Intervenous immunoglobulins as modulators of immune response : effect on T cell polarisation, pathogenicity and trafficking

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Modulation de la réponse immunitaire par les immunoglobulines intraveineuses : Effets sur la polarisation, la pathogénicité et le trafic des lymphocytes T

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Abbreviations

Ab	Antibody
Ag	Antigen
AHR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
BCR	B cell receptor
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CIDP	Chronic inflammatory demyelinating polyneuropathy
CNS	Central nervous system
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTL	Cytotoxic T lymphocyte
CVID	Common variable immunodeficiency
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3- grabbing non-integrin
DLN	Draining lymph node
DNA	Deooxribonucleic acid
EAE	Experimental autoimmune encephalitis
ELISA	Enzyme link immunosorbent assay
$F(ab•')_2$	Fragment antigen binding
Fc	Fragment crystallizable
FcγR	Fc gamma receptor
FcRn	Neonatal FcR
FCS	Foetal Calf Serum
FITC	Fluroescein isothiocyanate
Foxp3	Forkhead box p3
GBS	Guillain Barrée syndrome
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IBD	Inflammatory bowel disease
iTreg	Induced regulatory T cell
ITP	Immune thrombocytopenic purpura
IVIg	Intravenous immunoglobulin
LAG-3	Lymphocyte activation gene-3
LPS	Lipopolysaccharide
MHC	Major Histocompatibility complex
mAb	Monoclonal antibodies

Mo	Monocyte
Mφ	Macrophage
MS	Multiple sclerosis
NAbs	Natural antibodies
NFAT	Nuclear factor of activated T cell
NK cell	Natural Killer cell
nTreg	Natural regulatory T cells
PBS	Phosphate buffer saline
RA	Rheumatoid Arthritis
RORC/ RORγt	Retinoic acid-related orphan receptor
SEM	Standard error of mean
SLE	Systemic lupus erythematosus
STAT	Signal transducing and activating transcription factor
T-bet	T box transcription factor
TCR	T cell receptor
Tfh	Follicular helper T cells
TGF-β	Transforming growth factor β
Th cell	T helper cell
TLR	Toll like receptor
Tregs	Regulatory T cells
TNF	Tumor necrosis factor

Résumé

La signalisation au niveau de la synapse immunitaire entre les cellules présentatrices d'antigènes et les lymphocytes T est une phase critique de la réponse immune adaptative. Les lymphocytes T CD4+ naïfs sont polarisés en différentes populations (Th1, Th2, Th17, iTrgs) et les décisions majeures, telles que le choix entre l'homéostasie immunitaire et l'activation du système immunitaire, la tolérance au soi, l'auto-immunité, sont prises à ce moment. Dans certaines conditions, un déclenchement dérégulé de la réponse immunitaire provoque un déséquilibre entre les fonctions effectrice et régulatrice des lymphocytes T, conduisant à des phénomènes d'auto-immunité et d'inflammation systémique. Des doses élevées d'immunoglobulines intraveineuses (IVIg) sont fréquemment utilisées pour traiter ces maladies. Le mécanisme d'action des IVIg sur la polarisation des lymphocytes T reste à ce jour inexploré. Ainsi, j'ai recherché les effets de doses élevées d'IVIg sur la polarisation des lymphocytes T dans le cadre de l'encéphalomyélite auto-immune expérimentale (EAE), une maladie auto-immune mettant en jeu les lymphocytes T et un modèle animal de la sclérose en plaques. L'EAE a été induite chez des souris C57BL/6J en combinant le peptide MOG35-55 avec l'adjuvant complet de Freund (CFA). L'administration d'IVIg a retardé l'apparition de l'EAE et diminué significativement l'intensité des signes cliniques. Les IVIg ont inhibé la différenciation des lymphocytes T CD4+ naïfs en sous-populations effectrices (Th1 et Th17) et ont, de manière concomitante, provoqué la prolifération des lymphocytes T régulatrices CD4+ Foxp3+. En outre, les IVIg ont rendu les lymphocytes T effecteurs moins pathogéniques, en diminuant l'expression de molécules encéphalitogéniques telles que le GM-CSF et la podoplanine. Les IVIg ont diminué l'expression du récepteur à la sphingosine-1 phosphate (S1P1) à la surface des lymphocytes CD4+, séquestrant ces cellules dans les ganglions lymphatiques et diminuant l'infiltrat de l'organe cible (le système nerveux central) en Th1, Th17 et Trég. Le récepteur inhibiteur FcγRIIB n'est pas indispensable pour la modulation des sous-populations de lymphocytes T CD4+ effecteur et régulateur induite par les IVIg in vivo. D'autre part, les portions F(ab)'2 des IVIg ont conservé la fonction modulatrice associée aux IVIg. La cible de la rapamycine chez les mammifères (mTOR) est une kinase qui intègre de multiples signaux de l'environnement et qui est impliqué dans la régulation des réponses effectrice et régulatrice des lymphocytes T. Les IVIg ou les F(ab)'2 ont diminué l'activité de mTOR, rétablissant à nouveau l'équilibre entre les sous-populations de lymphocytes T régulateurs et de lymphocytes T helper pro-inflammatoires. Pris ensemble, ces résultats constituent une base cellulaire et moléculaire,

qui sous-tend l'effet bénéfique des IVIg dans certaines maladies auto-immunes et inflammatoires.

Summary

Signaling at the immune synapse between antigen presenting cells and T cells is the critical phase of the adaptive immune response. Naïve CD4 T cells are polarized into various subsets (Th1, Th2, Th17 and iTregs) and crucial decisions such as immune homeostasis versus immune activation; and tolerance to self-versus autoimmunity are made at this point. Dysregulated activation leads to pathogenic immune response to self-antigens and inflammatory pathologies. High dose therapy of intravenous gammaglobulin (IVIg) is widely used in the treatment of several T-cell and autoantibody-mediated autoimmune diseases. However, the comprehension of the mechanisms underlying its therapeutic benefit has remained a major challenge. In particular the mechanisms of action of IVIg in terms of T cell polarization *in vivo* have remained unexplored. Therefore, I have investigated the effect of high dose IVIg on T cell polarization using actively induced experimental autoimmune encephalomyelitis (EAE), a T cell-mediated autoimmune condition and an animal model of multiple sclerosis. EAE was induced in C57BL/6J mice using MOG_{35-55} emulsified in complete Freund's adjuvant (CFA). Concomitant administration of IVIg delays the onset of EAE and significantly decreases severity of the disease. IVIg inhibited the differentiation of naïve CD4 T cells into effector subsets (Th1 and Th17 cells) and concomitantly induced expansion of $Foxp3^{+}CD4$ cells. Further, IVIg rendered effector T cells less pathogenic by decreasing expression of encephalitogenic molecular players such as GM-CSF and podoplanin. IVIg decreased the expression of sphingosine-1 phosphate receptor (S1P1) on CD4 cells, thus sequestering these cells in the draining lymph nodes and decreasing infiltration of Th1, Th17 and Tregs to the target organ (central nervous system). Inhibitory FcγRIIB appeared dispensable for IVIg-mediated reciprocal modulation of effector and regulatory CD4 subsets *in vivo.* $F(ab')_2$ fragments of IVIg also retained the reciprocal CD4 T cell modulatory functions of IVIg. Mammalian target of rapamycin (mTOR) is a kinase which integrates various environmental signals and is involved in regulation of effector and regulatory T cell responses. IVIg or $F(ab')_2$ decreased activity of mTOR thus restoring the equilibrium between regulatory T cells and pro-inflammatory T helper subsets. Thus, these findings provide a novel cellular and molecular basis underlying the beneficial effect of IVIg in certain T-cell mediated autoimmune and inflammatory conditions.

Introduction

Intravenous immunoglobulins as modulators of immune response: Effect on T cell polarization, pathogenicity and trafficking

1. Introduction

1.1. Immune system

The immune system comprising innate and adaptive part is a network of interacting molecules and cells evolved to fight against the invading pathogens and regulate aberrant tissue responses such as cancer. Cells of the innate immune compartment include monocytes (Mo), macrophages (MΦ), dendritic cells (DC), mast cells (MC), neutrophils, eosinophils, natural killer (NK) and basophils. The complement system comprises of proteins in the serum that act by opsonizing and inducing cytolysis in pathogens and infected cells. Invasion of host tissues by pathogens leads to cell stress, hypoxia, necrosis, temperature shifts and tissue destruction [1]. Local injury at the site of infection or tissue damage releases various microbial and endogenous products from damaged or dying cells [2]. Molecules originating from pathogens are called as pathogen associated molecular patterns (PAMP), whereas those from insulted cells/tissues are designated as danger associated molecular patterns (DAMP). The cells of the innate immunity residing in various tissue of body are endowed with specialized germline-encoded pattern recognition receptors (PRRs) and multi-protein complexes like inflammasomes. Innate cells recognize PAMPs and DAMPs through these receptors at the site of tissue injury and initiate the early steps of mounting an immune response.

Information about local insult to tissues by pathogens or aberrant tissue responses is relayed to the adaptive arm of the immune system to initiate antigen-specific immune response [3]. Adaptive immunity mainly consists of lymphocytes of thymic (T cells) and bone marrow origin (B cells). T cells provide most of the cell-mediated immune response and B cells produce glycoproteins called antibodies that effectors of the humoral immune response. T cells are further classified as $CD4^+$ helper T cells (Th) and $CD8^+$ cytotoxic T cells (CTL). A minor population of γδ T cells and natural killer cells (NKT) also exist which share properties of innate and T cells. In addition to the antigen specific effector responses adoptive immunity also maintains a state of immunological memory to the antigens encountered. This immunological memory helps the host to initiate faster and robust antigen-specific immune response to eliminate the pathogens. Immune responses are constantly checked and regulated at various levels to ensure that body does not attack and destroy the self-organs and tissues. However, under particular conditions a dysregulated immune system might lead establishment of inflammatory conditions and damage to the self-tissues.

1.2. Immune homeostasis and activation

Activation of Th cells marks the key point in initiation of any immune response. Antigen presenting cells (APC) like dendritic cells, B cells and macrophages capture antigen process and present it to CD4 T cells in MHCII- restricted manner. Progress made in intra-vital multi-photon microscopy has revealed many aspects of dynamics of *in vivo* crosstalk between APCs and T cells involved in the initiation of an immune response [4]. Under homeostatic conditions, a naïve Th cell enters secondary lymphoid

organs such as draining lymph nodes (DLN) and arrives at the T cell rich zone. It scans the APCs for cognate antigens during the motion strategy termed as “random walk” at 12 µm/min [5]**.** Since, the APCs under homeostatic conditions present low levels of self-antigens in a non-stimulatory context; these naïve T cells are leave DLN inactivated to enter into circulation before arriving at another DLN **(Figure 1A).**

In case of infection or injury, tissue resident immature dendritic cells sense presence of foreign antigens and danger signals through PAMPs and DAMPs; and carry the captured antigen to the nearby DLN. These antigen-primed DCs enter a developmental program called “maturation” during which they decrease their endocytic capacity, and process the captured antigen. The antigen is presented in context of unregulated MHC. Along with antigen presenting molecules mature-DCs also up-regulate surface expression of various co-stimulatory and adhesion molecules like CD28, CTLA-4, PD-1, ICOS, OX40, CD80/CD86, CD40, ICAM-3binding C type lectin and DC-SIGN [6-8]. Additionally mature DCs secrete various cytokines like IL-12, IL-23, IL-2 and interferon-α. Naïve Th cells encountering an antigen loaded, matured APC in DLN changes its motility from “random walk” to “brief contacts” that last for hours. After brief contacts there is a phase of “sustained contact” between T cell and APC which lasts for 18-20 hour before the first division of T cell [5] (Figure 1B). Naïve T cells recognizing antigen though TCR initiate CD3 signaling and simultaneously integrate various cues from APCs. The period of “sustained contact” is the key phase in T cell activation, as APCs instruct T cells about the nature of antigen encountered in the periphery by presenting the processed antigen together with a combinatorial code of co-stimulatory molecules and cytokines [9, 10].

