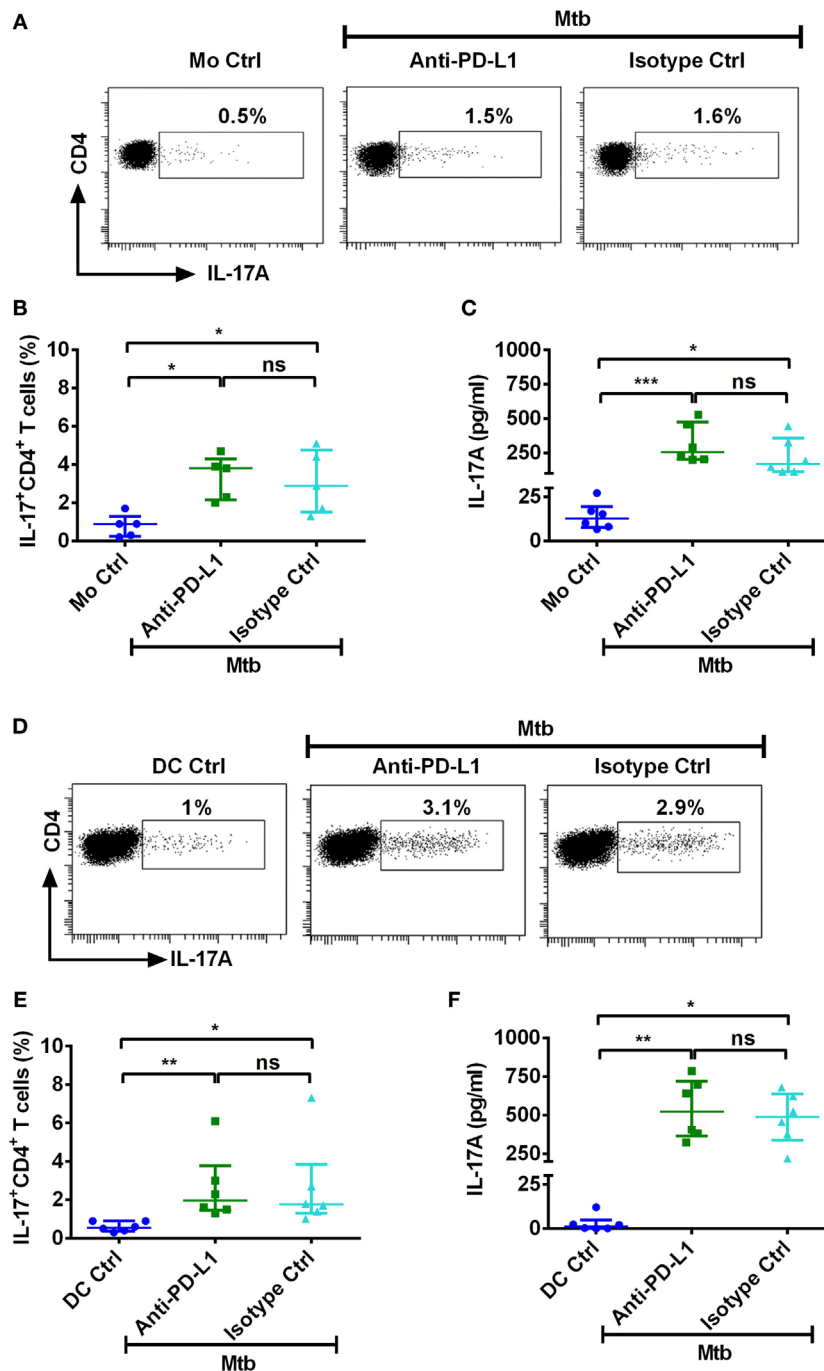


FIGURE 5 | PD-L1 on human innate cells is dispensable for regulating Th17 response to *M. tuberculosis*. Monocytes or DCs were cocultured with autologous CD4⁺ T cells either alone or with γ -irradiated *M. tuberculosis*. After 18 h, PD-L1 blocking mAb or isotype control mAb were added, and cultures were maintained for 5 days. Th17 cells were analyzed by flow cytometry by combination of surface staining for CD4 and intracellular staining for IL-17A. IL-17A in the cell-free supernatants was quantified by ELISA. (A–C) Representative dot plots showing the frequencies of CD4⁺IL-17A⁺ T cells (A), frequencies of CD4⁺IL-17A⁺ T cells (mean \pm SEM, $n = 5$) (B), and amounts of IL-17A production (mean \pm SEM, $n = 6$) (C) in monocyte–CD4⁺ T cell cocultures. (D–F) Representative dot plots showing the frequencies of CD4⁺IL-17A⁺ T cells (D), frequencies of CD4⁺IL-17A⁺ T cells (mean \pm SEM, $n = 6$) (E), and amounts of IL-17A production (mean \pm SEM, $n = 6$) (F) in DC–CD4⁺ T cell cocultures. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant; as determined by one-way ANOVA.

evidenced by the fact that PD-L1-deficient and PD-1-deficient mice are susceptible to *M. tuberculosis* infection and display exacerbated inflammation (66, 67).

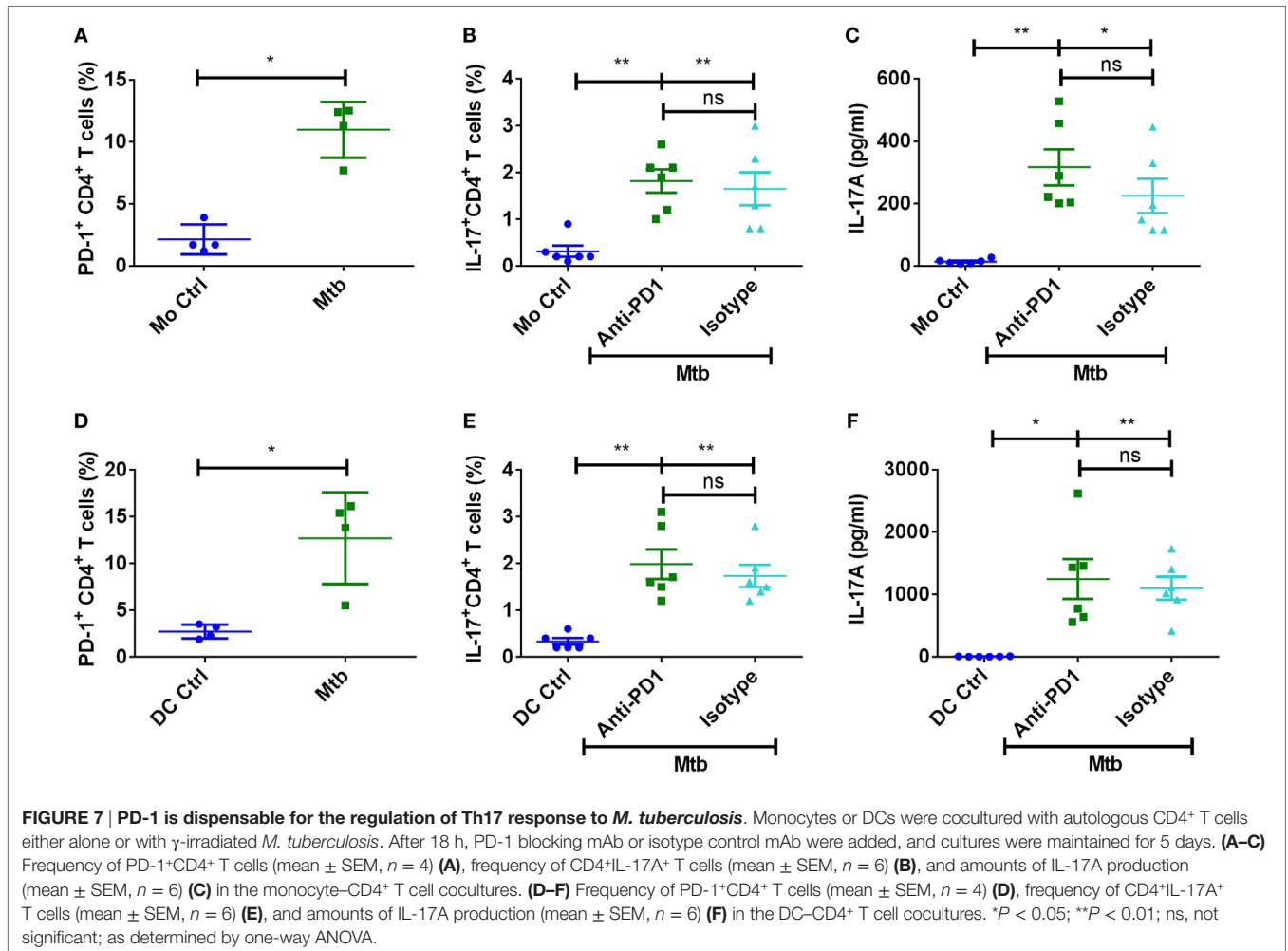
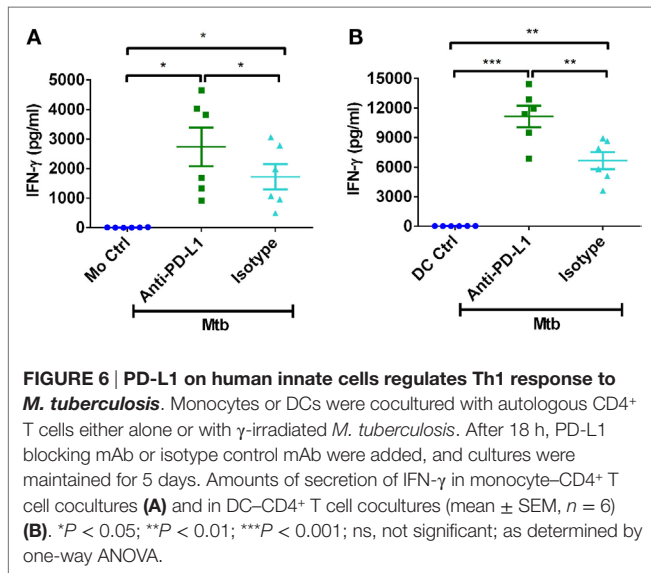
In the present study, we found that monocytes stimulated with *M. tuberculosis* and its antigen fractions significantly induce PD-L1 expression on human monocytes. On the contrary, only

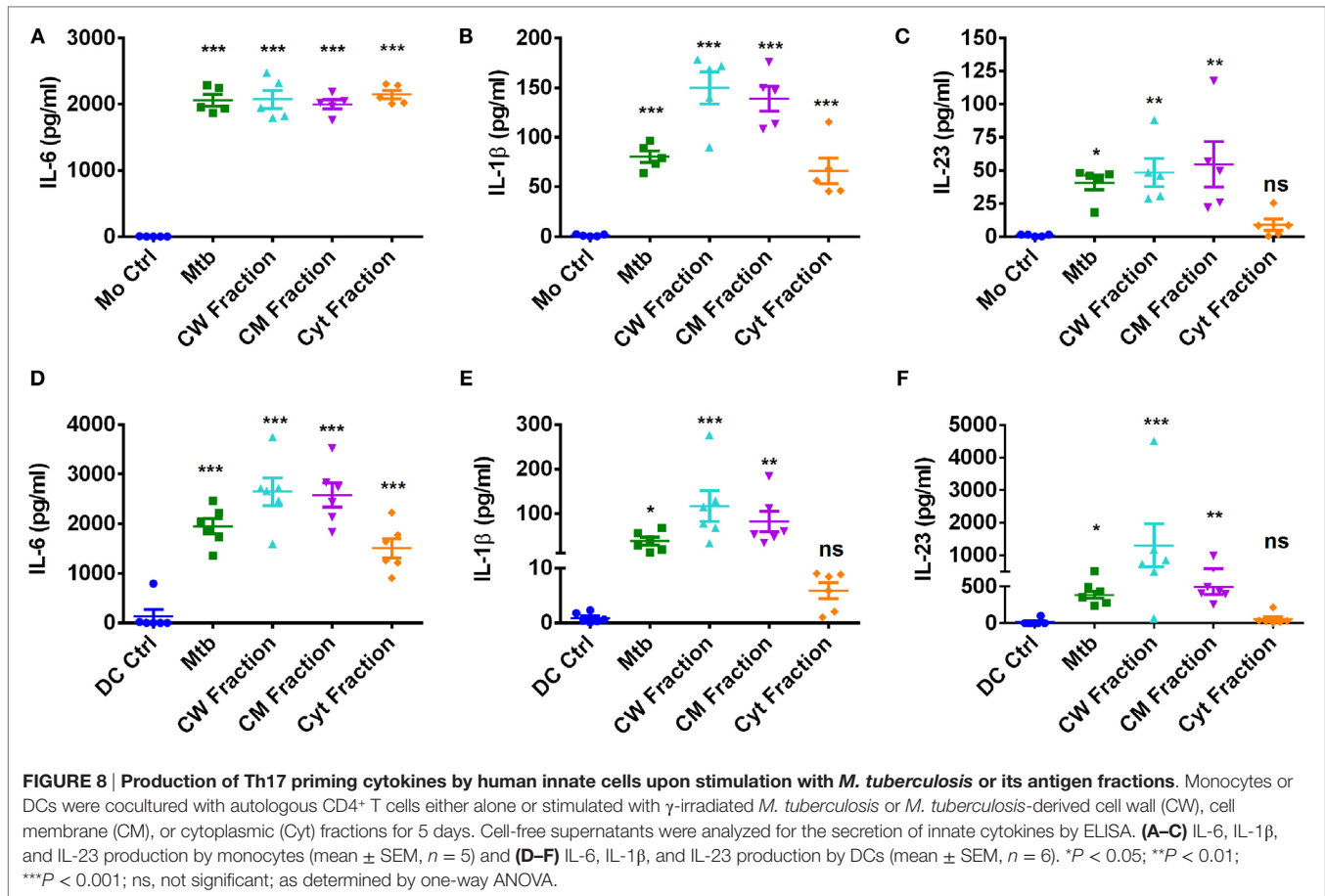
cell wall and cell membrane fractions induced PD-L1 expression on DCs, whereas the cytoplasmic fraction failed to enhance PD-L1 on DCs. PD-L2 was not expressed on both monocytes

and DCs under any stimulatory conditions. The inability of cytoplasmic fraction to induce PD-L1 on DCs was not due to its lack of stimulatory capacity as cytoplasmic fraction induced DC maturation similar to that of cell wall and cell membrane fractions.

To decipher the role PD-L1 in regulating Th17 response to *M. tuberculosis*, we employed blocking antibodies to PD-L1. Our results demonstrate that PD-L1 blockade did not significantly alter the frequencies of Th17 cells. On the other hand, IFN- γ production was significantly enhanced when PD-L1 was blocked, thus confirming the previous data on role of PD axis in regulating Th1 responses (31, 32). However, we did observe a marginal (~50 pg/ml) but insignificant increase in IL-17 production upon PD-L1 blockade. As Th17 cells and Tregs are reciprocally regulated and moreover Tregs are known to suppress Th17 response (66, 68, 69), it is likely that blockade of PD-L1 can indirectly favor Th17 response to a certain extent. This could explain minimal augmentation of IL-17A production that we observed upon PD-L1 blockade.

In addition to PD-L1, *M. tuberculosis* significantly enhanced PD-1 on CD4⁺ T cells. However, PD-1 blockade had no repercussion on either monocyte- or DC-mediated Th17 response. Our data contradict a recent report that indicated that



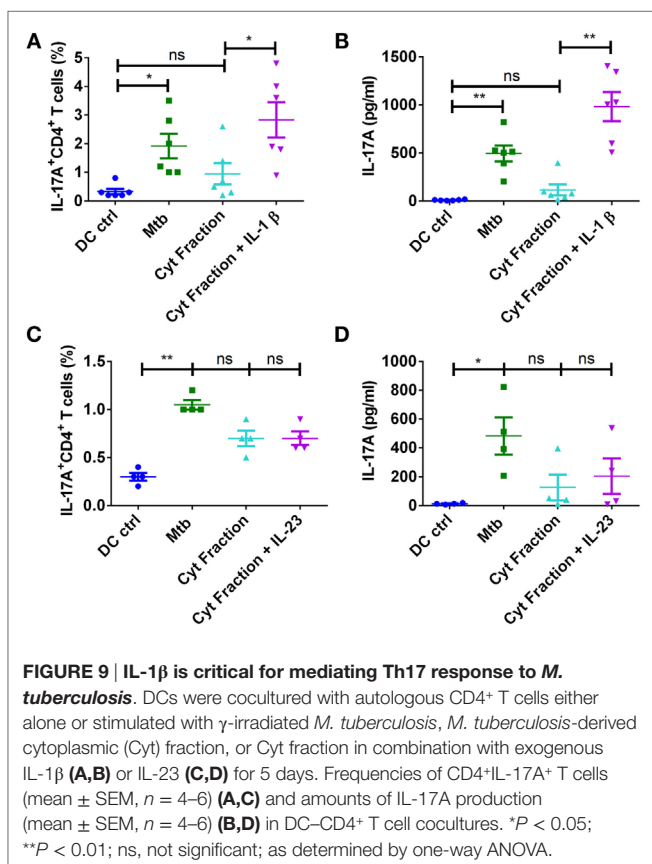


Mycobacterium-induced PD-1 orchestrates suppression of Th17 response in tuberculosis patients (28). This discrepancy might be due to the fact that previous report focused on active disease where prolonged infection can enhance additional co-stimulatory molecules that might not be present during the earlier phases of stimulation. For example, APCs from active disease express both the co-stimulatory molecules PD-L1 and PD-L2 (31), whereas our current data and previous reports show that DCs from healthy individuals stimulated with *M. tuberculosis* both at the transcript level as well as the protein level induce only PD-L1 and not PD-L2 (30, 33). It is essential to note that we have used γ -irradiated *M. tuberculosis* or *M. tuberculosis*-derived cell wall, cell membrane, or cytoplasmic fractions in our experiments, and these conditions may not completely mimic *in vivo* situation of infection with live bacteria. Therefore, further work is necessary to decipher the role of PD-1–PD-L1/PD-L2 pathway in tuberculosis. Nevertheless, our report indicates that PD pathway does not contribute to negative regulation of human Th17 response to *M. tuberculosis*. Also, cytoplasmic fraction of *M. tuberculosis* failed to induce Th17 response despite lack of induction of PD-L1 on DCs.

To decipher the possible mechanism that explains inability of cytoplasmic fraction to promote DC-mediated Th17 response, we analyzed key Th17-polarizing cytokines secreted by monocytes and DCs. Our results indicate that cytoplasmic fraction failed to induce IL-1 β , the key cytokine that promotes

Th17 response. The amounts of secretion of IL-1 β and Th17 responses were significantly correlated. Importantly, exogenous supplementation of IL-1 β was sufficient to significantly augment Th17 response by the cytoplasmic fraction. These results indicate that inability of cytoplasmic fraction to induce the production of IL-1 β from DCs resulted in its failure to prime a robust Th17 response. Further work on mechanisms underlying the differential production of IL-1 β from monocytes and DCs upon stimulation with cytoplasmic fraction is warranted (70). Distinct expression of pattern-recognition receptors that sense cytoplasmic fraction on monocytes and DCs might have resulted in the differential IL-1 β secretion upon stimulation with the cytoplasmic fraction. Therefore, antigens that come in direct contact with innate immune cells, such as the cell wall antigens, are attractive candidates for future tuberculosis vaccines. It is important to note that IL-1 β is a critical mediator of immunity to *M. tuberculosis* (71, 72). Our results suggest that this function of IL-1 β is in part *via* enhancement of Th17 responses. As tuberculosis patients were reported to exhibit lower antigen-specific Th17 response (28), supplementing IL-1 β might enhance Th17 response.

Taken together, our report demonstrates that DCs have differential capacity to mediate Th17 response to various antigen fractions of *M. tuberculosis*. Therefore, cell wall antigens of *M. tuberculosis* constitute potential subunit vaccine candidates.



Importantly, we demonstrate that PD pathway is critical for regulating Th1, but not Th17, response to *M. tuberculosis*. IL-1 β however is necessary for promoting a prominent Th17 response to *M. tuberculosis*.

AUTHOR CONTRIBUTIONS

ES-V and JB designed the research. ES-V, VS, MD, AK, CS, ML, and CG performed the research. ES-V, VS, MD, AK, CS, ML, CG, SK, and JB contributed to data analyses and data interpretation. ES-V and JB wrote the manuscript. ES-V, VS, MD, AK, CS, ML, CG, SK, and JB revised the manuscript critically for important intellectual content and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2016.00465>.

FIGURE S1 | *M. tuberculosis* and its antigen fractions enhance costimulatory molecules on monocytes. Monocytes (0.5×10^6 /ml) were either cultured alone or stimulated with γ -irradiated *M. tuberculosis* (Mtb) or *M. tuberculosis*-derived cell wall (CW), cell membrane (CM), or cytoplasmic (Cyt) fractions for 24 h. (A–D) Surface expression of CD80 [mean fluorescence intensity (MFI)], CD86 (% positive cells), CD40 (MFI), and HLA-DR (MFI)] (mean \pm SEM, $n = 5$) was analyzed by flow cytometry. ** $P < 0.01$; ns, not significant; as determined by one-way ANOVA.

FIGURE S2 | *M. tuberculosis*-derived cytoplasmic fraction induce maturation of DCs. DCs (0.5×10^6 /ml) were either cultured alone or stimulated with γ -irradiated *M. tuberculosis* (Mtb) or *M. tuberculosis*-derived cell wall (CW), cell membrane (CM), or cytoplasmic (Cyt) fractions for 24 h. (A–D) Surface expression of CD80 [mean fluorescence intensity (MFI)], CD86 (% positive cells), CD40 (MFI), and HLA-DR (MFI)] (mean \pm SEM, $n = 4$) was analyzed by flow cytometry. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; as determined by one-way ANOVA.

FIGURE S3 | Production of Th17-polarizing cytokines by human innate cells upon PD-L1 blockade. Monocytes or DCs were cocultured with autologous CD4⁺ T cells either alone or stimulated with γ -irradiated *M. tuberculosis* (Mtb). After 18 h, PD-L1 blocking mAb or isotype control mAb were added, and cultures were maintained for 5 days. Cell-free supernatants were analyzed for the secretion of innate cytokines by ELISA. (A–C) IL-6, IL-1 β , and IL-23 production by monocytes and (D–F) IL-6, IL-1 β , and IL-23 production by DCs (mean \pm SEM, $n = 7$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant; as determined by one-way ANOVA.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cross-presentation of antigens by dendritic cells: role of autophagy

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Commentary on: Mintern et al. Differential use of autophagy by primary dendritic cells specialised in cross-presentation. *Autophagy*. 2015; 11:906-17.