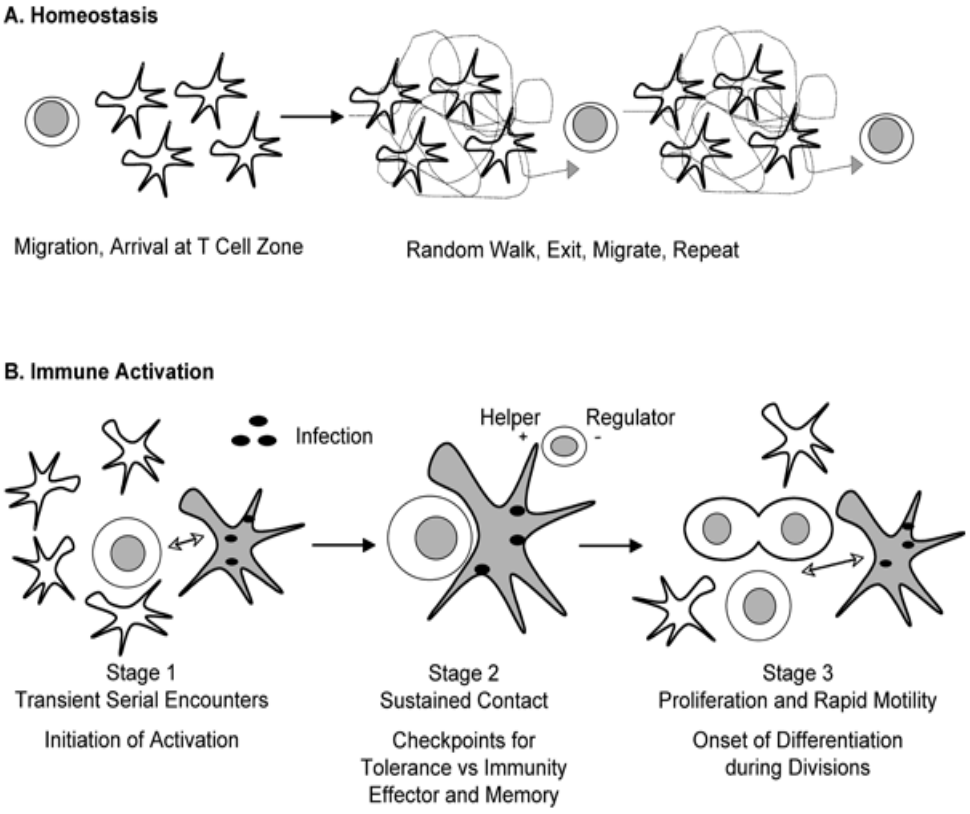

Figure 1: APC and T cell interaction during homeostasis and immune activation

A. Naïve T cells enter the DLN and interact with APCs residing in the T cell rich zone and do a 'random walk' and scan for APC for activation cues. Since APCs are in immature phase they T cells leave the LN to enter into circulation to enter another LN.
B. Under conditions of immune activation APC bring antigen from periphery and present it with higher amount of co-stimulatory and adhesion molecules on their surface. Antigen-specific naive T cells make transient serial encounters of 8-10 min with APCs which lasts for 8-10 hrs. Next step is sustained contact between these cells which lasts for 18-30 hours after which T cells enter phase of cell cycle and differentiation. (Adapted from Paul W, *Fundamental Immunology 6th Edition* 2010)

The activated CD4 T cells are polarized to various subsets, which in turn shape the further course of immune response by providing help to B and CD8 T cell. B cells secrete family of glycoproteins called immunoglobulins which defend the body against invading pathogens. CD8 T cells are involved in clearance of infected cells. Crucial decisions such as immune homeostasis versus activation; memory versus effector response; and tolerance to self-versus autoimmunity are made at this point of T cell activation and polarization.

Different Th lineages generated in immune response include Th1, Th2, Th17, and induced regulatory T cells (iTregs) which are identified by their ability to secrete distinct set of cytokines [11-13]. In addition to these, a different lineage of natural regulatory T cells (nTregs) exist which emerge from thymus [14-16].

1.3. **T cell polarization and Th cells subsets**.

Th cell polarization occurs in response to activation by APCs and cytokine profile in the milieu of activation. The specific set of cytokines involved in various Th cell polarizations are Th1: IL-12/IFN-γ; Th2: IL-4/ (IL-2); Th17: TGF-β, IL-6, IL-23, IL-21 and IL-1β; iTregs: TGF-β/IL-2. Transforming transcription factors which are the master regulators of differentiation for these lineages are: T-bet/STAT4 for Th1, GATA3/STAT6 for Th2, RORγt (RORC in human)/STAT3 for Th17 and Foxp3/STAT5 for Tregs **(Figure 2).**

Other putative Th cell lineages include TGF-β-producing Th3cells [17]**,** IL-10-producing Tr1 cells [18]**,** IL-9-producing Th9 cells [19, 20]**,** the follicular helper cells

(Tfh) which migrate to follicular regions of lymph nodes and spleen to help B cells [21-23]; and IL-22-producing Th22 cells [24, 25]. Whether these subsets represent lineages distinct from the known Th subsets needs to be further explored, as many of these cytokine are also produced by Th1/Th2/Th17/Tregs.

1.3.1. Th1 cells

TLR activated APCs in presence of type I IFNs, IFNγ or CD40L produce large quantity of IL-12 [26]. IL-12 is the key cytokine in induction of Th1 cells. Signaling through the IL-12 receptor β2 (IL-12Rβ2) results in STAT4-mediated promotion of IFNγ expression which sustains the expression of IL-12Rβ2. Th1 cells initially express IFNγ which acts in autocrine/paracrine manner to activate STAT1. Activated STAT1 strongly promotes expression of T-bet, which increases the transcription of IFN.

T-bet then enhances the transcriptional competence of the IFNγ gene leading to increased production of IFN-γ [27-30]. T-bet, the Th1 master regulator prevents Th2 differentiation by inhibiting GATA3. Thus co-ordinated signaling with IFN-γ and IL-12 leads to full differentiation of Th1 cells. IFN-γ, lymphotoxin-α (LTα), tumor necrosis factor (TNF)-α and IL-2 are the principal cytokines produced by Th1 cells.

IFN-γ plays critical role in innate and adaptive immunity against viral and intracellular bacterial infections. LTα, which is the marker for disease progression in multiple sclerosis (MS) patients, is a potent lymphangiogenesis mediator [31]. TNF-α enhances infiltration of MΦ and neutrophils to a site of infection. CD4+ T cell survival and memory $CD4^+$ and $CD8^+$ T cell generation requires IL-2 production from Th1 cells [32, 33]. Th1 cells are mainly implicated in immune response against intracellular

pathogens like mycobacterial infections [34, 35] and also in the pathogenesis of some autoimmune diseases.

1.3.2. Th2 cells

Naïve Th cells activated in presence of IL-4 activate intracellular STAT6 and *gata3* genes [36]. The IL-4/STAT6 pathway also induces growth factor independent-1 (Gfi-1), which plays an important role in promoting selective growth of GATA-3^{high} cells [37, 38]. Reorganization of chromatin structure in the Th2 locus enhances the transcription of *Il4*, *Il5*, and *Il13* genes [39]. Feed forward activity of IL-4 further enhances Th2 cell differentiation. Differentiation into Th1 is inhibited by GATA-3 which down regulates expression of IL-12Rβ2 and STAT4. NKT cells, basophils and mast cells are the sources of IL-4 *in vivo.* Th2 cells are implicated in promoting humoral immunity and promote B cells to up-regulate antibody production to fight extracellular organisms.

1.3.3. Th17 cells

Combination of TGF-β and IL-6 is required to polarize naive Th cells into IL-17 producing Th17 cells [40-42]. Intriguingly TGF-β is an immune regulatory cytokine and IL-6 is pro-inflammatory cytokine. Naïve Th cell activation in presence of TGF-β alone leads to activation of Smad pathway, which generally down regulates immune responses[43]. However in presence of IL-6/IL-21, STAT 3 is also activated. STAT 3 triggers functional expression of retinoic acid-related orphan nuclear receptor (RORγt), the lineage-specific transcription factor for Th17 cells [41, 44]. Th17 cells secrete IL-21 which acts in autocrine manner to enhance differentiation of Th17 cells [45]. IL-1 β and

IL-23 are also required for differentiation of Th17 cells [46, 47]. IL-1β is important to enhance the expansion of differentiated Th17 cells and IL-17 production [48]. Th17 cells are stabilized by IL-23, which mediates further expression of IL-22 [49].

T cells are themselves the source of TGF-β for the differentiation of Th17 cells [40, 44, 50]. Cytokines such as IL-1β, TNF, platelet-derived growth factor (PDGF), IL-3, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-17 act on DCs, monocytes, MΦ, mast cells, and B cells to produce IL-6 [51, 52]. IL-21 is produced by activated and memory T cells and NKT cells, but not by APC [53]. IL-23 is predominantly produced by cells of the innate immune system, including DC and MΦ [54]. IL-23 stabilizes the differentiating Th17 cells to the Th17 lineage but is not involved in the initial differentiation of Th17 cells [49, 55]. Th17 cells participate in immune responses against extracellular bacteria and fungi [56]. Th17 cells are also responsible for many organ-specific autoimmune diseases. Th17 cells exert their effector functions by secreting IL-17A, IL-17F, IL-21, and IL-22 [49]. These effector cytokines target many immune and non-immune cells to induce the production of many pro-inflammatory mediators. Th17 cells use IL-22 as a mediator to communicate with non-immune tissues; such as to enhance the production of protective acute-phase reactants in hepatocytes and β-defensins in keratinocytes thus, enhancing the immune barrier function of epithelium [57].