Dendritic cells (DCs) are a heterogeneous population of antigen presenting cells (APCs) that initiate and orient immune responses. They sense the pathogens and pathogen-derived antigenic molecules via pattern recognition receptors (PRRs). They process and present extracellular antigens in the context of major histocompatibility complex (MHC) class II molecules to CD4⁺ T helper cells and intracellular antigens via MHC class I molecules to CD8⁺ T cells. DCs can also process and present extracellular antigens on MHC class I via the process of cross-presentation. Two main intracellular pathways for cross-presentation have been reported: cytosolic and vacuolar [1].

Macroautophagy, hereafter referred to as autophagy, is an important biological process involving lysosomal degradation of damaged cellular components and misfolded proteins. In this process, targeted cytoplasmic molecules will be segregated from the rest of the cellular components within autophagosome, a double-membraned vesicle structure. A series of autophagy-related proteins (ATG) and several kinases play an important role in the regulation of autophagy. Autophagy was initially investigated as a process mainly implicated in the cell survival and in the maintenance cellular homeostasis. It can also directly intersect with, and impact, major pathways of various other cellular processes including immune recognition and response. More specifically, autophagy has been identified as a route of delivering cytoplasmic and nuclear antigens to MHC class II for their presentation to the CD4⁺ T cells [2].

Role of autophagy in cross-presentation of antigens by DCs is controversial. Several reports have suggested that cross-presentation is an autophagy-dependent process while others proposed it to be autophagy-independent. These discrepancies might be attributed to various factors: experimental set-up and particularly exploring the autophagy either in DCs as such or in antigen donor cells; heterogeneity in DC subsets; and the nature of antigens used in the experiments.

The initial report demonstrated the importance of autophagy in antigen donor cells for the MHC class I cross-presentation of antigens by DCs both *in vitro* and *in vivo* [3]. The authors showed that induction of autophagy in ovalbumin (OVA)-expressing HEK 293T cells or

gp100-expressing melanoma cell lines lead to enhanced cross-presentation of model antigens by murine splenic DCs to antigen-specific CD8⁺ T cells. Whereas, inhibition of autophagy in antigen donor cells greatly hampered the cross-presentation [3]. Notably, this report suggested that autophagosomes could efficiently act as antigen cargo for cross-presentation by professional APCs.

The role of autophagy in DCs towards antigen-cross-presentation met with debates. Data from CD11c⁺ conventional murine DCs where *Atg5*, a key gene implicated in autophagy was conditional deleted, shown that autophagy is dispensable for the innate recognition, endocytosis, phagocytosis and migration of DCs. Confirming the previous reports, autophagy was essential for the MHC class II presentation of TLR agonists-containing phagocytosed antigens. However, no defect was observed in *Atg5*^{-/-} DCs to cross present antigens (OVA) to MHC class I [4]. Contrary to above report, α -alumina (Al₂O₃) nanoparticles induced efficient cross-presentation of conjugated antigens in bone marrow-derived DCs in an autophagy-dependent manner [5].

Several murine and human DC subsets differ in their phenotype, ontogenesis and functional properties. Not all DCs but, only particular DC subsets, can cross-present antigens efficiently. Mouse CD8⁺ and CD103⁺ DC subsets are the dominant cross-presenters compared to CD8⁻ and CD103⁻ subsets. On the other hand, CD1a⁺ DCs are dominant cross-presenting subtypes than CD14⁺ in human [6]. It is thus expected that contribution of autophagy in cross-presentation is determined by subtypes of DCs that are capable of cross presenting or not.

The aforementioned pitfalls are recently addressed in an elegant way. Villadangos and colleagues report that autophagy has a role in antigen cross-presentation only in particular DC subtypes that are specialized in cross-presentation [7]. They showed that human CD1a⁺ subtype and mouse CD8⁺ and CD103⁺ DC subtypes following maturation have higher autophagy compared to CD1a⁻, CD8⁻ and CD103⁻ subtypes respectively. The autophagic flux with the accumulation of DC aggresome-like structures that contain ubiquitinated cargo and sequestosome 1/p62, one of the substrate anchors for autophagy are also enhanced in cross-presenting DCs. The authors generated *Atg 7*^{-/-} conditional knockout in CD11c⁺

DCs to assess the role of autophagy in cross-presentation by CD8⁺ DCs. The absence of autophagy did not impact the expression of MHC I, MHC II and CD86 in CD8⁺ DCs, suggesting that autophagy is dispensable for DC homeostasis in steady state. Interestingly, they found that the role of autophagy in efficient cross-presentation depends on the type of antigen. Only soluble OVA cross-presentation was impaired by autophagy deficiency, while cross-presentation of cell-associated antigens and those delivered via endocytic receptors (such as DEC-205) was unaffected. These data thus suggest that autophagy contributes to efficient cross-presentation of soluble antigens that are internalized by micropinocytosis or pinocytosis, but not by phagocytosis and receptor-mediated endocytosis.

In summary, role of autophagy in antigen cross-presentation by DCs is determined by particular subset of DCs and nature of the antigens.

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Heme oxygenase-1 is dispensable for the anti-inflammatory activity of intravenous immunoglobulin

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Intravenous immunoglobulin G (IVIG) is used in the therapy of various autoimmune and inflammatory conditions. The mechanisms by which IVIG exerts anti-inflammatory effects are not completely understood. IVIG interacts with numerous components of the immune system including dendritic cells, macrophages, T and B cells and modulate their functions. Recent studies have reported that heme oxygenase-1 (HO-1) pathway plays an important role in the regulation of inflammatory response in several pathologies. Several therapeutic agents exert anti-inflammatory effects via induction of HO-1. Therefore, we aimed at exploring if anti-inflammatory effects of IVIG are mediated via HO-1 pathway. Confirming the previous reports, we report that IVIG exerts anti-inflammatory effects on innate cells as shown by the inhibitory effects on IL-6 and nitric oxide production and confers protection in experimental autoimmune encephalomyelitis (EAE) model. However, these effects were not associated with an induction of HO-1 either in innate cells such as monocytes, dendritic cells and macrophages or in the kidneys and liver of IVIG-treated EAE mice. Also, inhibition of endogenous HO-1 did not modify anti-inflammatory effects of IVIG. These results thus indicate that IVIG exerts anti-inflammatory effects independent of HO-1 pathway.

Initially used as replacement therapy in immune deficiencies, IVIG is also widely used for the treatment of a number of autoimmune and systemic inflammatory diseases^{1–5}. Despite its therapeutic use for more than three decades, the precise mechanism by which IVIG exerts its beneficial effect is not fully understood. Exploration of mechanisms of IVIG is useful to define the dosage, to identify an appropriate window and duration of treatment, and to delineate biomarkers of therapeutic response. IVIG interacts with numerous components of the immune system including dendritic cells (DCs), macrophages, T and B cells and modulate their functions^{6–21}. These mechanisms of IVIG also reflect the functions of circulating IgG in the maintenance of immune homeostasis.

Recent studies in various experimental models such as sepsis, cardiovascular pathologies, experimental autoimmune encephalomyelitis (EAE) and transplantation, and infection models such as *Mycobacterium tuberculosis* have highlighted the biological significance of heme oxygenase-1 (HO-1) enzymatic pathway and the reactive products of this pathway in regulating the inflammation and in the adaptation of the pathogens to the host microenvironment^{22–28}. HO-1 catalyzes the degradation of heme, resulting in the liberation of equimolar amounts of iron, carbon monoxide (CO) and biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Congenital defects in HO-1 expression in mice and human are associated with systemic inflammation²⁹. HO-1 inhibits ovalbumin-induced airway inflammation by enhancing the biological activity of regulatory T cells (Tregs) in an IL-10-dependent manner³⁰. Nevertheless, development, maintenance and the functions of Tregs under physiological conditions are not dependent on the activity of HO-1³¹.

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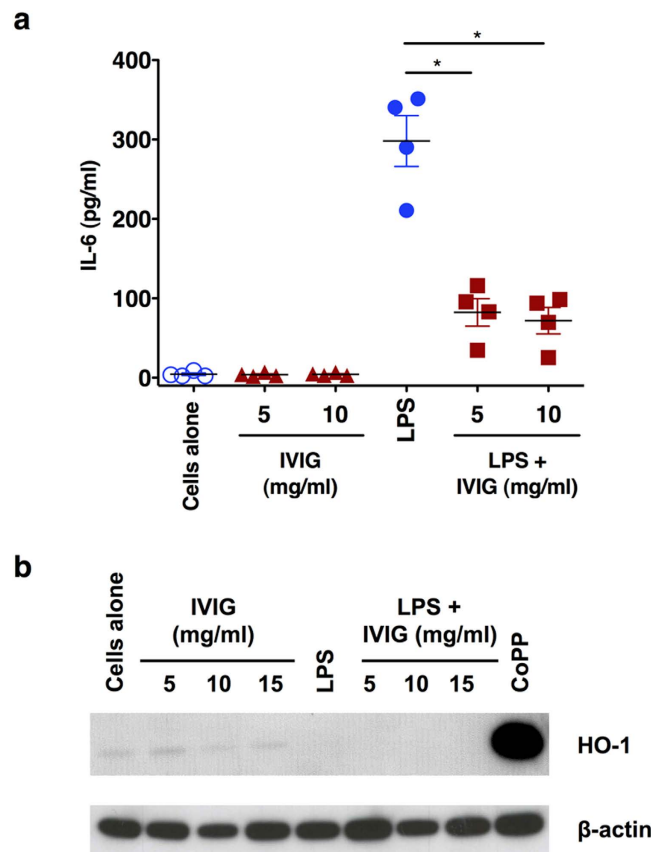


Figure 1. Anti-inflammatory effects of IVIG on human monocytes are not associated with induction of HO-1. (a) IVIG suppresses LPS-induced IL-6 production in human monocytes. Human peripheral blood monocytes were cultured in RPMI-1640 medium with 10% fetal calf serum either alone (cells alone) or with IVIG (5 and 10 mg/ml) for 24 hours. In some conditions, after 24 hours of culture, monocytes were exposed to LPS for additional 24 hours. IL-6 in the culture supernatants was measured by ELISA (n = 4). * $p < 0.05$, One-way ANOVA (b) Expression of HO-1 in human monocytes treated with IVIG (5, 10 or 15 mg/ml) alone or with LPS during last 24 hours of culture. CoPP was used as a positive control to induce HO-1. Images have been cropped for presentation and full-size blots are presented in Supplementary Figure S2.

CO and biliverdin have potent anti-inflammatory, anti-proliferative, anti-apoptotic, and antioxidant activities and exert their effects on many cell types, including cells of the immune system³². CO suppresses the pro-inflammatory response and promotes the anti-inflammatory programs of macrophages, DCs and monocytes^{33,34}. Thus, either overexpression of HO-1 in innate cells or exposure to CO leads to inhibition of pro-inflammatory cytokines and enhancement of IL-10. CO also inhibits the lipopolysaccharide (LPS)-mediated maturation of DCs^{35,36}.

Thus, in view of the common anti-inflammatory role exerted by both HO-1 and IVIG, we investigated if mechanisms of action of IVIG both *in vitro* and *in vivo* implicate HO-1 pathway.

Results

Anti-inflammatory effects of IVIG on human monocytes are not associated with induction of HO-1.

It is known that IVIG exerts anti-inflammatory effects on innate cells such as monocytes, DCs and macrophages leading to suppression of inflammatory cytokines^{8,37–39}. By analysing the production of IL-6, we first confirmed the anti-inflammatory action of IVIG. Unstimulated monocytes produced insignificant amount of IL-6. However, upon stimulation with LPS, a TLR4-agonist, monocytes produced large amounts of IL-6. Importantly, IVIG significantly reduced the production of IL-6, thus validating the anti-inflammatory effects of IVIG (Fig. 1a). The inhibition however was not dependent on the dose of IVIG.

We then examined the effect of IVIG on the expression of HO-1. Untreated monocytes expressed marginal amount of HO-1 and was not modified by IVIG. Even under inflammatory conditions, IVIG failed to induce the expression of HO-1 (Fig. 1b) in all tested concentrations. The lack of expression of HO-1 in IVIG-treated monocytes was not due to technical errors or non-functioning of HO-1-detecting antibodies as treatment of monocytes with cobalt protoporphyrin IX (CoPP), an inducer of HO-1, strongly induced HO-1. These results thus suggest that anti-inflammatory effects of IVIG on monocytes are independent of HO-1 pathway.

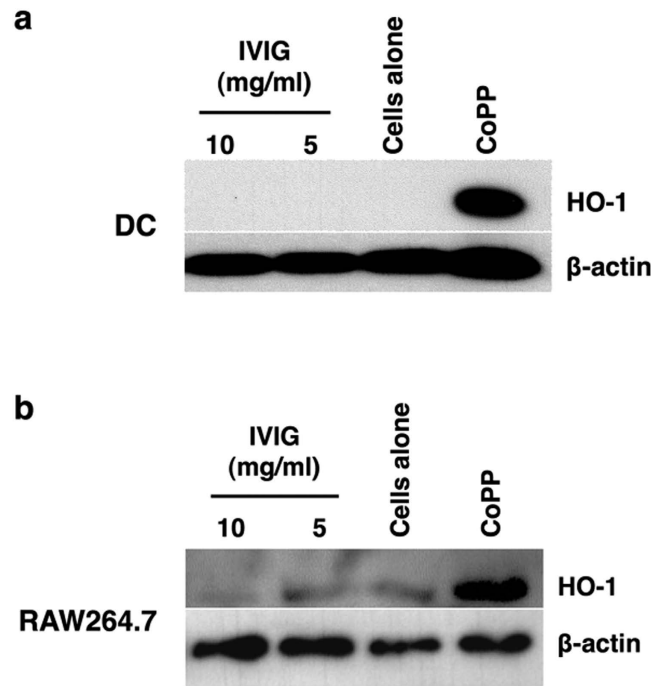


Figure 2. IVIG lacks the capacity to induce HO-1 in dendritic cells and macrophages. (a) Human peripheral blood monocyte-derived DCs or (b) RAW264.7 macrophages were cultured in the medium alone or with IVIG (5 and 10 mg/ml) for 24 hours. Expression of HO-1 was detected by western blot. CoPP was used as a positive control to induce HO-1. Images have been cropped for presentation.

Inability of IVIG to induce HO-1 in dendritic cells and macrophages. We investigated the effect of IVIG on the expression of HO-1 in other innate cells. Consistent with the results obtained with monocytes, IVIG did not induce HO-1 both in monocyte-derived human DCs as well as RAW264.7 macrophage cell line (Fig. 2a,b). CoPP, the positive control, induced HO-1 in both the cell types. These results thus indicate that inability of IVIG to induce HO-1 is not restricted to particular innate cell.

Inhibition of endogenous HO-1 is not coupled with reduced functioning of IVIG. As innate cells express basal level of HO-1 (Fig. 1b, 2b), we wondered whether inhibition of this endogenous HO-1 is associated with reduced anti-inflammatory action of IVIG. Peritoneal macrophages from C57BL/6J mice and RAW264.7 cells were treated with IVIG for 24 hours and followed by stimulation with LPS. The activation of macrophages by LPS leads to oxidation of L-arginine via nitric oxide synthase and produce nitric oxide (NO). As shown in Fig. 3a,b, IVIG significantly inhibited LPS-induced NO production. However, treatment of cells with tin-mesoporphyrin (SnMP) to suppress enzyme activity of the endogenous HO-1 did not inhibit anti-inflammatory effects of IVIG on NO production (Fig. 3a,b).

The protective effect of IVIG in experimental autoimmune encephalomyelitis is independent of induction of HO-1 *in vivo*. In order to validate the non-involvement of HO-1 pathway in IVIG-mediated anti-inflammatory effects *in vivo*, we resorted to EAE model. EAE was induced in C57BL/6J mice using MOG₃₅₋₅₅. Confirming the previous reports, treatment of mice with IVIG significantly delayed the onset of EAE and the severity of the disease (Fig. 4a)⁴⁰⁻⁴². However, consistent with *in vitro* results, this protection was not associated with an induction of HO-1 irrespective of the organs examined (liver and kidney) (Fig. 4b). Western blot analysis of lungs and spleen also showed same results. Naive mice injected with CoPP (20 mg/kg) were used as positive control for the expression of HO-1. In fact, expression of HO-1 was confirmed in the liver and kidneys of these mice 24 hours following CoPP injection (Fig. 4b).

Discussion

It was suggested that HO-1 functions as a “therapeutic funnel”³². Several reports in experimental models have recently demonstrated that HO-1 pathway and its products could be used for the prevention or treatment of immune-mediated disorders. These protective effects are mediated by multiple functions of HO-1 that include immunosuppression, anti-inflammatory, anti-apoptosis and anti-oxidant effects. The anti-inflammatory mechanisms of HO-1 are mainly via modulation of activation of immune cells including antigen presenting cells and lymphocytes³². In fact, IVIG has been demonstrated to modulate the functions of both innate cells and T cells. Thus, IVIG was reported to suppress the activation of DCs, macrophages and monocytes and secretion of inflammatory cytokines while enhancing anti-inflammatory mediators like IL-10 and IL-1ra. In addition, IVIG also inhibits APC-mediated effector T cell activation, proliferation. Recent reports further demonstrate that IVIG inhibits Th1 and Th17 subsets, which are pathogenic in various autoimmune and inflammatory diseases, and

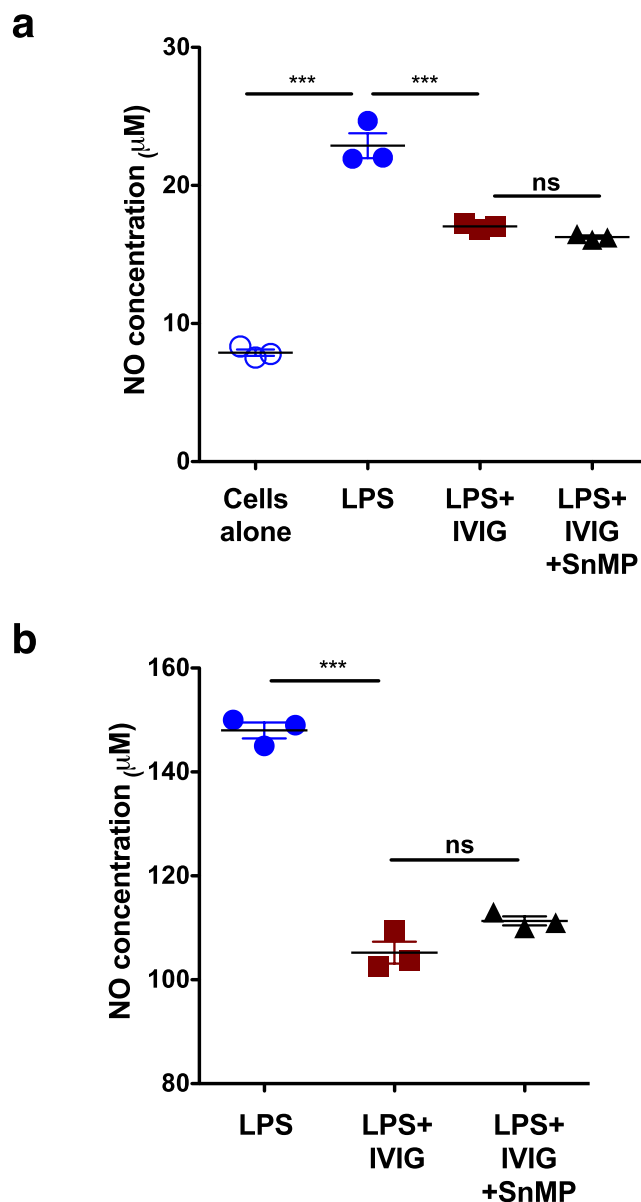


Figure 3. Inhibition of endogenous HO-1 is not associated with loss of anti-inflammatory action of IVIG. (a) Peritoneal macrophages from C57Bl/6j mice (n = 3) or (b) RAW264.7 macrophages (n = 3) were treated with IVIG (10 mg/ml) for 24 hours. They were then exposed to either LPS alone or LPS and SnMP for additional 24 hours. Production of NO was evaluated by Griess method. ***p < 0.001, One-way ANOVA and ns, not significant.

reciprocally expands Tregs^{40–48}. Importantly, HO-1 appears to be required for the action of several therapeutic molecules. For example, rapamycin appears not to exert its anti-proliferative effects on smooth muscle cells unless HO-1 is present⁴⁹. All these different lines of evidence underscore the importance of dissection of HO-1 pathway in the anti-inflammatory effects of IVIG.

Previous reports have shown that innate cells express HO-1 in steady state and inhibit toll-like receptor-mediated (such as LPS) activation and secretion of pro-inflammatory cytokines^{35,36,50}. Although, the expression of HO-1 in monocytes, DC and RAW264.7 was not prominent, we could detect basal expression of protein by western blot. However, IVIG did not modulate the basal expression of HO-1. Thus, suppression of LPS-mediated IL-6 and NO production by IVIG were independent of HO-1 pathway. It could be argued that IVIG-mediated suppression of IL-6 and NO might be due to passive neutralization of LPS by antibodies as we pre-treated the innate cells with IVIG before stimulation with LPS. However, as analyzed by the expression of CD80 and CD86, IVIG-mediated anti-inflammatory effects was well-preserved in the monocytes even if cells were stimulated with LPS followed by treatment with IVIG (Supplementary Fig. S1), thus ruling out passive neutralization of LPS as a mechanism of anti-inflammatory effect of IVIG. As activation stimuli such as LPS were reported to inhibit the expression of HO-1³⁵, we opted for examining if IVIG-preconditioning results in HO-1

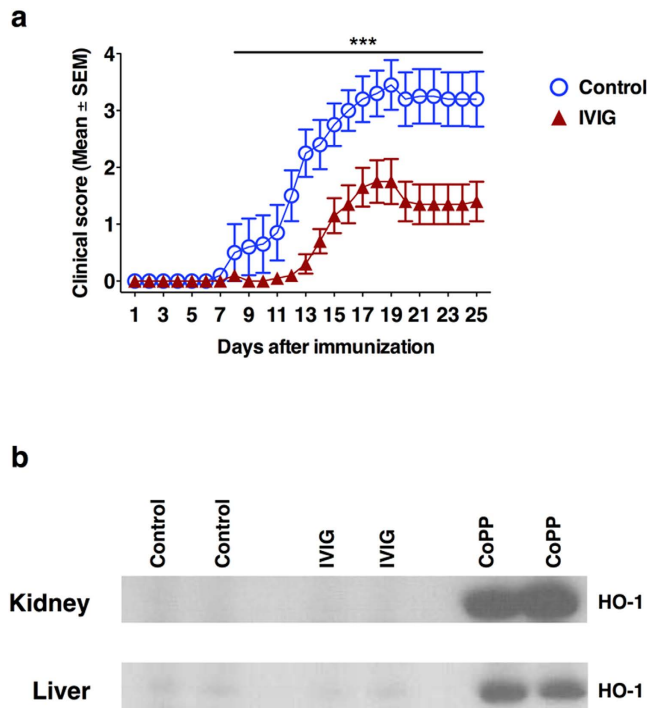


Figure 4. The protective effect of IVIG in EAE is independent of induction of HO-1 *in vivo*. (a) Treatment of mice ($n = 10$) with IVIG significantly delays the onset of EAE and the severity of the disease in C57BL/6J mice. Control mice ($n = 10$) received 0.2M glycine while IVIG group received 0.8 g/kg IVIG daily from day 0 to 19. Mean clinical scores (\pm SEM) are presented. *** $p < 0.001$, two-way ANOVA with Bonferroni post-t-test. (b) Expression of HO-1 in kidney and liver of IVIG-treated EAE mice. The mice treated with CoPP were used as a positive control. Images have been cropped for presentation.

expression in innate cells, which in turn inhibits LPS-mediated activation of cells. Our data however clearly demonstrates that IVIG or otherwise, normal circulating antibodies lack the capacity to induce HO-1 and even upon inhibition of endogenous HO-1, IVIG-mediated anti-inflammatory effects were not altered.