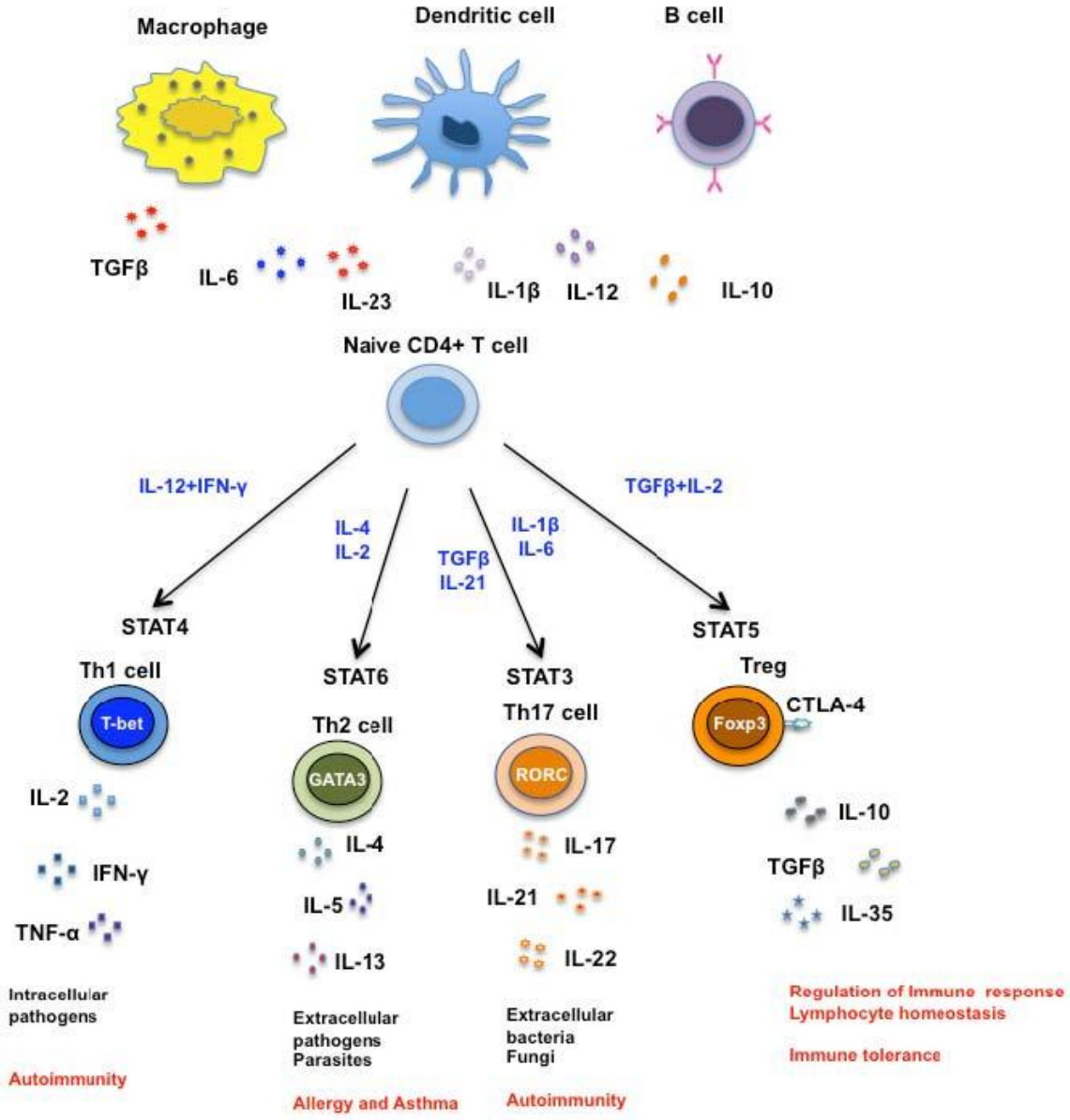

Figure 2: Current schedule of T helper cell differentiation.

The activated naïve Th cells can develop into distinct lineage based on the cytokine milieu in the local environment. A distinct set of cytokines promotes the differentiation processes for each lineage: IL-12/IFN¬γ for Th1; IL-4/(IL-2) for Th2; TGFβ, IL-6, IL-21, IL-1β, IL-23 for Th17 and TGFβ/IL-2 for iTregs. These Th cell lineage express unique set of transcription factor and, cytokines which are critical for exerting effector functions important in host defense as well as in immune-mediated diseases. (Adapted from Maddur et al, 2010 PLos Pathogens)

1.3.4. Regulatory T cells

CD4 T cells expressing CD25 and transcription factor Foxp3 are called as Tregs. Population of Tregs includes thymus derived natural (nTregs) and peripherally induced Tregs (iTregs). High affinity interaction between self-peptide-MHC complex and TCR on the developing T cells in thymus leads to selection of nTregs. Cortical and medullary thymic epithelial cells and thymic DC are implicated in the development of Tregs. Engagement of TCR and IL-2R signaling in developing T leads to activation of STAT5, which up regulates Foxp3 gene [58].

iTregs are generated from naïve CD4 T cells if, activated in presence of TGF-β and IL-2 [55]. Activation of Smad3 in presence of TGF-β and NFAT activation by TCR stimulation are the early signaling events in induction of iTregs. Smad3 and NFAT remodel the chromatin structure in Foxp3 enhancer region which facilitates the expression of Foxp3 [59]. IL-2–mediated STAT5 activation is also critical for the induction of Foxp3 expression [60-62] .

Self-reactive T cells are dominantly controlled by nTregs, contributing to the maintenance of immunologic self-tolerance [63, 64]. Tregs are antigen specific but upon activation can suppress T cells non-specifically. Tregs can inhibit functions of $CD4^+$, $CD8^+$ T cells, DC, B cells, MΦ, monocytes, mast cells, NK cells and NKT cells [65-67]. Tregs use several contact dependent and cytokine mediated mechanism to exert their suppressive functions **(Figure 3)** [65]. Tregs can secrete suppressor cytokines like TGF-β, IL-10 and IL-35 which suppress responder T cells. Tregs can cause cytolysis through granzyme and perforin. Tregs inhibit the interaction of naive T cells with DC,

thereby terminating the T cell activation [68]. Tregs down regulate expression of co-stimulatory molecules CD80 (B7-1), CD86 (B7-2) and CD40, and the MHC-peptide complexes on DC through cytotoxic T lymphocyte antigen 4 (CTLA-4) [69-71]. Treg-modulated DCs produce decreased amount of inflammatory cytokines IL-12, IL-1β, IL-6 and IL-8 and more of anti-inflammatory cytokine IL-10. Treg-express surface CD39, which degrades extracellular ATP to AMP thus, inhibits the ATP-mediated activation of DC [63] [67]. Tregs decrease autoantibody production by B cells by inhibiting T cell-dependent B cell responses. Strategies like increasing Treg numbers and/or enhancing their suppressive function has been proven to be beneficial for treating autoimmune diseases and preventing allograft rejection [72-74].

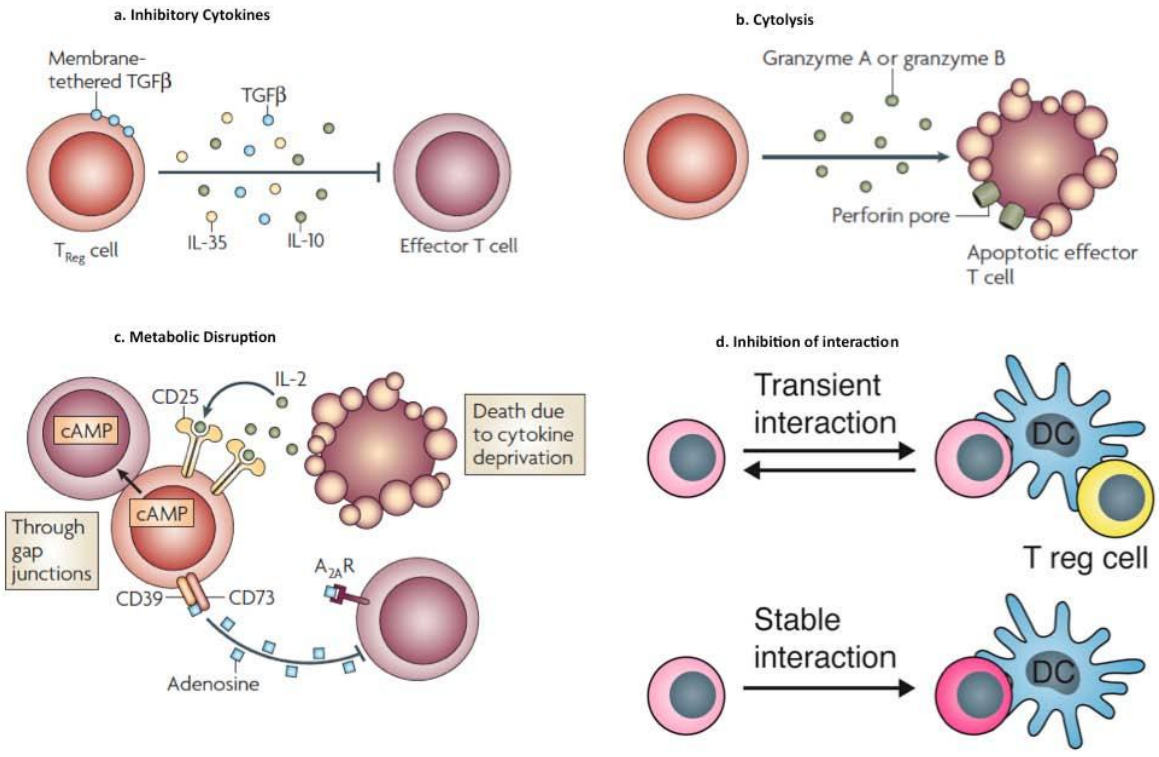

Figure 3. Mechanisms of suppression by Tregs

(a) Tregs produce inhibitory cytokines include IL-10, IL-35 and TGFβ which can suppress the effector T cell functions.

(b) Target cell can be attached by granzyme (A or B) and perforin dependent cytolysis.

(c) CD25 (high affinity IL-2 receptor) expressed on Tregs might consume the cytokine which induces apoptosis in cells nearby. CD39 is involved in cyclic AMP (cAMP)-mediated inhibition.

(d) Tregs can interfere with DC-T cell interaction leading to premature termination of T cell activation and induction of anergy. (Adapted from Amsen et al., 2009; Curr Opin Immunol. and Rudensky and Campbell, 2006; J Exp Med)

1.4. mTOR integrates multiple signals during T cell activation

The differentiation of naïve CD4 cells into specific Th cell type *in vivo* is a complex process which requires these cells to integrate various environmental cues delivered by APCs in the secondary lymphoid organs [4, 75]. Naïve T cell interacting with APCs often encounter combination of cytokines with both pro and anti-inflammatory activity. There is also a considerable variation in the strength of the signal delivered by APCs in terms of avidity of interaction with MHC-II. How naïve T cells integrate all these signals and enter lineage commitment is a fascinating problem to be understood. The question how biological systems integrate multiple signals is still a “black-box”, However mammalian target of rapamycin (mTOR) has emerged as a kinase which is involved in integration various environmental signals and regulation of cells energy demands for growth and development [76]. mTOR consists of two protein complexes mTORC1 and mTORC2; mTORC1 is activated by PI3-kinase, Akt and Rheb whereas, mTORC2 is activated by PI3-K and enhances the phosphorylation of Akt **(Figure 4)** [77]. mTORC1 is rapamycin sensitive and mTORC2 is resistant to rapamycin [76]. Probing into the role of mTOR in immune cells has revealed many exciting observations. With respect to T cell biology, genetic deletion of mTOR resulted in impaired development of Th1, Th2 and Th17 cells. This effect was due to inability of these cells to activate STAT pathway upon stimulation. Interestingly mTOR deficient T cells developed into Foxp3 expressing Tregs independent of TGGβ [78, 79]. Interestingly mTOR keeps a check on expression of Foxp3 in Tregs [80], thus mTOR plays crucial role in differentiation naïve Th cells into effector Th cells (Th1, Th17 and Th2) and negatively regulates expression of Foxp3 in Tregs.