Tregs play an important role in the prevention of autoimmune and inflammatory responses⁵¹. Initial reports have indicated that HO-1 in Tregs is critical for their immune suppression functions⁵². However, subsequent reports have contradicted this data and showed that HO-1 expression in Tregs is not key for immunoregulatory functions of these cells both in human and mice^{31,53}. As stimuli from DCs are crucial in Treg expansion, subsequent report showed that HO-1 expression in DCs mediated suppressive functions of Tregs⁵⁴. Despite induction of DC-mediated Treg expansion by IVIG as reported recently by us and others, we could not observe induction of HO-1 in DCs by IVIG suggesting that IVIG targets different pathways for the expansion of Tregs. In fact, several lines of evidence suggest that IVIG could expand Tregs via numerous mechanisms, which might act either independent or inter-linked^{18,19,40,42,48}. The modulation of DC functions upon recognition of IVIG via DC-SIGN or DICER constitutes a major event^{40,48}. This interaction leads to expression of COX-2-dependent PGE2 production in DCs, which in turn expands Tregs. The role of PGE2 in IVIG-mediated Treg expansion was documented both *in vivo* in animal models and in autoimmune patients treated with IVIG^{40,45}.

Experimental models have shown that HO-1 pathway inhibits pathogenic T cell responses. EAE has been used as an experimental model for multiple sclerosis and that induction of HO-1 pathway suppress neuro-inflammation in EAE²³. Induction of HO-1 also suppressed IFN- γ and TNF- α responses of CNS-infiltrating T cells. Suppression of Th1 responses by HO-1 was also reported in type 1 diabetes model in NOD mice²⁵. Although modulation of Th17 responses by HO-1 in EAE was not analyzed in the previous report, it is likely that HO-1 suppresses Th17 responses as anti-inflammatory functions of HO-1 in non-eosinophilic asthma were associated with inhibition of Th17 responses⁵⁵.

Several therapeutic strategies including injection of tolerogenic cells, recombinant proteins, monoclonal antibodies to inflammatory cytokines, pharmacological agents and oral tolerance have been explored in EAE^{56,57}. However, long-term safety issues and particularly in pregnant and lactating women are of major concern with currently used therapies for MS⁵⁸. Promising clinical results in relapsing-remitting multiple sclerosis prompted dissection of cellular and molecular mechanisms of action of IVIG in EAE⁵⁸. We have recently reported that IVIG inhibits both Th1 and Th17 responses in EAE model⁴¹ and similar to HO-1 induction model of Chora *et al.*²³, significantly inhibited CNS infiltration of T cells. Confirming the *in vitro* results, the protection rendered by IVIG in EAE was not associated with HO-1 induction. It was however not surprising given that endogenous expression of HO-1 had no consequence on EAE and daily injection of CoPP was required to induce HO-1 in mice and to protect from EAE²³. Current data show that without activating HO-1, therapeutic benefits could be obtained in EAE. Thus, our results exemplify the multi-faceted mechanisms of IVIG to exert anti-inflammatory

effects independent of HO-1 pathway. These results could be further consolidated by using HO-1-deficient mice. However, data from such mice would be difficult to interpret due to complex interactions of the HO-1 pathway products with various immune and non-immune cells.

IVIG products are not uniform and display variations with respect to formulation, stabilizing agents and source of plasma of healthy donors. Although these variations could influence the outcome of therapy of primary immunodeficiency patients, all IVIG products appear to have similar therapeutic benefits in autoimmune patients. Of note, when different IVIG preparations were examined for their effect on DC activation, endothelial functions, and Th17 differentiation and expansion, all tested IVIG preparations exerted similar effects^{37,59}. In this report, we found that lack of induction of HO-1 by IVIG is not restricted to particular innate cell or IVIG preparation. In addition to Gamunex[®], other three IVIG preparations (Sandoglobulin[®], Tegeline[®] and Gammagard[®]) were also inefficient to induce HO-1. Thus, our data indicate that inability of IVIG to induce HO-1 is a universal phenomenon irrespective of preparations.

Methods

Human Cell culture. Buffy coats from the healthy donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang (EFS), Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (Institut National de la Santé et de la Recherche-EFS ethical committee permission N°12/EFS/079) and experiments were performed in accordance with the approved guidelines of INSERM. Peripheral blood mononuclear cells (PBMCs) were purified from the buffy coats by density gradient centrifugation using Ficoll-paque PREMIUM (GE healthcare, France). CD14⁺ monocytes were isolated from PBMCs by positive selection with CD14 microbeads (Miltenyi Biotec, France). They were cultured for 6 days in RPMI-1640 medium plus 10% fetal calf serum containing GM-CSF (1000 IU/million cells) and IL-4 (500 IU/million cells) to obtain monocyte-derived DCs⁶⁰.

Murine macrophages. All animal studies were approved and performed according to the guidelines of Charles Darwin ethical committee for animal experimentation (Université Pierre et Marie Curie Paris) at the pathogen-free animal facility of Centre de Recherche des Cordeliers, Paris. Murine peritoneal macrophages were extracted from C57BL/6J mice (purchased from Janvier Laboratories, France) by intraperitoneal lavage and cultured in Dubelcco's Modified Eagle's Medium supplemented with 1% penicillin, 1% streptomycin, 5% amino acids and 5% fetal bovine serum.

Murine RAW264.7 macrophages were maintained in Dubelcco's Modified Eagle's Medium supplemented with 1% penicillin, 1% streptomycin, 5% amino acids and 5% fetal bovine serum.

All cells were maintained at 37 °C in humidified air containing 5% CO₂.

Preparations of IVIG. Gamunex[®] (Grifols Bioscience, USA) was used throughout the study. In addition, Gammagard[®], Sandoglobulin[®], and Tegeline[®] were also used for *in vitro* experiments. They were dialysed against a large volume of PBS followed by RPMI-1640 medium at 4 °C for 18 hours to remove the stabilizing agents. IVIG was used at concentrations of 5, 10 or 15 mg/ml/0.5 million cells.

Animals and EAE. All animal studies were approved and performed according to the guidelines of Charles Darwin ethical committee for animal experimentation (Université Pierre et Marie Curie Paris) at the pathogen-free animal facility of Centre de Recherche des Cordeliers, Paris.

Ten-week old C57BL/6J mice (purchased from Janvier Laboratories, France) were injected intraperitoneal with CoPP 20 mg/kg. After 24 hours, liver and kidney were recovered and snap-frozen for western-blot analysis to check the HO-1 expression.

To induce EAE, C57BL/6J mice (10/group) were immunized with 200 µg MOG₃₅₋₅₅ peptide emulsified in complete Freund's adjuvant (1:1 by volume containing 800 µg of nonviable desiccated *Mycobacterium tuberculosis* H37Rv. In addition, 300 ng of pertussis toxin was given intravenously on the same day and 2 days later. Clinical signs of EAE were assessed daily based on the following scoring system: 0, no signs; 1, tail paresis; 2, hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; and 5, moribund. From the day of the immunization until the peak of the disease (day 19), mice received daily intraperitoneal injections of 16 mg (0.8 g/kg) IVIG (Gamunex[®]). The control groups received equal volumes of 0.2 M glycine, the excipient of Gamunex[®].

Detection of HO-1 by Western blot. Human monocytes, DCs and RAW264.7 macrophages (0.5 million cells per ml) were treated with different IVIG preparations and with CoPP 25 µM, the activator of HO-1. After 24 hours, supernatants were removed and cells were lysed with a lysis buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris, 10% glycerol and 10 mM Na Fluoride, pH = 6.8). In another set of experiments, following 24 hours treatment of monocytes with IVIG, LPS (10 ng/ml; Sigma-Aldrich, France) was added to the cells to stimulate the monocytes and to induce inflammatory cytokines. After 24 hours, supernatants were removed and cells were lysed.

Liver and kidneys from EAE mice at the peak of the disease (day 19 following induction of EAE) or from the mice injected with CoPP were lysed with the lysis buffer.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred to polyvinylidene fluoride membrane. HO-1 was detected using an anti-HO-1 rat monoclonal IgG (R&D Systems, France), a horseradish peroxidase-conjugated rabbit anti-rat IgG, and the enhanced chemiluminescence kit. β-actin was detected with a mouse anti-β-actin antibody (Sigma-Aldrich).

Phenotype analysis of monocytes treated with IVIG. Peripheral blood monocytes from the healthy donors were stimulated with LPS for 30 min. They were then exposed to IVIG for 48 hours. The expression of

CD80 and CD86 was analyzed by flow cytometry using PE-conjugated MAbs to CD80 and FITC-conjugated MAbs to CD86 (both from BD Biosciences).

Cytokine analysis. IL-6 in the cell-free culture supernatants was quantified by ELISA (Ready-SET-Go, eBioscience, France). The detection limit was 2 pg/ml.

Measurement of NO production. Peritoneal macrophages from C57Bl/6J mice and RAW264.7 cells were treated with IVIG (10 mg/ml) for 24 hours. They were then exposed to either LPS alone or LPS and SnMP (25 μ M; Frontier Scientific, USA) for additional 24 hours. Production of NO was evaluated by Griess method.

Statistical analysis. Two-way analysis of variance (ANOVA) with Bonferroni's post-test was used to analyze daily clinical score. One-way ANOVA was used to determine the statistical significance of the *in vitro* data. *P* value of less than 0.05 was considered significant.

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Author Contributions

C.G., P.H., M.D., E.S.-V., F.C., M.M. and V.K.S. performed the experiments, C.G., P.H., J.D.D., S.V.K. and J.B. analyzed the data, C.G. and J.B. wrote the paper and all authors reviewed and approved the manuscript.

Additional Information

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Article

Differential Effects of *Viscum album* Preparations on the Maturation and Activation of Human Dendritic Cells and CD4⁺ T Cell Responses

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Abstract: Extracts of *Viscum album* (VA); a semi-parasitic plant, are frequently used in the complementary therapy of cancer and other immunological disorders. Various reports show that VA modulates immune system and exerts immune-adjuvant activities that might influence tumor regression. Currently, several therapeutic preparations of VA are available and hence an insight into the mechanisms of action of different VA preparations is necessary. In the present study, we performed a comparative study of five different preparations of VA on maturation and activation of human dendritic cells (DCs) and ensuing CD4⁺ T cell responses. Monocyte-derived human DCs were treated with VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A. Among the five VA preparations tested VA Qu Spez, a fermented extract with a high level of lectins, significantly induced DC maturation markers CD83, CD40, HLA-DR and CD86, and secretion of pro-inflammatory cytokines such as IL-6, IL-8, IL-12 and TNF- α . Furthermore, analysis of T cell cytokines in DC-T cell co-culture revealed that VA Qu Spez significantly stimulated IFN- γ secretion without modulating regulatory T cells and other CD4⁺ T cytokines IL-4, IL-13 and IL-17A. Our study thus delineates differential effects of VA preparations on DC maturation; function and T cell responses.

Keywords: *Viscum album*; innate cells; dendritic cells; maturation; cytokines; T cell response; IFN- γ ; Th17; Th1; Th2; regulatory T cell

1. Introduction

Extracts of *Viscum album* L. (VA) or European mistletoe, a semi-parasitic plant, are traditionally used for the complementary therapy of cancer and other disorders [1–4]. Several lines of evidence indicate that VA improves patient survival, reduces the damage caused by conventional cancer therapies and increases patients' quality of life [1,5,6]. Depending on the concentration used for treatment, mistletoe extracts induce tumor cell death and exert direct necrotic effects or apoptosis [2]. VA preparation is a heterogeneous mixture of several bio-active molecules, but the major components are lectin and viscotoxin. Mistletoe lectin (ML) consists of two subunits, the A chain (29 KDa) and B

chain (34 kDa). The A chain is responsible for ribosome inactivation, whereas the B chain helps in binding to terminal galactoside residues on cell membrane [7,8].

Dendritic cells (DCs) are antigen presenting (APCs) and involved in mounting and modulating the immune response. Being sentinels of the immune system, DCs bridge innate and adaptive immunity. Thus, DCs are potential targets for the therapeutic intervention in immune-mediated conditions. Immature DCs expressing low MHC II on their surface are specialized in uptake of antigens. Upon receiving activation signals, DCs undergo maturation and induce distinct CD4⁺ T cell responses. The mature DCs express high level of MHC II and co-stimulatory molecules and secrete a large array of cytokines that mediate inflammation and CD4⁺ cell polarization [9–14]. However, in the absence of danger signals, presentation of self-antigens by immature DCs promotes immune tolerance by silencing the effector and autoreactive T cells and enhancing CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) or T regulatory type 1 cells [9,15–18].

As DCs have a central role in anti-tumor immune responses, efficient functioning of these cells is crucial for the success of cancer immunotherapy [19]. DCs are immature and functionally defective in cancer patients and tumor-bearing animals, possibly due to insufficient danger signals in the tumor microenvironment [20]. Further, several reports indicate that tumor cells hamper the maturation process of DCs and their capacity to prime protective T cell responses [21–24].

Our previous report demonstrates that VA Qu Spez, one of the VA preparations, induces activation of human DCs, and DC-mediated CD4⁺ T cell proliferation and tumor-specific CD8⁺ T cell responses as measured by IFN- γ and TNF- α secretion [25]. However, several therapeutic preparations of VA are currently available. Each VA preparation is heterogeneous in its chemical composition and is influenced by the host tree, harvest season and extraction method [26–28]. Therefore, the therapeutic outcome of a particular VA preparation might not be similar to that of other preparations [29,30]. An insight into the mechanisms of action of different VA preparations is therefore necessary to provide guidelines for the correct therapeutic use of VA preparations.

In the present study, we performed a comparative study of five different preparations of VA (VA Qu Spez, VA Qu Erf, VA M Spez, VA P and VA A) on the maturation and activation of human DCs and ensuing CD4⁺ T cell responses. Our data show that among five preparations tested, VA Qu Spez is the most potent inducer of DC maturation and secretion of DC cytokines. Furthermore, VA Qu Spez significantly stimulated IFN- γ secretion without modulating Tregs and other CD4⁺ T cytokines IL-4, IL-13 and IL-17. Our study thus delineates differential effects of VA preparations on DC maturation, function and T cell responses.

2. Results

2.1. Effect of Different VA Preparations on the Maturation of DCs

Immature DCs of 5 day old were either untreated or treated with five VA preparations at four different concentrations: 5, 10, 15 and 20 $\mu\text{g}/\text{mL}/0.5 \times 10^6$ cells for 48 h. DCs were analysed for the expression of various maturation-associated surface molecules (Figure 1A–F). We found that among five VA preparations, only VA Qu Spez was able to significantly enhance the intensity of expression of antigen presenting molecule HLA-DR, co-stimulatory molecules CD86 and CD40 and % of expression of terminal maturation marker CD83. The induction of DC maturation by VA Qu Spez was observed only at higher concentrations i.e., 15 and 20 μg . Further, the effect of VA Qu Spez on maturation of DCs was dose-dependent. The expressions of CD40 and HLA-DR were 100% on control DCs and were not altered by VA Qu Spez. VA Qu Spez also did not alter % expression of CD1a and intensity of expression of CD83.

We observed that HLA-DR expression on VA Qu Spez (20 μg) and LPS (positive control, 10 ng/0.5 million cells)-stimulated DCs was similar. However, induction of CD40 and CD86 by VA Qu Spez was 2-fold lesser and CD83 was 4-fold lesser than LPS. In line with our previous report on stimulation of tumor-antigen-specific cytotoxic T cell responses by VA Qu Spez-stimulated DCs [25], we found

that these DCs expressed higher levels of HLA class I molecules ($13.6\% \pm 1.1\%$ on control DCs vs. $20.6\% \pm 3.2\%$ on VA Qu Spez-stimulated DCs, $n = 3$). However, VA Qu Frf, VA M Spez, VA P and VA A did not significantly modify the expressions of any of maturation-associated molecules on DCs. These results suggest that among all preparations tested; only VA Qu Spez is able to induce maturation of DCs.

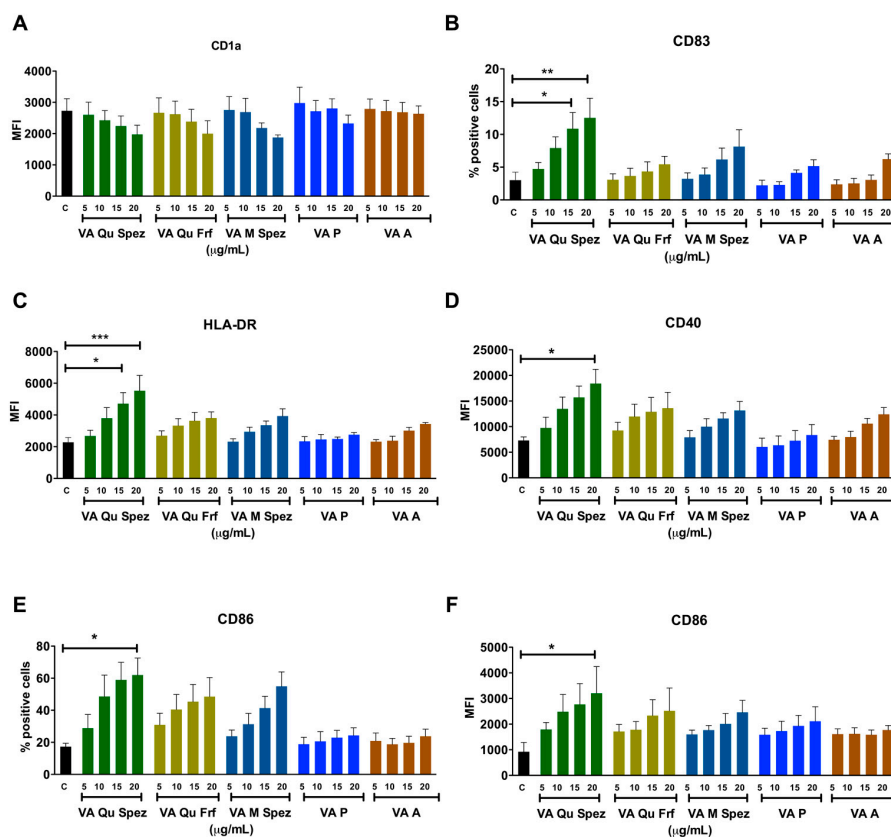


Figure 1. Differential effects of VA preparations on the phenotype of human DCs. Immature DCs were treated with medium alone (control, labelled as ‘C’) or with five preparations of VA (VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A) at indicated concentrations for 48 h. Expressions (mean \pm SEM, ≥ 4 independent donors) of (A) CD1a; (B) CD83; (C) HLA-DR; (D) CD40; (E,F) CD86 on DCs were analysed by flow cytometry. The data are presented either as % positive cells or MFI of indicated markers. X-axis denotes concentrations of VA preparations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.2. VA Qu Spez but Not Other VA Preparations Stimulate the Secretion of DC Cytokines

It is well reported that DC-derived cytokines play a critical role in regulating the immune responses and in polarizing distinct CD4⁺ T cell responses. We analysed the differential effects of various VA preparations on the secretion of DC cytokines such as IL-6, IL-8, IL-12, IL-10 and TNF- α . As VA Qu Spez significantly induced maturation of DCs, it was likely that this effect is associated with modulation of DC cytokines. In fact, compared to control DCs, VA Qu Spez-treated DCs showed significantly increased secretion of IL-6, IL-8, IL-12 and TNF- α (Figure 2A–C,E). Control DCs secreted 4.7 ± 5.1 pg/mL of IL-6 and was enhanced to 156.9 ± 105.1 pg/mL by VA Qu Spez. In case of IL-8, control DCs secreted 102.2 ± 78.5 pg/mL, whereas VA Qu Spez at the highest concentration induced 612.1 ± 20.4 pg/mL. The Th1-polarizing cytokine IL-12 was secreted at 3.3 ± 4.9 pg/mL by control DCs and was increased to 10.4 ± 6 pg/mL by VA Qu Spez-treated DCs. TNF- α secretion by untreated DCs was 3.2 ± 2.1 pg/mL, and with VA Qu Spez treatment, this cytokine was increased to 135.7 ± 37.9 pg/mL. We could observe a moderate but insignificant induction of the aforementioned DC cytokines by VA Qu Frf and VA M Spez. However, VA P and VA A did not modulate any of the DC cytokines (Figure 2A–C,E). These results show that VA Qu Spez is the most potent

preparation that induces both maturation and cytokines by DCs. Of note, production of IL-10, an immunosuppressive cytokine was unaltered upon VA Qu Spez treatment (Figure 2D). Together, our data suggest that VA Qu Spez significantly induces several pro-inflammatory cytokines without modulating immune-suppressive cytokine IL-10.