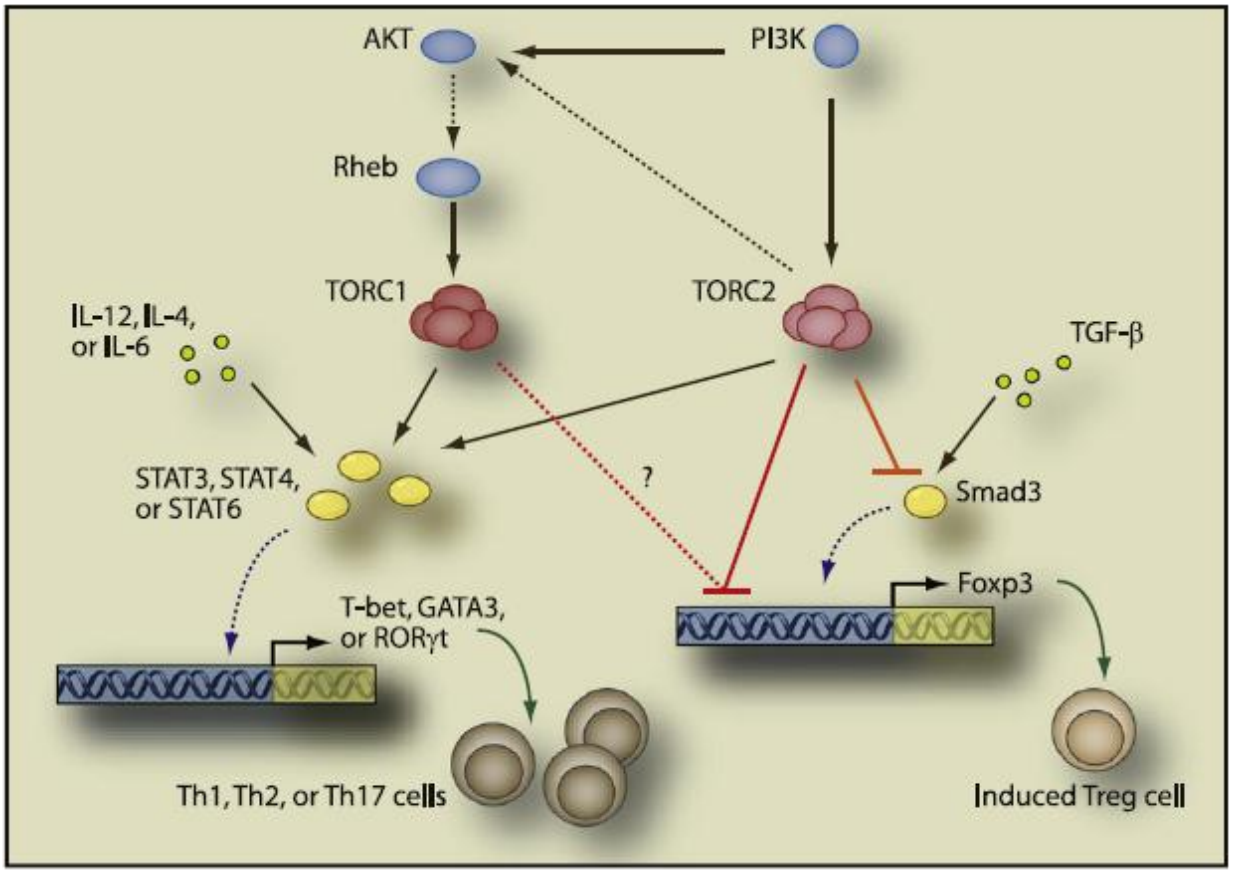

Figure 4. mTOR pathway in regulating the effector and regulatory Th Cells

mTORC1 and mTORC2 are the key components of mTOR. mTORC1 is activated by Akt, whereas mTORC2 enhances the phosphorylation of Akt. mTOR facilitates the differentiation of effector Th cells by augmenting the strength of respective STAT signal. Reciprocally, mTOR suppresses the expression of Foxp3 in differentiating Th cells by attenuating TGF-b signaling and also via a TGF-b independent mechanism. The negative effect of mTOR on the induction of Foxp3, although sensitive to rapamycin, can be mediated by mTORC2. But it remains unclear which mTOR complex is responsible for supporting the differentiation of effector Th cells. In contrast to its role in the differentiation of iTreg cells, mTOR probably has little influence on the development of nTreg cells. (Adapted from I-Cheng Ho, Immunity 2009)

2. T cells in autoimmunity : shifting paradigms from Th1/Th2 to Th17/Tregs

2.1. Th1/Th2 hypothesis and the emergence of Th17 cells

Initially autoimmunity and many immunological disorders were explained on the basis of imbalance of Th1/Th2 responses. Th1 cells were considered to be pathogenic while Th2 cells were attributed with inhibitory functions [81]. IFNγ, the principle cytokine of Th1 cells was found in the target tissues of at the peak of EAE and CIA [82-84]. Adoptive transfer of Th1 cells was sufficient to induce disease in mouse models of type1 diabetes and EAE [85]. Administration of IFNγ in MS exaggerated the disease [86]. Mice deficient in T-bet and STAT4 were unable to produce IFNγ and were resistant to development of experimentally induced EAE [87, 88]. Administration of anti-IL12 was beneficial in EAE and CIA [86]. All together, these studies supported hypothesis that self-antigen specific IFNγ-producing Th1cells are the pathogenic cells in many autoimmune conditions.

However, some key experiments performed in EAE required the revision of this theory. The flaws of the theory include: IFNγ injections protected against EAE, antibodies to IFNγ worsened EAE, IFNγ knockouts mice were more susceptible to EAE, and TNF knockouts had an exaggerated EAE and administration of TNF protected mice from EAE **(Table 1)** [89-94].

These contradictory findings and the experiments to understand the role of the cytokine IL-23 in EAE have helped to decipher the paradox of Th1/th2 hypothesis. IL-23 is a heterodimeric cytokine with p40 and p19 subunit. The p40 subunit is common to

Th1 inducing IL-12 and IL- 23. Mice deficient in IL-23 were resistant to various animal models of autoimmunity like IBD, Collagen arthritis and EAE [95, 96].

Table 1. Predictions and outcomes of TH1/TH2 hypothesis in EAE

Prediction	Outcome
Administration of γ-IFN would worsen EAE	Administration of γ-IFN protected from EAE
γ-IFN knockouts would be resistant to EAE	EAE worse in γ-IFN knockouts
Antibody to γ-IFN would protect in EAE	Antibody to γ-IFN worsened EAE
TNF knockouts would be resistant to EAE	TNF knockouts had worsened EAE
Administration of TNF would worsen EAE	Administration of TNF protected from EAE

Adapted from Steinman L, Nature Medicine 2007 [97]

Further, IL-23 plays critical role in amplification of myelin-specific Th17 cells which induce more severe form of EAE than IL-12-driven Th1 cells [98]. Treatment of mice with antibodies to IL-23 protected them from EAE [99]. IL-17 produced by Th17 cells has pro-inflammatory effect and is involved in tissue damage and autoimmune diseases [100]. Increased level of IL-17 in clinical samples are associated with RA, MS, inflammatory bowel disease, psoriasis and asthma [49]. Several lines of evidences confirm that Th17 cells are the main pathogenic cells in autoimmunity and systemic inflammatory diseases [73, 101-116]. The fact that Th1 cells can also transfer organ specific autoimmunity [117], the present consensus is that both Th1 and Th17 cells are involved in T cell mediated pathology. Th17 cells which are generated early are known to reach the site of inflammation and initiate migration of other inflammatory cells (example Th1) which propagates the tissue damage [118]. Cytokines produced by Th17 cells such as IL17A, IL17F, IL-21, IL-22 and GM-CSF are involved in recruitment of other inflammatory and effector immune cells at the site of inflammation thus leading to

tissue destruction [49, 119, 120]. Fate mapping studies in EAE revealed that Th17 are the main cells infiltrating CNS and subsequently produce IFNγ shutting off the production of IL17 [121]. Th17 express podoplanin which mediates formation of ectopic lymphoid structures which are the hallmarks of many chronic autoimmune and inflammatory conditions [122]. Altogether, Th17 cells are indispensible for induction of autoimmune and inflammatory diseases.

2.2. Dysregulated equilibrium between pathogenic and regulatory T cells leads to immune disease

IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) is a condition resulting from mutations in *foxp3* gene resulting dysfunctional Tregs [15]. Compromised functions of Tregs are known to be associated with many autoimmune diseases such as MS, autoimmune polyglandular syndrome type II, SLE, type 1 diabetes, psoriasis, myasthenia gravis, RA, and chronic ITP [66, 123-126]. Depletion of Tregs before and after induction of autoimmune disease leads to exaggerated disease with increased cellular and humoral responses [127]. Adoptive transfer of Tregs in the animal models of autoimmunity decreased severity of the disease [14, 18]. Recovery phase of many inflammatory diseases is associated with increase in number of Tregs in the target organs. Thus, Tregs actively regulate autoimmunity throughout the lifespan and are indispensible for maintenance of immune homeostasis [16]. Reprogramming of Tregs by pathogenic T cells and various inflammatory agents renders them less suppressive. Pathogenic Th17 cells are known to develop at the cost of Tregs under severe inflammatory situations of increased IL-6 [41, 128]. Thus, balance between

regulatory and effector T cells functions appears crucial for homeostasis and is intricately controlled by various pro and anti-inflammatory mediators [58, 73].

3. Intravenous Immunoglobulin

3.1. Composition, pharmacology and indications of IVIg

IVIg is a therapeutic concentrate of polyclonal IgG obtained from pools of plasma of a large number of healthy blood donors. Preparations of IVIg contain at least 96% of IgG with traces of IgA and IgM **(Table 2)**. The repertoire of IVIg is relatively wide as it obtained from large number of donors [129]. IVIg has a high content of self-reactive NAbs (Natural antibodies) which can bind to various self-antigens and pathogen specific antibodies [130]. Initially used as replacement therapy for patients with immune deficiencies, IVIg is now widely used for the treatment of a large number of autoimmune and systemic inflammatory diseases including ITP, neuromuscular and neuro-immunological diseases such as acute Guillain–Barré syndrome, myasthenia gravis, acute or chronic inflammatory demyelinating polyneuropathy, or stiff person syndrome [131] **(Table 3)**. IVIg is also proven valuable for refractory dermatomyositis or multifocal motor neuropathy. The efficacy of IVIg in relapsing–remitting multiple sclerosis (RRMS) is not as extensively documented as for other disease modifying drugs, but available data suggest its beneficial effects in this condition and IVg is used as second line drug for patients not responding or not supporting first-line treatment. [132]. Dose regimen of IVIg is according to the therapeutic goals, As a replacement therapy IVIg is used at 300-500 mg/kg body weight every 3-4 weeks. As an immunomodulatory/anti-inflammatory therapy it is used at 1-2 g/kg, administered at once or divided into 5 daily doses; additional maintenance dose at 4-6 week interval [133, 134]. IgG plasma concentration

of 12-14 mg/ml and 20-35 mg/ml is reached after replacement and high dose therapy, respectively [135].

Table 2 : Composition of different IVIg preparations

Company	CSL-Behring			Octapharma	LFB	Talecris		Baxter	
IVIg	Sandoglobuline	Sandoglobuline	Privigene	Octagam	Tegeline	Gamunex	Endobuline	Gammagard	Kiovig
Sizes Available	1,2,3,6,12 g	120 mg/ml	100 mg/ml	50 mg/ml	12 g	100 mg/ml	10 g	100 mg/ml	100 mg/ml
IgG	>96	>96	>95	>96	>97	>98	95	90	98
IgG1	65,2	64,5	67,8	65	58,8	50-80	56,9	56,9	56.9
IgG2	28,3	32,4	28,7	30	34,1	20-50	16	26,6	26,6
IgG3	4,15	2,3	2,3	3	5,4	-	3,3	3,4	3,4
IgG4	2,4	0,8	1,2	2	1,7	1,0-3,0	0,3	1,7	1,7
IgA	40g/g	15mg/L	25ug/ml	100ug/ml	17ug/ml	460mg/L	1mg/g	3ug/ml	140ug/ul
Aggregates	2	2	12	3	-	-	-	-	-
Excipient	Sucrose	Nicotinamide-Isoleucine - Proline	Proline	Maltose	Saccharose	Glycine	Glucose NaCl	Albumin, Glycine, NaCl, Glucose	Glycine

Adapted from Seite et al., 2008; Autoimmunity Rev [129]

Table 3: Clinical use of IVIg in autoimmune and inflammatory conditions

Licensed	Off-Label
Idiopathic thrombocytopenic purpura	Acquired immune thrombocytopenia
Guillain Barre syndrome (GBS)[a]	Autoimmune neutropenia
Chronic inflammatory demyelinating polyneuropathy (CIDP)	Autoimmune hemolytic anemia
Kawasaki Disease (KD)[a]	Parvovirus B19-associated red cell aplasia
Multifocal motor neuropathy (MMN)[a]	Anti-Factor VIII autoimmune disease
Birdshot retinochoroidopathy (BSRC)	Multiple sclerosis
	Myasthenia Gravis[a]
	Lambert Eaton myasthenic syndrome
	Stiff person syndrome
	ANCA-positive systemic vasculitis
	Polymyositis
	Dermatomyositis[a]
	Antiphospholipid antibody syndrome
	Rheumatoid arthritis and Felty's syndrome
	Systemic lupus erythematosus (SLE)
	Juvenile idiopathic arthritis (JIA)
	Toxic epidermal necrolysis (TEN)
	Autoimmune skin blistering diseases (BP, PF, PV)
	Steroid-dependent severe atopic dermatitis
	Graft versus host disease[a]
	Antibody-mediated rejection (AMR) of the graft
	Sepsis syndrome
	Steroid-dependent severe atopic dermatitis
	Asthma

a Indicates diseases in which evidence for the effects of IVIg has been obtained in controlled trials.

Adapted from Ravetch et al., 2008; Annu Rev Immunol [136]

3.2. Immunoregulatory mechanisms of IVIg in autoimmune and inflammatory diseases

Several mutually non-exclusive immunoregulatory effects of IVIg have been described that apparently contribute in synergy to an effective therapy in various clinical settings [137]. IVIg contains a wide range of anti-idiotypic antibodies that regulate autoreactive B-cell clones and neutralize pathogenic autoantibodies [134, 138, 139]. IVIg saturates the IgG transport receptor [6, 140], which leads to accelerated catabolism of pathogenic auto-antibodies and modulates the affinity of FcγR on phagocytic cells. Studies based on animal models of antibody mediated autoimmunity show that IVIg up-regulates inhibitory FcγRIIB on splenic macrophages [141]. FcγRIIB mediated beneficial effect of IVIg is known to be dependent on α2,6-linked of sialic acid to galactose on the glycan at Asn^{297} in the CH_2 region of Fc fragment small fraction of IgG (sIgG). IVIg contains a small fraction (1-2%) of sIgG in it. sIgG fraction of IVIg **or** α2,6 sialylated recombinant human IgG1 Fc protein could reproduce the benefits of IVIg when used at much lower dose [142]. sIgG has been demonstrated to interact with the C-type lectin receptor (SIGN-R1) on myeloid cells that up-regulates the expression of FcγRIIB on 'effector MΦ' via TH2 pathway [143].

IVIg attenuates complement-mediated damage by scavenging to the activated C3b fraction of C3 [144, 145]. The interaction of IVIg with complement proteins, therefore, prevents the generation of the C5b–9-membrane attack complex and subsequent complement-mediated tissue damage in muscle microvasculature and brain [145-147]. IVIg modulates cytokine and chemokine production by various cell types: decreased

levels of the pro-inflammatory cytokine IL-1 and increased levels of IL-1R antagonist have been reported in patients following IVIg infusion; TGF-α1 was down-regulated in the muscles of dermatomyositis patients who responded to IVIg therapy; Other studies have shown reduced synthesis of IL-2, IL-3, IL-12, IL-22, and GM-CSF following IVIg therapy [147-150]. IVIg contains antibodies that interact with cytokines and membrane molecules such as the T-cell receptor, cytokine or chemokine receptors, CD4, CD40 and CD95, which have important roles in the balance between auto-reactivity and tolerance [151, 152]. Further, IVIg is shown to exert an impact on the cellular compartment of the immune system; IVIg directly interacts cells of adoptive immunity like B cells, T cells and that of innate immunity and modulate their functions [153]. The maturation state of key APCs like DC is known to regulate immune response and tolerance. Immature and semi-mature DC presenting antigens are known to maintain tolerance by inducing Tregs while mature DC induces strong immune response [154]. IVIg inhibits maturation and function of DC, also modulates the pattern of cytokines secreted by these cells. By down-regulating the interferon-γ-mediated differentiation of DCs, and by inhibiting the uptake of nucleosomes, IVIg might exert an immunoregulatory effect in patients with lupus [155, 156]. In addition, IVIg-treated DC ameliorates ongoing autoimmune disease *in vivo* upon adoptive transfer [157]. IVIg also modulates *in vivo* and *in vitro* T-cell responses by impairing antigen presentation [158].

3.3. Modulation of the Tregs by IVIg

The immune system is subject to multiple regulatory mechanisms to control undesired pathogenic immune response to self-antigens and inflammation. One of these regulatory mechanisms involves suppression of auto-reactive T cells by Treg. Treg express GITR, CCR4, CD62L, CTLA-4 and lineage-specific transcription factor Foxp3 [15, 67]. Treg actively regulate autoimmunity throughout an individual's life and an imbalance in their immune regulation might lead to autoimmunity. Maintenance of immune tolerance by Treg is not attributed to a single mechanism or target cell; rather it involves several pathways targeting multiple cell types. Thus, Treg inhibit the proliferation and cytokine production by conventional T cells and can also regulate the functions of natural killer cells, NKT cells and professional APC. The suppression of immune responses by Treg generally requires direct cell-cell contact implicating CTLA-4, CD39 and LAG-3, but soluble factors, particularly TGF-β and IL-10, have also been implicated. Ability of IVIg to modulate the functions of DC has opened the possibility that the tolerogenic effects of IVIg may implicate the Treg in correcting autoimmunity. Further, the FcγR-mediated effects of IVIg cannot entirely account for its benefit in a number of peripheral and central demyelinating diseases where auto-reactive T cells play critical role. Since the expression of FcγR on T cells has not been established unequivocally, the observed beneficial effects raise certain speculations, that is, if these effects could be attributed to a direct interaction of IgGs with T cells or as mentioned above, an indirect influence via DC. Indeed IVIg manifests its protective effect in T-cell-dependent pathologies via an early modulation of auto-reactive T cells. EAE is a

CD4+T-cell-mediated autoimmune disease affecting the CNS [159]. The prophylactic infusion of IVIg prevents the development of EAE [160-165], and this protection conferred by IVIg is associated with a peripheral expansion of CD4+CD25+Foxp3+Treg and amelioration of their functions [165].

The IVIg-expanded Treg were more efficient in suppressing the in vitro response of TCR-stimulated CD4+CD25-T cells as compared to Treg from control group; in adoptive transfer experiments, mice that were reconstituted with Treg from IVIg-treated mice developed milder EAE as compared to non-reconstituted mice; IVIg failed to protect against EAE in mice that were depleted of the Treg [165]. Expansion of Treg by IVIg, described above, suggests that IVIg imposes immune tolerance via modulation of T-cell subsets, in particular, the CD4+CD25+Foxp3+Treg compartment [166]. However, the identification of Th17 cell subset has raised more questions on the mechanisms of action of IVIg in inflammatory and autoimmune conditions. IVIg inhibits *in vitro*, differentiation and amplification of human Th17 cells [167]. In view of the critical role played by Th17 cells in inflammatory and autoimmune processes, an important question concerns the effect of IVIg on the differentiation and function of Th17 population *in vivo*.

4 Multiple sclerosis / EAE is an organ specific T cell mediated auto-immune disease

Multiple sclerosis (MS) is a demyelinating disease of central nervous system affecting more than 2.5 million people worldwide [168]. MS predominately affects younger women and depending on the region, incidence may rise up to 3 in 1000. MS occurs in various forms like benign, relapsing/remitting, secondary progressive and primary progressive. Perturbation in sensation, motor, autonomic, visual and cognitive systems are the main features of MS in addition to optical neuritis. MS is the primary inflammatory disease of the CNS, characterized by perivascular inflammation and massive leukocyte infiltration leading to axonal loss. Animal model for MS is experimental autoimmune encephalomyelitis (EAE). EAE can be induced in a various species of experimental animals by injection of a myelin peptide emulsified in complete Freund's adjuvant (CFA) subcutaneously [169].

The pathophysiology of MS is complex and heterogeneous; however some aspects of it are being understood recently. Several lines of evidence suggest that MS has an autoimmune etiology [170, 171]. T lymphocytes and antibodies reactive to myelin were found in the lesion in MS patients [172]. These T cells are mainly $CD4^+$ and specific for the various myelin proteins such as MBP (myelin basic protein), MOG (myelin oligodendrocyte glycoprotein), PLP (myelin proteolipid) and MAG (myelin-associated glycoprotein). Pathogen-associated proteins, mainly from hepatitis B virus (HBV) resemble myelin proteins and are antigenic suggesting that, molecular mimicry may be the underlying cause for onset [173]. Further, adoptive transfer of T cells specific for MBP or CNS antigens in the experimental animals leads to MS like

syndrome is the proof that MS is T cell mediated pathology [174].

Naïve Th cells reactive to various epitopes of MBP presented in the context of MHC-II by APCs are activated in the periphery. These activated cells migrate to CNS using adhesion molecules like LFA-1 and VLA-4 [175]. Secondary wave of T cell activation and amplification in the CNS is mediated by resident APCs in the CNS [176]. Initially, Th1 cells were thought to be pathogenic in MS. However, recent studies exploring the functions of IL-12 and IL-23 revealed that Th17 cells are the essential lymphocytes in pathogenesis of MS [95]. Th17 cells secrete IL-17 family cytokines like IL-17A, IL-17F and GM-CSF [49, 120]. These cytokines are key players in initiating early events of demyelination. Additionally Th17 cells express podoplanin, which mediates formation of ectopic lymphoid structures in CNS thus resulting in to amplified immune response. The released inflammatory cytokines IFN-γ, IL-23 and TNF-α activate microglia and astrocytes. Chemokines like RANTES, IL-8 recruit other immune cells such as monocytes, $CD8^+$ T cells and B cells from blood.

Mechanisms of demyelination include direct deposition of complement, antibody dependent cellular cytotoxicity, phagocytosis and probably progression to direct attack of axons by cytotoxic T cells, secretion of proteases by neutrophils and apoptosis of oligodendrocytes [177]. The inflammation lasts from few days to two weeks in case of EAE and longer in chronic form of MS. After the attack phase of the CNS, demyelinated axons and apoptotic oligodendrocytes and T lymphocytes were observed [178]. During the resolving phase of inflammation astrocytes proliferate and there is shift to Th2 cytokine profile including IL-10 and TGF-β [58]. Increase in Tregs number in the CNS is another hallmark of resolving inflammation [82].

5 Hypothesis and aims

Several lines of evidence show that Th17 cells might be the potent inducers and sustainers of inflammation and play critical role in the pathogenesis of autoimmune and inflammatory diseases. Cytokines produced by Th17 cells such as IL17A, IL17F, IL-21, IL-22 and GM-CSF are involved in recruitment of other inflammatory and effector immune cells at the site of inflammation thus leading to tissue destruction. Tregs on the other hand actively regulate autoimmunity throughout the lifespan of an individual and are indispensible for maintenance of immune homeostasis. Many autoimmune diseases are associated with reduced numbers of Tregs or defects in their functions. Thus, the balance between effector Th cells (Th17) and Treg cells might critically influence the outcomes of many human immune mediated diseases.