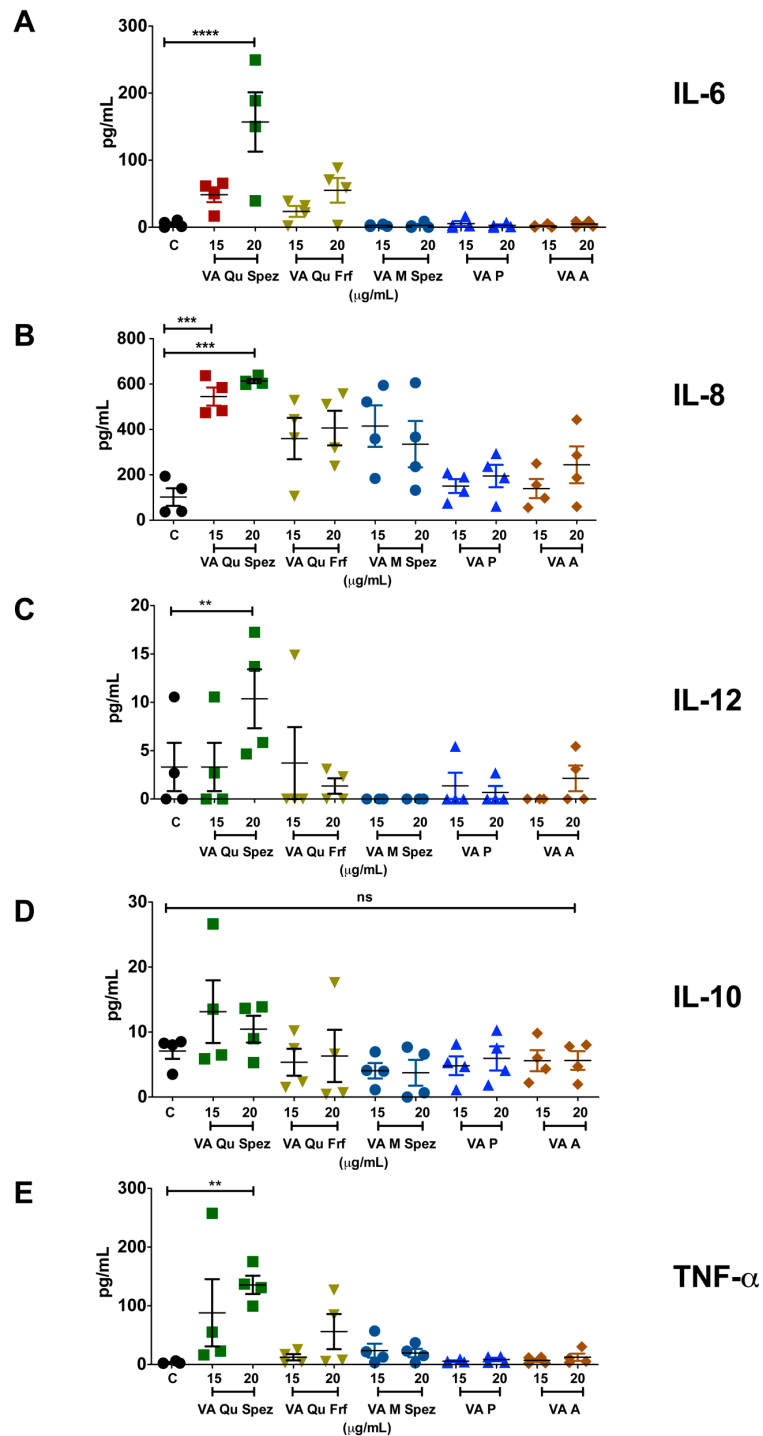


Figure 2. VA Qu Spez but not other VA preparations stimulate the secretion of DC cytokines. Immature DCs were untreated (control, labelled as ‘C’) or treated with five preparations of VA at various concentrations for 48 h. The amount (pg/mL, mean \pm SEM, four independent donors) of (A) IL-6; (B) IL-8; (C) IL-12; (D) IL-10; and (E) TNF- α in cell-free supernatants was measured. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

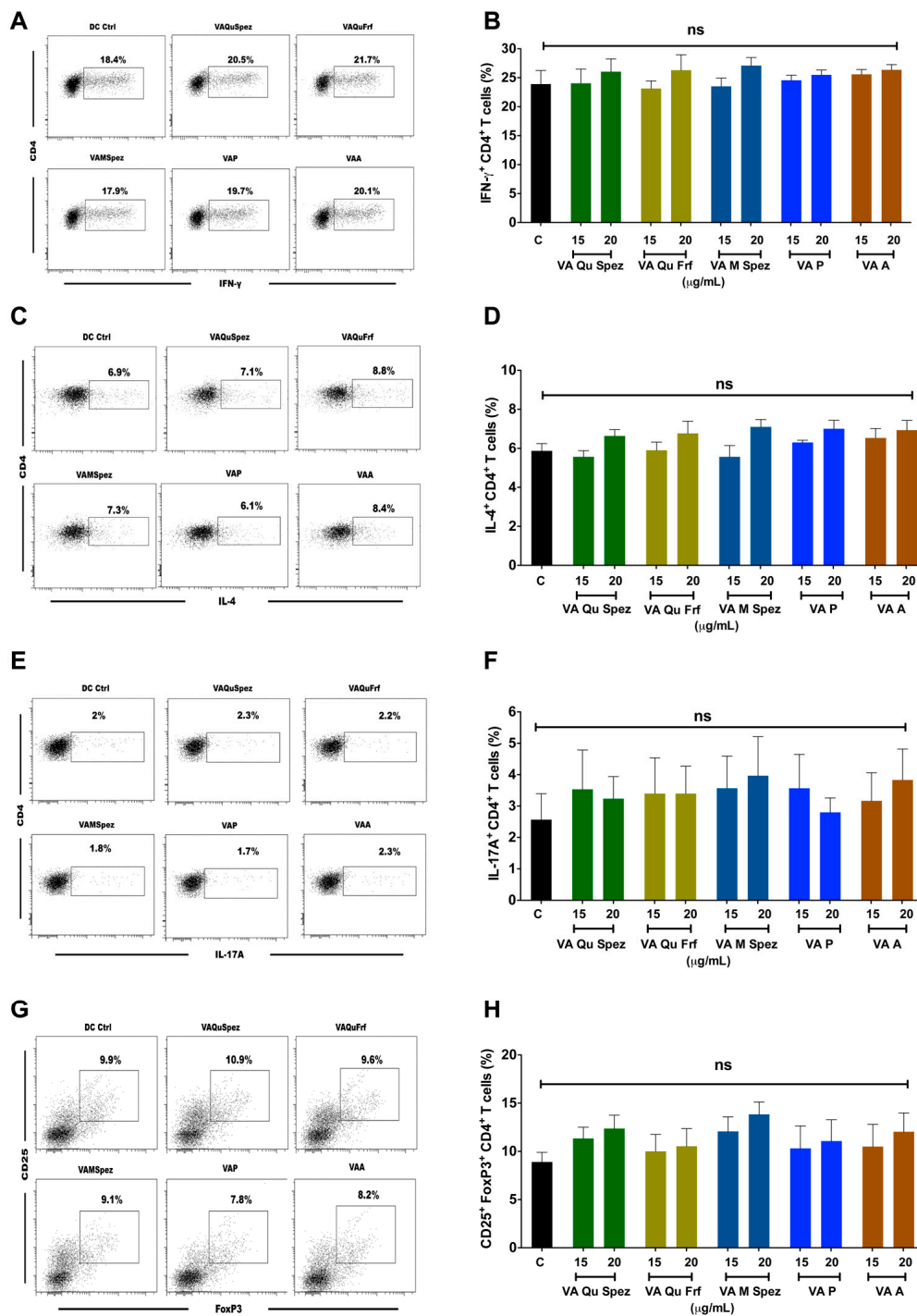


Figure 3. Effect of various VA preparations on the CD4⁺ T cell responses. DCs were treated with medium alone (DC Ctrl, labelled as ‘C’) or with five preparations of VA for 48 h. These DCs were co-cultured with CD4⁺ T cells at 1:10 ratio. After five days of co-culture, the cells were analysed for the various CD4⁺ T cell subsets by intra-cellular cytokines (IFN- γ , IL-4, IL-17A) or transcription factor (FoxP3) for Th1, Th2, Th17 and Tregs respectively. (A,C,E,G) representative dot plots showing the proportion of IFN- γ ⁺, IL-4⁺, IL-17A⁺ CD4⁺ T cell and CD4⁺CD25⁺Foxp3⁺ T cells respectively; (B,D,F,H) Percentage (mean \pm SEM, six independent donors) of IFN- γ ⁺ Th1, IL-4⁺ Th2, IL-17A⁺ Th17 and CD4⁺CD25⁺Foxp3⁺ Treg cells respectively. ns, non-significant.

2.3. Differential Effects of VA Preparations on the CD4⁺ T Cell Response

One of the key functions of APC is to promote CD4⁺ T cell responses. DCs primed with various preparations of VA were co-cultured with CD4⁺ T cells and Th1, Th2, Th17 and Treg responses were determined by flow cytometric analysis of intracellular IFN- γ (Th1), IL-4 (Th2), IL-17A (Th17), FoxP3 (Treg). Although VA Qu Spez induced maturation of DCs, this effect was not associated with the modulation of frequency of any of the T cell subsets (Figure 3A–H). However, analysis of amount of secretion of T cell cytokines in DC-CD4⁺ T cell co-culture revealed that VA Qu Spez significantly stimulated IFN- γ secretion (Figure 4A), without having any effect on the secretion of IL-4 (Figure 4B), IL-13 (Figure 4C) and IL-17A (Figure 4D). These results suggest that VA Qu Spez selectively favours Th1 responses without modulating Th2, Th17 and Treg responses. Other four preparations of VA did not alter either frequency of T cell subsets or secretion of various T cell cytokines. These results were in line with the fact that VA Qu Frf, VA M Spez, VA P and VA A did not induce maturation and activation of DCs.

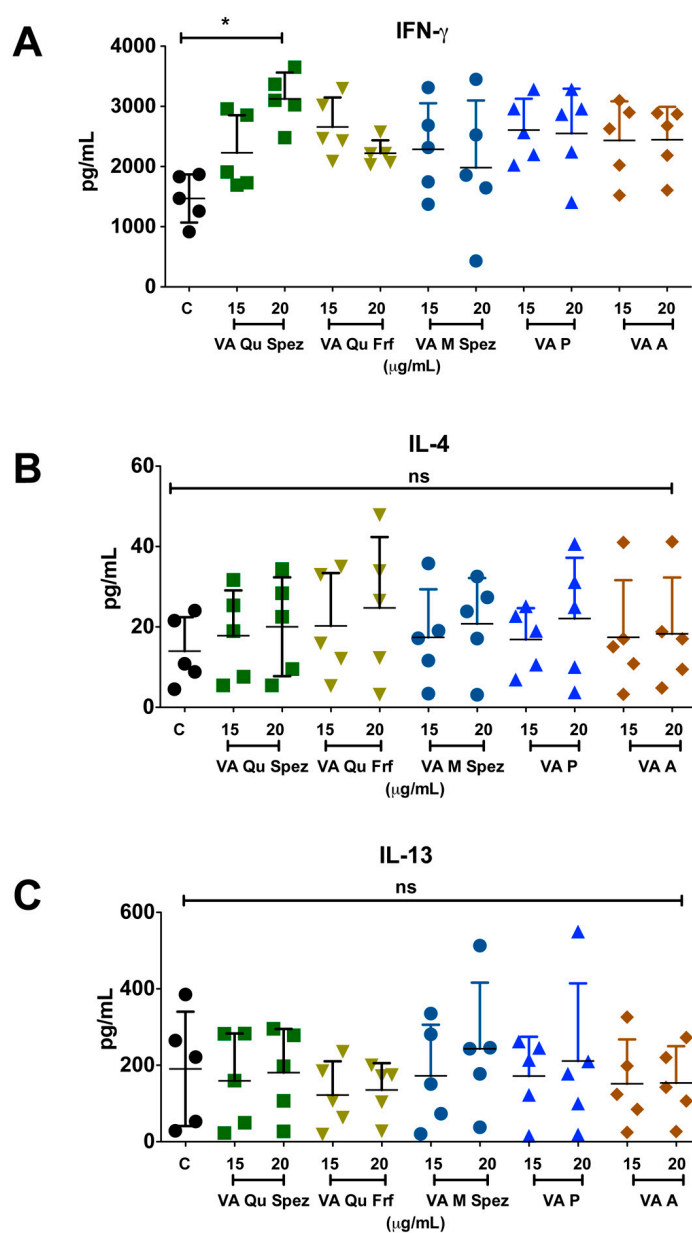


Figure 4. Cont.