IVIg is a therapeutic preparation of normal human polyclonal IgG obtained from pools of plasma from a large number of healthy blood donors. High dose therapy of IVIg is being widely used to treat various autoimmune and inflammatory conditions. Understanding the molecular mechanisms by which IVIg exerts its beneficial effects has been a challenge. IVIg is known to exert its beneficial effects by several mutually non-exclusive mechanisms. Many of these are based on animal models of antibody mediated autoimmunity and *in-vitro* studies. Nevertheless, IVIg is also widely used in much T cell-mediated autoimmune pathologies. The beneficial effect of IVIg in EAE, an animal model of T mediated pathology, is associated with a peripheral expansion of $CD4^{+}CD25^{+}Foxp3^{+}$Treg and amelioration of their functions. The emerging knowledge on Th17 cells as potent pathogenic T cells in autoimmunity and Th17 and Treg exercise

reciprocal regulatory effects has raised additional interesting questions on the mechanisms of IVIg *in vivo*. Although, IVIg is shown to interact directly with T cells and inhibit differentiation and amplification of human Th17 cells *in vitro,* effect of IVIg on differentiation of Th17 cells *in vivo* remains unexplored. I therefore, hypothesise that IVIg inhibits the Th17 cell development and regulates the balance between Th17 and regulatory T cells *in vivo*. I have studied this hypothesis with the following objectives:

- Extend the knowledge on the protective effect of IVIg in a T cell-mediated pathology
- Understand whether beneficial effect of IVIg in EAE is associated with modulation of Th17 cells along with Th1, Th2 and Tregs
- Investigate the molecular mechanisms involved in modulation of T cells by IVIg *in vivo* in an animal model of T cell mediated autoimmunity

I used active EAE as *in vivo* model of autoimmune disease to accomplish the objectives. EAE was induced in mice by injecting MOG_{35-55} peptide emulsified in CFA with additional PTX. Effect of IVIg on frequency of Th1, Th17, Th2 and Tregs in EAE was measured by using cell specific markers Th1: IFN γ; Th17: IL-17; Th2: IL-4 and Tregs: Foxp3.

Results

6 Results

Intravenous gammaglobulin reciprocally regulates effector and regulatory CD4 T cell functions *in vivo* independent of FcγRIIB

(In communication)

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Abstract

Despite an increasing use of high dose therapy of intravenous gammaglobulin (IVIg) in the treatment of T-cell and autoantibody-mediated inflammatory and autoimmune diseases, comprehension of the mechanisms underlying its therapeutic benefit has remained a major challenge. Using actively-induced experimental autoimmune encephalomyelitis (EAE) model, a T cell-mediated autoimmune condition, we demonstrate that IVIg inhibits the differentiation of naïve CD4 T cells into effector subsets (Th1 and Th17 cells) and concomitantly induces an expansion of Foxp3$^+$ regulatory cells. Further, IVIg renders effector T cells less pathogenic by decreasing the expression of encephalitogenic molecular players like GM-CSF and podoplanin. IVIg decreases the expression of sphingosine-1 phosphate receptor (S1P1) on CD4 cells, thus sequestering these cells in the draining lymph nodes and decreasing infiltration of Th1, Th17 and Treg to the central nervous system. Intriguingly and contrary to the current arguments, the inhibitory FcγRIIB is dispensable for IVIg-mediated reciprocal modulation of effector and regulatory CD4 subsets *in vivo.* Additionally, F(ab')$_2$ part of IVIg also retained this function of IVIg. IVIg or F(ab')$_2$ fragments decreases the activity of mTOR kinase thus restoring the equilibrium between regulatory T cells and inflammatory CD4 T cell subsets. Together, our results provide cellular and molecular basis underlying the beneficial effect of IVIg in certain autoimmune and inflammatory conditions.

INTRODUCTION

High dose therapy of IVIg is being widely used to treat various autoimmune and inflammatory conditions[1-3]. IVIg is a therapeutic preparation of normal human polyclonal IgG obtained from pools of plasma from a large number of healthy blood donors[4]. Understanding the cellular and molecular mechanisms by which IVIg exerts its anti-inflammatory effects in highly diverse pathological situations incriminating autoantibodies, pathogenic T cells, complement-mediated tissue damage or dysregulated cytokine network has rendered the area particularly challenging. Indeed, IVIg exercises a therapeutic effect in idiopathic thrombocytopenic purpura, Kawasaki disease, myasthenia gravis, dermatomyositis, pemphigus, anti-neutrophil cytoplasmic antibody–associated vasculitis and a number of other diseaseses[5].

IVIg exerts its beneficial effects by several mutually non-exclusive mechanisms[6-10]. Many of these effects are deduced based on animal models of antibody-mediated autoimmunity and *ex vivo* studies[11]. IVIg impacts significantly on both innate and adaptive immune compartments[7]. More recently, it is shown that IVIg up-regulates inhibitory FcγRIIB on macrophages through a Th2 pathway in a K/BxN serum transfer arthritis model[12]. However, therapeutic benefit of IVIg is also clearly established in several T cell-mediated autoimmune pathologies[2,13-15]. The beneficial effect of IVIg in EAE, an animal model of multiple sclerosis and a T cell-mediated pathology, is associated with a peripheral expansion of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Treg)

and a significant amelioration of their functions[16]. Further, IVIg interacts directly with T cells and inhibits differentiation and amplification of human Th17 cells *in vitro*[17]. Hence, emerging knowledge that Th17 cells as potent pathogenic T cells in autoimmunity and that Th17 and Treg exercise reciprocal regulatory effects[18], have raised additional interesting possibilities on the mechanisms of IVIg *in vivo*.

We surmised that IVIg restores the dysregulated equilibrium between Th17 cells and Treg in T cell-mediated pathologies. In the present study using actively-induced EAE, we thus set out to investigate whether IVIg modulates T cell polarization *in vivo* and tilts the balance from pathogenic effector T (T_{eff}) cells towards Treg. We found that IVIg exerted its beneficial effect in EAE by inhibiting the differentiation of Th17 and Th1 cells and simultaneously increasing Treg. Further, IVIg inhibited encephalitogenic potential of pathogenic T cells and interfered with their trafficking to the target organ. Interestingly, FcγRIIB was dispensable for this effect and $F(ab')_2$ fragments of IVIg recapitulated the effect of intact IgG molecules in reciprocally modulating T_{eff} cells and Treg. This reciprocal regulation involved modulation mammalian target of rapamycin (mTOR) kinase in CD4 cells by IVIg.

RESULTS

IVIg delays the onset of EAE and decreases severity of the disease by inhibiting Th17 and Th1 cells and increasing Treg

To understand the modulation of effector and regulatory CD4 subsets by IVIg in an autoimmune set up, EAE was induced in WT C57BL/6J mice using MOG_{35-55} emulsified in CFA. From the day of immunization to the peak of the disease (day16-18), mice in the control and IVIg group were treated with 0.2M Glycine and IVIg (Gamunex ®) respectively. Control mice started to display clinical signs from day 7 and mean score at the peak was 3.5. IVIg significantly delayed the onset of EAE (day 11) and decreased severity of the disease as shown by clinical signs **(Fig. 1a)**. MOG_{35-55}-specific naïve CD4 cells differentiating into Th17 and Th1 cells are known to be the pathogenic in EAE[19,20]. To investigate whether IVIg affects differentiation of Th17 and Th1 cells in EAE, mice in each group were sacrificed 9 days after EAE induction (onset) and analyzed for their signature cytokines (IL-17 in Th17; IFNγ in Th1) by flow cytometry. We observed decrease in Th17 and Th1 cells in inguinal draining lymph nodes (DLN) of IVIg-treated mice (**Fig. 1b, top panel and 1c**). Similar trend was also observed in spleens of IVIg-mice (**Fig. 1b, lower panel and 1d**). Inhibition of Th17 and Th1 cells by IVIg *in vivo* was further confirmed by the profile of cytokine secretion. Cells from DLN and spleen on day 9 were stimulated *ex vivo* with MOG_{35-55} for 24 hours and cell-free supernatants were analyzed for cytokines. Cells from IVIg-treated mice secreted decreased amounts of IL-17 and IFNγ (**Supplementary Fig. 1a, b**) as compared to control. IVIg did not affect the $CD4^+Foxp3^+$ Treg in DLN (**Supplementary Fig. 2a, b**). However, we observed concomitant increase in $CD4^+Foxp3^+$ Treg in the spleens of IVIg-treated mice (**Supplementary Fig. 2c, d**), which is in consistent with our previous report[16]. Thus, IVIg reciprocally modulates T_{eff} and Treg in EAE.

IVIg decreases infiltration of lymphocytes to the CNS by inhibiting their egress from the DLN

Protective effect of IVIg treatment in EAE is associated with decreased number of lymphocytes and absence of inflammatory foci in the CNS[14,16]. It has been previously shown that IVIg interferes with leucocyte recruitment to the CNS in a α4-integrin-dependent manner[21]. However, Th17 and Th1 cells use different strategies to invade CNS[22,23]. To investigate the effect of IVIg on trafficking of Th17 and Th1 in EAE, brain and spinal cords were analyzed on the day of onset (Day 9). IVIg inhibited infiltration of Th1, Th17 and Treg into the CNS (**Fig. 2a-c**). Accordingly, there was an overall decrease in absolute number of CD4 cells found in the CNS (**Fig. 2d**). Surprisingly, DLN of IVIg-treated EAE mice were bigger in size and contained two to five-fold more number of CD4 cells than the untreated EAE mice **(Fig. 2e**). We also observed decrease in total number of CD4 cells circulating in the blood **(Fig. 2f)**. These data suggested that, T cell entry into DLN of IVIg-treated EAE mice was intact; however, their exit from DLN was affected. To further probe into the molecular mechanisms involved in sequestering of CD4 cells into the DLN, we investigated sphingosine 1 phosphate (S1P)–S1P receptor1 (S1P1) axis, which controls the trafficking and migration of lymphocytes[24,25]. IVIg treatment in EAE mice for 6 days decreased $CD4^+S1P1^+$ cells in the DLN (**Fig. 2g, h**) and MFI of S1P1 on CD4 cells (**Fig. 2i**). These results suggest that, IVIg down-regulates S1P1 on CD4 cells leading to inhibition of their egress from DLN, thus explaining the increase in the size of the DLN and decrease in infiltration of lymphocytes to CNS.

IVIg down-regulates the expression of GM-CSF and podoplanin (PdP) in CD4 T cells

Both Th17 and Th1 cells are involved in EAE, however Th17 cells have emerged as the main pathogenic mediators[26]. Signature cytokines of Th17 cells like IL-17A, IL-17F, IL-21 and IL-22 are dispensable for the induction of EAE[27]. Encephalitogenic potential of Th17 cells in EAE has been attributed to molecules like GM-CSF[28] and podoplanin[29]. Injected to EAE mice, IVIg significantly decreased $CD4^+GM\text{-}CSF^+$ cells in DLN (**Fig. 3a, b**). Additionally, there was a decrease in the absolute number of these cells in CNS (**Fig. 3c**). Similarly IVIg also down-regulated the expression of podoplanin on CD4 cells in EAE (**Fig. 3d-f**). Thus, in addition to inhibiting the differentiation of Th17 cells, by down-regulating GM-CSF and PdP, IVIg may render these cells less encephalitogenic.

FcγRIIB is dispensable for IVIg-mediated reciprocal modulation of effector and regulatory CD4 subsets *in vivo*.

Anti-inflammatory effect of IVIg in several animal models of antibody-mediated pathology is attributed to the inhibitory Fc receptor FcγRIIB[12,30-32]. The role of FcγRIIB in protection of EAE mice by IVIg is however unexplored. To examine whether reciprocal modulation of CD4 subsets by IVIg in EAE is dependent on FcγRIIB, we induced EAE in $FcγRIIB^{-/-}$ mice under C57BL/6J background and analyzed various CD4 subsets 9 days after immunization. IVIg inhibited Th17 and Th1 cells in $FcγRIIB^{-/-}$-EAE mice (**Fig. 4a,**

b). Concomitantly, we observed an increase in the percent of Treg in the spleen (**Fig. 4c**). There was a decreased infiltration of Th17, Th1 and Treg to CNS (**Fig. 4d-f**), which was due to inhibition of trafficking of CD4 cells from the DLN by IVIg in FcγRIIB$^{-/-}$-EAE mice (**Fig 4g, h**). Further, IVIg treatment decreased the expression of GM-CSF and podoplanin in FcγRIIB $^{-/-}$ mice rendering them less encephalitogenic (**Fig 4i, j**). Altogether, these data reveal that FcγRIIB is dispensable for the reciprocal modulation of effector and regulatory CD4 subsets by IVIg in EAE.

F(ab)'$_2$ part of IVIg recapitulates the capacity to reciprocally modulate effector and regulatory CD4 subsets in EAE

As Fc part of IVIg is implicated in the FcγRIIB-dependent functions of IVIg, our results on dispensable role of FcγRIIB on IVIg-mediated regulation of T cell populations raises a possibility that F(ab')$_2$ part of IgG molecule should be equally effective as that of intact IgG. To explore this, EAE mice were treated with IVIg or equimolar concentration of F(ab')$_2$ fragments. Mice were sacrificed on day 9 and analyzed for CD4 T cell populations by flow cytometry. Interestingly, F(ab')$_2$ also inhibited Th17 and Th1 cells, and enhanced the number of Treg similar to intact IVIg (**Fig. 5a-c**). MOG$_{35-55}$ specific cytokine secretion assay revealed decreased amounts of IL-17 and IFNγ from F(ab)'$_2$ or IVIg-treated mice (**Supplementary Fig. 3a, b**). This is in consensus with previous reports from our *in vitro* results[17]. Similar to IVIg, F(ab')$_2$ retained the ability to inhibit infiltration of Th1, Th17 and Treg to the CNS (**Fig. 5d-f**). Modulation of lymphocyte trafficking and sequestration of CD4 cells in DLN was also consistent in F(ab')$_2$-treated EAE mice (**Fig. 5g, h**). Further, F(ab')$_2$ treatment also decreased the expression of GM-CSF and podoplanin in CD4 cells (**Fig. 5i, j**). These results demonstrate that *in vivo* reciprocal modulation of CD4 subsets by IVIg is F(ab')$_2$-dependent. These results in addition to the above-mentioned dispensable nature of inhibitory FcγRIIB for the modulatory effect of IVIg in EAE, precluded us from examining the direct role of Fc fragments.

Reciprocal regulation of T effectors and Treg by IVIg implicate mTOR kinase pathway

The differentiation of naïve CD4 cells into specific Th cell type involves integration of various environmental cues delivered by antigen presenting cells (APCs) in the secondary lymphoid organs[33,34]. mTOR integrates these signals and controls T_{eff} and Treg responses[35,36]. To investigate whether IVIg-mediated reciprocal modulation of effector and regulatory cells involves mTOR pathway, mice were sacrificed 6 days after immunization. We analyzed the activity of mTOR in CD4 cells from DLN by estimating the level of phospho-S6 ribosomal protein (ser240/244)[37]. CD4 cells from IVIg-treated mice displayed reduced phosphorylation of S-6 ribosomal protein as compared to the control (**Fig. 6a**). IVIg-treatment decreased the activity mTOR in both T_{eff} and Treg (**Fig. 6b**). These data suggest that by inhibiting activity of mTOR kinase in CD4 cells, IVIg can decrease the differentiation of naïve CD4 cells to effector subsets while simultaneously increasing the number of Treg.

DISCUSSION

Activation of naïve CD4 T cells and their differentiation into various subsets is the crucial event in the initiation of an adaptive immune response. Dysregulated functions of immune cells leads to undesired pathogenic immune response to self-antigens and inflammatory pathologies[38]. Several lines of evidence suggest that Th1 and Th17 play critical role in the initiation and progression of many autoimmune and inflammatory diseases. On the other hand, Treg expressing the transcription factor Foxp3 are involved in the suppression of auto-reactive T cells and regulation of immune response[39]. Several autoimmune diseases are associated with reduced number of Treg or defects in their functions[40-43]. Thus, balance between Treg and T_{eff} including Th17 and Th1 determines the course of immune-mediated disorders[44]. Th17 are the one of the major pathogenic cells in immune-mediated tissue damages such as multiple sclerosis[45], chronic inflammatory bowel disease[46], psoriasis[47], SLE[48], asthma, allergic contact dermatitis, dermatomyositis, pemphigus, allergic rhinitis, anti-neutrophil cytoplasmic antibody–associated vasculitis and rheumatoid arthritis[49]. Although IVIg is beneficial in the treatment of several of these complications[50], the precise mechanisms governing the Th cell polarization and the balance between Treg and Th17 cells by IVIg have not been identified. We demonstrate that administration of IVIg to EAE mice significantly reduces the severity of disease by inhibiting differentiation of Th17 and Th1 cells. . Further, *ex-vivo* stimulation of the lymphocytes with MOG_{35-55} showed decreased secretion of IL-17 and IFNγ. In consensus with our previous results, we observed an expansion of Treg[16]. Thus, the protective effect of IVIg in EAE underscores the reciprocal modulation of effector CD4 cells (Th1 and Th17) and Treg.

In addition, IVIg decreases the expression of GM-CSF and podoplanin in CD4 T cells. GM-CSF produced by Th17 cells potentiates neuroinflammation by attracting myeloid cells to the CNS and up-regulating their surface MHCII[28]. Podoplanin expressed on Th17 cells is involved in the formation of deleterious ectopic lymphoid structures in CNS[29,51]. By reducing the expression of these molecules in CD4 T cells, IVIg may thus inhibit the formation of inflammatory foci. The relevance of these findings in other autoimmune conditions and in patients treated with IVIg needs to be examined. Furthermore, IVIg effectively inhibited the infiltration of Th1, Th17 and Treg to CNS[14,16,21]. We observed an increased accumulation of CD4 cells in the DLN by IVIg and their concomitant decrease in circulation. We provide mechanistic evidence for this interference of lymphocyte trafficking. Differentiated T cells exit from the DLN by using a gradient of S1P across the lymphoid tissue, lymph and blood through S1P1[24,25]. By down regulating S1P1 on CD4 cells, IVIg inhibits the lymphocyte egress leading to sequestration of these cells in the DLN. We did not observe any changes in spleen with respect to total number of CD4 cells (data not shown), this is similar to the effect of FTY720, a known modulator of S1P1-S1P axis of lymphocyte trafficking[52]. However, the role of IVIg in modulating the levels of circulating S1P warrants further investigation.

Up-regulation of inhibitory FcγRIIB is implicated in the beneficial effect of IVIg[30,31]. Using EAE, we show that IVIg is able to inhibit Th17 and Th1 cells independent of FcγRIIB. Additionally, increase of Treg, inhibition of GM-CSF and

podoplanin and modulation of CD4 T cell trafficking by IVIg does not require FcγRIIB. We also report that $F(ab')_2$ fragments of IVIg retained the capacity to reciprocally regulate CD4 subsets similar to intact IVIg. These results also indicate that α2,6 sialylation on Fc portion of IgG is not implicated in the modulation of CD4 T cells by IVIg in actively-induced T-cell mediated autoimmune and inflammatory conditions. However, exploring the role of SIGNR1 in this regulatory shift of CD4 subsets by IVIg is an interesting perspective.

The complex process of T cell activation and differentiation *in vivo* is regulated by multiple cues in the microenvironment of secondary lymphoid organs. mTOR is a kinase that integrates these signals and plays a critical role in the T_{eff} and Treg responses[53,54]. In view of the fact that IVIg can directly interact with T cells[17] and influence the TCR signaling[55], we reasoned that reciprocal modulation of T cell responses by IVIg may involve mTOR signaling pathway. Accordingly, we observed a decreased activity of mTOR kinase in both conventional T cells and Treg. By inhibiting mTOR signaling, IVIg may inhibit the differentiation of effector cells (Th17 and Th1) while favoring the Treg, although the underlying mechanisms remain unclear. Whether Treg activating Tregitopes in IVIg play any role in the reciprocal regulation of effector and Treg remains speculative[56]. Additionally, sequestering of CD4 cells in DLN by IVIg may be a consequence of affecting mTOR-pathway as S1P-mTOR axis is implicated in lymphocyte trafficking[57].

Pathogenesis of EAE involves activation and differentiation of naive neuroantigen-specific CD4 cells into Th17 and Th1 cells in the secondary lymphoid organs. These pathogenic T cells migrate to the CNS and potentiate axonal destruction by facilitating infiltration of other myeloid effector cells through GM-CSF and formation of ectopic lymphoid structures mediated by podoplanin. Our results show that IVIg-mediated inhibition of EAE implicates multiple targets acting at different phases of immune response. Thus, IVIg can inhibit the initiation of pathogenic immune response by inhibiting the polarization of naïve T cell into Th17 and Th1 cells. This process is associated with a concomitant expansion Tregs *in vivo.* IVIg can circumvent neuronal degeneration by inhibiting the infiltration of CD4 T lymphocytes to the target organ by restraining their exit from the DLN through S1P-S1P1 pathway. Further, IVIg may decrease the tissue damaging potential of pathogenic T cells by down regulating key molecules such as GM-CSF and podoplanin. Together, our results provide a cellular and molecular basis underlying the beneficial effect of IVIg in certain T cell-dependent autoimmune and inflammatory conditions.

EXPERIMENTAL PROCEDURES

Animals

All animal studies were performed according to the guidelines of Charles Darwin ethical committee for animal experimentation (UPMC Paris) at the pathogen-free animal facility of Cordelier research center, Paris. FcγRIIB$^{-/-}$ mice (8 weeks old) on C57BL/6

background were obtained from Taconic Farms. Wild type C57BL/6J mice were purchased from Janvier Laboratories, France.

Induction of EAE, assessment and IVIg treatment

EAE was induced in 10 week old female C57BL/6J or FcγRIIB$^{-/-}$ mice as previously reported [16]. Briefly, 200 µl of emulsion was injected subcutaneously at 2 sites over the flank region. Each mouse received 200 µg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK PolyPeptide laboratory Strasbourg, France) emulsified in Complete Freund's Adjuvant (Sigma-Aldrich) containing 880 µg of nonviable *Mycobacterium tuberculosis* H37RA (Difco Laboratories). Additionally 300 ng of pertussis toxin (List Biologic Laboratories) was injected intravenously on the day of immunization and 48hr later. Mice were daily assessed for the development of clinical signs according to the following scoring pattern: 0-No signs, 1-tail paresis, 2-hindlimb paresis, 3- hind limb paralysis, 4 – tetraplegia, 5-moribound. IVIg (Gamunex® 10% w/v, Talecris Biotherapeutics) was given at 0.8 g/kg i.p from the day of the immunization until the peak of the disease (day 16-18). Mice in $F(ab')_2$ group received 0.55 g/kg BW (equimolar concentration). Control mice received equal volumes 0.2 M Glycine (excipient used in Gamunex®).