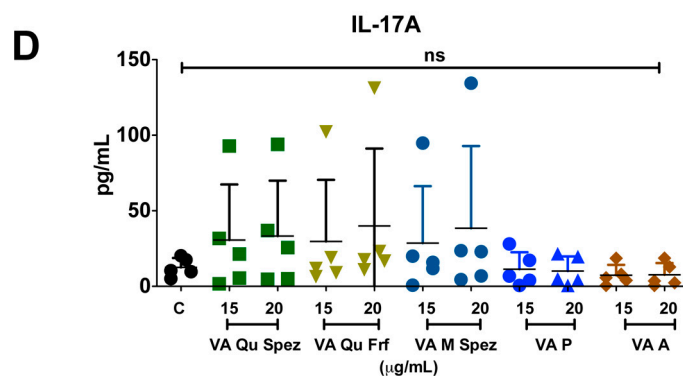


Figure 4. VA Qu Spez-educated DCs significantly induce the secretion of Th1 cytokine IFN- γ in DC-CD4⁺ T co-cultures. Immature DCs were treated with medium alone (control, labelled as 'C') or with five preparations of VA for 48 h. These DCs were co-cultured with CD4⁺ T cells for five days. Amount (pg/mL, mean \pm SEM, five independent donors) of secretion of (A) IFN- γ ; (B) IL-4; (C) IL-13; and (D) IL-17A in the cell-free supernatants from the above co-cultures was presented. * $p < 0.05$.

3. Discussion

Currently available mistletoe extracts are highly heterogeneous due to differences in the host trees, nutritional source, season of harvest, and extraction methods [4,26–28]. Therefore, VA preparations could exert divergent biological activities. However, comparative study of immunomodulatory properties of different VA extracts on immunocompetent cells such as DCs has not been performed to date. The present data therefore provide guidelines for the therapeutic use of VA preparations.

IFN- γ plays an important role in mediating the protective immune response against cancer, viral and intracellular bacterial infections [31]. IFN- γ enhances MHC class I expression on tumor cells and MHC class II expression on APCs like DCs, which in turn link innate and adaptive immunity [32]. IFN- γ responsiveness of tumor cell is important for the successful immune recognition. Indeed, it has been demonstrated that mice that are non-responsive to IFN- γ develop more tumors as compared to wild-type mice. Studies have shown that cross-talk between lymphocytes and IFN- γ /STAT1 signalling pathway plays an important role in maintaining the immune competitiveness of the host [33]. Idiotype-specific CD4⁺ Th1 cells can achieve tumor apoptosis directly by Fas/Fas L interaction and indirectly by IFN- γ production [34]. Thus, IFN- γ pathway is considered as an extrinsic tumor-suppressor mechanism [35]. We found that VA Qu Spez significantly enhances IFN- γ production without modulating Treg subsets and production of other T cell cytokines IL-4, IL-13 and IL-17A. This selective enhancement of Th1 cytokine strongly supports the use of VA as an immune modulator.

The success of DC-based cancer immunotherapies is dependent on the maturation status of DCs, their migration capacity and ability to mount protective T cell responses [36]. DC immunotherapy for cancer in humans though shown promises, it has not met with great success as compared to therapeutic molecules that target immune checkpoints. The reasons are multiple including poor survival of transferred DCs, limited number of DCs reaching the secondary lymphoid organs, heterogeneity in the DC subtypes and immune suppressive environment created by the tumor. Previous reports have shown that PGE₂ produced by DCs mediate Treg expansion [37–39], which might help in tumor evasion. Vaccination of cancer patients with 'PGE₂-educated DCs' also induced Treg expansion in the patients [40]. We observed that VA Qu Spez did not modulate Treg responses suggesting that VA Qu Spez selectively induces IFN- γ responses. Although not examined in DCs, we have recently shown that VA Qu Spez inhibits COX2-mediated PGE₂ in epithelial cell line [41,42]. Therefore, it is likely that VA Qu Spez-mediated suppression of COX-2 in DCs might be responsible for nonmodulation of Tregs in the present study. As these data are from the in vitro experiments, further work is necessary to validate these results from the patients treated with VA. Of note, through enhancement of Fas/FasL

expression and caspase activation, IFN- γ has been shown to enhance apoptotic response to ML II in human myeloid U937 cells [43].

MLs are the active components of mistletoe extracts and have several functions. The cytotoxicity of mistletoe is attributed majorly to its lectin contents [44,45] and lectin internalization is required for ML-I-mediated apoptosis [46]. MLs are responsible for stimulating cells of the innate and adaptive immune system such as DCs, macrophages, natural killer cells, and B and T lymphocytes. This function of MLs might represent one of the mechanisms responsible for the anti-tumoral and immunomodulatory effects of mistletoe extracts. It is known that ML-I B chain causes Ca^{2+} influx in Jurkat cells and is mediated by its interaction with surface glycoprotein receptors [47]. Chemical labelling of the lectin revealed that it binds to surface of peripheral and intra-tumoral monocytes [48].

A recent study shows that 3D structure of ML-A chain shares structural homology with shiga toxin from *Shigella dysenteriae* and provides an explanation for the strong immune stimulatory capacity of ML [49]. It is also demonstrated that Korean mistletoe lectin (KML) induces activation of innate cells by TLR4-mediated signalling [50]. The nature of the receptor(s) on DCs that recognizes ML and mediates activation is not known. Since Korean ML and European ML share 84% sequence identity [51], it is presumable that European ML might signal DCs via TLR [49]. However, we found that not all VA preparations are stimulatory on DCs. VA Qu Frf, an unfermented preparation containing the highest concentration of lectin and viscotoxin was unable to activate DCs. Other VA preparations, which are fermented and contain low lectin, were also unable to stimulate DCs, whereas VA Qu Spez, a fermented preparation that contains the second highest concentration of lectin ($785 \pm 10\%$ ng/mL) efficiently activated DCs and promoted Th1 response. These results suggest that mere lectin content in a VA preparation does not necessarily determine its immunostimulatory capacity. The methodology of preparation, i.e., fermented vs unfermented, might be crucial for conferring the stimulatory properties to VA. Alternatively, the fermentation process might modify the structure of the lectins of the VA preparation.

To conclude, our study delineates the differential effects of various VA preparations on DC maturation, function and T cell responses. These results reveal that VA Qu Spez is the most potent preparation in activating DCs and promoting Th1 response. The current evidence to support mistletoe therapy in oncology is weak [52]. Thus, this study along with other reports on mistletoes [53–60] provides a rationale for examining the use VA as an immune modulator. Such mechanistic studies are also important to undertake randomised clinical trials to improve level of evidence for the use of VA in complementary therapy of cancer.

4. Materials and Methods

4.1. VA Preparations

Five clinical grade preparations of VA (VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A) obtained from Hiscia Institute, Verein für Krebsforschung (Arlesheim, Switzerland) were used. These preparations were free from endotoxins and were formulated in 0.9% sodium chloride isotonic solution as 5 mg/mL vials. The chemical compositions of the VA preparations are provided in Table 1.

Table 1. Composition of VA preparations.

Preparation Concentration	Host Trees	Lectin Content (ng/mL)	Viscotoxin Content (μ g/mL)	Method of Preparation
VA Qu Spez 10 mg	Quercus (Oak)	$785 \pm 10\%$	$5 \pm 5\%$	Fermented
VA Qu Frf 10 mg	Quercus (Oak)	$2391 \pm 10\%$	$19 \pm 5\%$	Unfermented
VA M Spez 10 mg	Malus (Apple)	$548 \pm 10\%$	$4 \pm 5\%$	Fermented
VA P 10 mg	Pinus (Pine)	$28 \pm 10\%$	$6 \pm 5\%$	Fermented
VA A 10 mg	Abies (Fir)	$23 \pm 10\%$	$19 \pm 5\%$	Fermented

4.2. Human DCs

Human monocyte-derived DCs were used as a source of DCs. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors. The buffy coats were purchased from Centre Necker-Cabanel (EFS, Paris, France). Ethics committee approval for the use of such material (Institut National de la Santé et de la Recherche-EFS Ethical Committee Convention N°12/EFS/079) was obtained and experiments were performed in accordance with the approved guidelines of INSERM. Circulating monocytes were isolated using CD14 microbeads (Miltenyi Biotec, Paris, France) and were cultured for 5 days in RPMI 1640 containing 10% fetal calf serum, rhIL-4 (500 IU/10⁶ cells) and rhGM-CSF (1000 IU/10⁶ cells) to obtain immature DCs [61].

4.3. *Viscum Album* Treatment of DCs

Immature DCs were washed and cultured in rhIL-4 and rhGM-CSF and treated with VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A at four different concentrations: 5, 10, 15 and 20 µg/mL/0.5 million cells for 48 h. Cell culture supernatants were collected for analysing the cytokines and DCs were analysed for the phenotype by flow cytometry.

4.4. DC: CD4⁺ T Cell Co-Cultures

CD4⁺ T cells were isolated from the PBMC using CD4 microbeads (Miltenyi Biotec). VA-treated DCs were washed extensively and seeded with 1 × 10⁵ responder allogeneic CD4⁺ T cells at DC: T cell ratio of 1:10. On 5th day, CD4⁺ T cell responses were analysed by intra-cellular staining for specific T cell cytokines (IFN-γ, IL-17A and IL-4) and transcription factor (FoxP3). The cell-free culture supernatants were analysed for the cytokines secreted.

4.5. Flow Cytometry

For surface staining, following Fc receptor blockade, antibodies against surface molecules were added at pre-determined concentration and incubated at 4 °C for 30 min. FITC-conjugated monoclonal antibodies (MAbs) to CD1a, CD86, HLA-DR, and CD25; PE-conjugated MAbs to CD83 (all from BD Biosciences, Le Pont de Claix, France), CD40 (Beckman Coulter, Villepinte, France) and Alexa Fluor[®] 700-conjugated MAbs to CD4 (eBioscience, Paris, France) were used for the analysis of surface phenotype.

For intra-cellular staining, cells were stimulated with phorbolmyristate acetate (50 ng/mL; Sigma-Aldrich, St. Quentin Fallavier, France) and ionomycin (500 ng/mL; Sigma-Aldrich) at 37 °C for 5–6 h in the presence of golgi-stop (BD Biosciences) during the last 2 h. Cells were fixed and permeabilised using Foxp3 Fixation/Permeabilization kit (eBioscience) and incubated at 4 °C with FITC-conjugated MAbs to IFN-γ (eBioscience), PE-conjugated MAbs to IL-17A and IL-4 (eBioscience), and APC-conjugated MAbs to FoxP3 (eBioscience). Live-dead cells were differentiated by PO-Fixable Viable dye (eBioscience).

Cells were acquired on LSR II and processed with FACS DIVA software (BD Biosciences) and analysed by Flowjo. The data were presented as % positive cells for indicated markers or mean fluorescence intensities (MFI) of their expression.

4.6. Cytokine Assay

IL-6, IL-8, IL-10, IL-12, TNF-α, IL-4, IL-13, IFN-γ and IL-17A in the cell-free culture supernatants were quantified by Ready-SET-Go enzyme-linked immunosorbent assay (ELISA) kits (eBioscience).

4.7. Statistical Analysis

The significant differences between samples were determined by One-way ANOVA Tukey's multiple comparison test using Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Values of $p < 0.05$ were considered statistically correlated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

5. Conclusions

Our study demonstrates the differential effects of various VA preparations on human DC activation and ensuing CD4⁺ T cell responses. Our data reveal that VA Qu Spez is the most potent VA preparation in activating DCs and promoting Th1 response.

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