Generation of $F(ab')_2$ fragments

$F(ab')_2$ fragments were generated by digesting IVIg with pepsin (Sigma Aldrich) at 50:1 ratio for 18hrs in 0.2 M sodium acetate buffer pH 4.1. $F(ab')_2$ was extensively dialyzed against sterile PBS and filtered through 0.22 µ membrane before injecting to the mice. Purity of $F(ab')_2$ was verified by SDS-PAGE and coomassie blue staining.

Isolation of cells from blood, spleen, draining lymph nodes and CNS

On the day of the sacrifice, blood was collected from mice under Xylazinane / Ketamine anesthesia. Mice were perfused with 40 ml of 0.2 mM EDTA in PBS through the left ventricle and spleen, draining lymph nodes (inguinal), brain and spinal cord were collected. Single cell suspensions were obtained by mechanical disaggregation and passing the cells through 70 µm nylon membrane filter. Mononuclear cells from CNS were isolated using 37.5% Percoll gradient centrifugation (GE health care). Red blood cells were lysed using ACK lysis buffer.

Flow cytometry

To detect the intracellular cytokines, 1.5×10^6 cells were stimulated in with 25 ng of phorbol 12-myristate 13-acetate (PMA) and 1 µg ionomycin (Sigma) in 10% FCS/RPMI for 4 hours at 37 °C. Monensin (Golgistop® BD biosciences) was added according to manufacturer's instructions to block the protein transport. Cells were surface labeled with anti-mouse CD4-Paciifc Blue (Clone RM4-5, BD biosciences), podoplanin-PE (Clone 8.1.1 Biolegend) and S1P1- APC (R&D Systems) antibody after the blocking Fc-receptors with anti-mouse CD16/32 antibody (BD Fc Block). Surface

stained cells were washed, fixed, permiabalised using FoxP3 staining buffer set (eBioscience). Antibodies to detect intracellular cytokines, IL-17A-A488 (clone 11B11, BD), IFNγ-APC (Clone XMG1.2, BD), Foxp3-PE (clone FJK16s, eBioscience), GM-CSF PE (Clone: MP1-22E9 eBioscience), and phospho-S6-A488 (Cell signaling Technology) were used in permeabilization buffer (eBioscience). Cells were acquired and analyzed using BD LSR II and FACS Diva software.

Ex-vivo stimulation of cells and cytokines assays

Single cell suspension was prepared from DLN and spleen was stimulated with 10 µg/ml of MOG_{35-55} for 24 hour in RPMI-1640 culture medium supplemented with 100 IU/ml penicillin, 100 ug/ml streptomycin (Gibco) and 10% fetal calf serum. Cytokine concentration in the supernatant and serum was estimated by using cytometric bead array (Mouse Th1/Th2/Th17 cytokine CBA kit; BD Biosciences).

Statistical analysis

Two way analysis of variance (ANOVA) with Bonferroni's post-test was used to analyze daily clinical score. Mann–Whitney's U test was used to compare parameters between control and IVIg group. Values of p obtained are indicated in Figure legends. Graph-pad prism was used to analyze and plot the data.

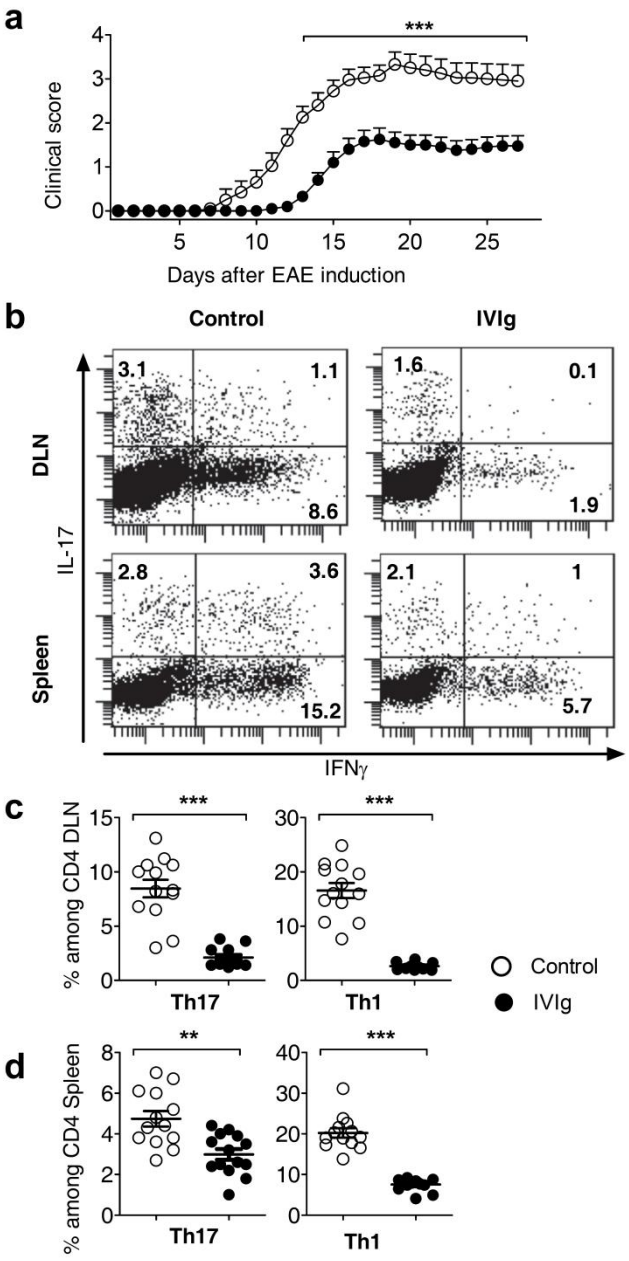


Figure 1 IVIg protects mice against EAE by decreasing Th17 and Th1 cells. (a) IVIg delays onset and decreases severity of EAE: EAE was induced in 10-12 week female Wt C57BL/6J mice. From day 0 to day 18, mice in control (open circles) and IVIg (filled circles) group received intraperitonial injections of 0.2 M Glycine and IVIg (0.8 g/kg Gamunex®) respectively. Development of clinical signs was monitored daily as described in "Experimental procedures". Mean clinical scores from two independent experiments are presented (n=20). Error bars represent SEM. Significance of difference in the EAE scores between the control and IVIg groups was analyzed by Two way ANOVA with Bonferroni's post t test, ***p<0.001. (b) Representative dot plots showing CD4 cells which are positive for IL-17 (Y-axis) and IFNγ (X-axis). Number in each quadrant represents the percent of cells among CD4 population. Mice were sacrificed on the day of onset (day 9). DLN and Spleen were obtained for flow cytometry. DLN (Inguinal, upper panel) and spleen (bottom panel) from control (left) and IVIg (right) mice are shown. (c) And (d) Plots show frequency of Th17 (Left) and Th1 (right) from control (Open circles) and IVIg (filled circles) group. Cells from DLN (c) and spleen (d) are shown. Data are from two in independent experiments (n>12). Mean value is depicted as a horizontal line among the symbols and error bars represent SEM, **p<0.01 and ***p< 0.001 determined by Mann Whitney U test.

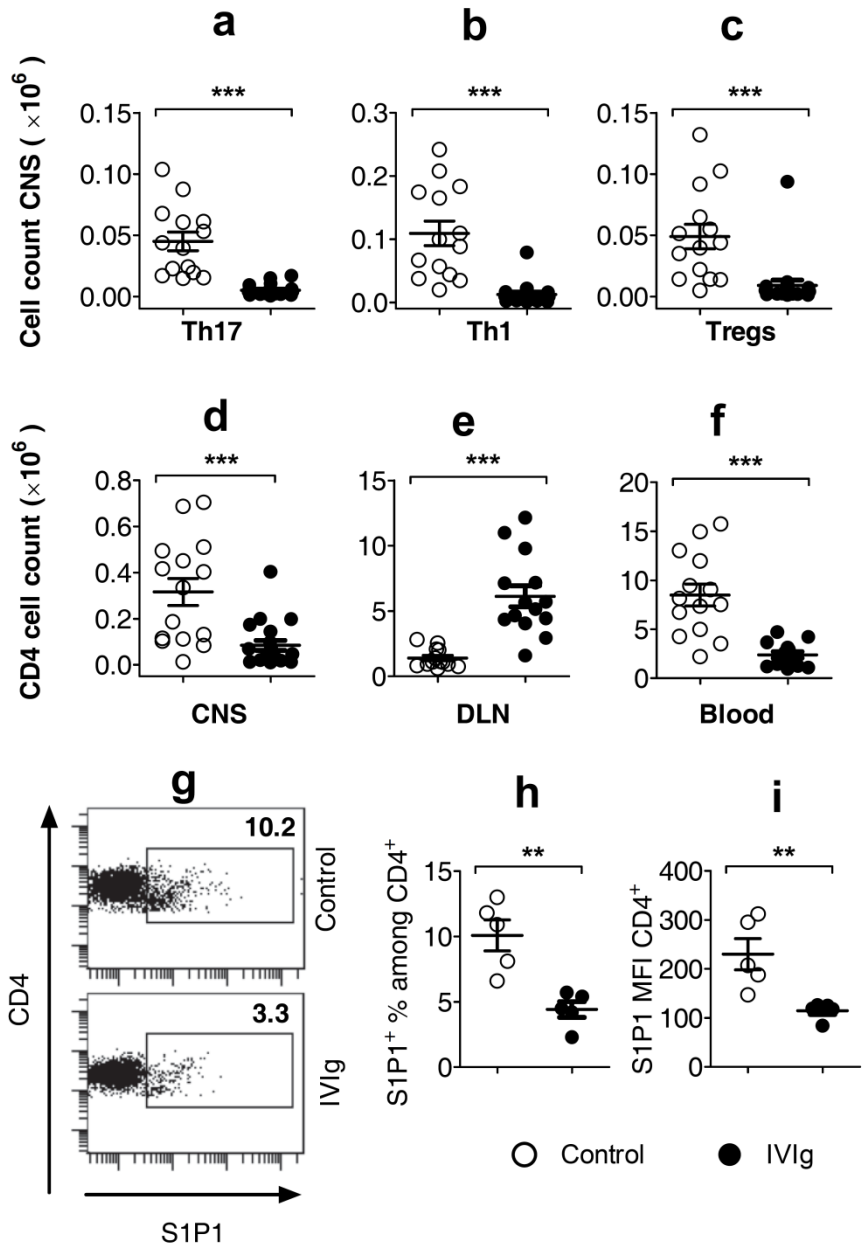


Figure 2 IVIg decreases infiltration of CD4 lymphocytes to the CNS by inhibiting their egress from DLN. Control and IVIg treated EAE mice were sacrificed on day 9. To analyze the total number of circulating cells in the blood, mice were infused with PBS/EDTA and cells were collected. Cells from Blood, DLN, spleen and CNS were analyzed by flow cytometry. Absolute numbers of each type of cells in various organs were estimated by extrapolating data from flow cytometry. Open circles represent control mice and filled circles represent IVIg treated mice. Mean value is depicted as a horizontal line among the symbols and error bars represent SEM. Plots show total number of Th17 (a), Th1 (b) and Treg (c) in CNS (n>10). Absolute number of CD4 cells in CNS (d), in DLN (e), and in blood (f), (n>10) are plotted. (g) Representative dot plots from DLN showing CD4 (Y-axis) and S1P1 (X-axis) from control (top) and IVIg group (bottom). Numbers represents the percent of $S1P1^+$ cells gated among the CD4 population. Percent of $CD4^+$ $S1P1^+$ (h), MFI of S1P1 receptor on CD4 cells in DLN (i) are shown (n=5), ** p<0.01, ***p< 0.001 determined by Mann Whitney U test.

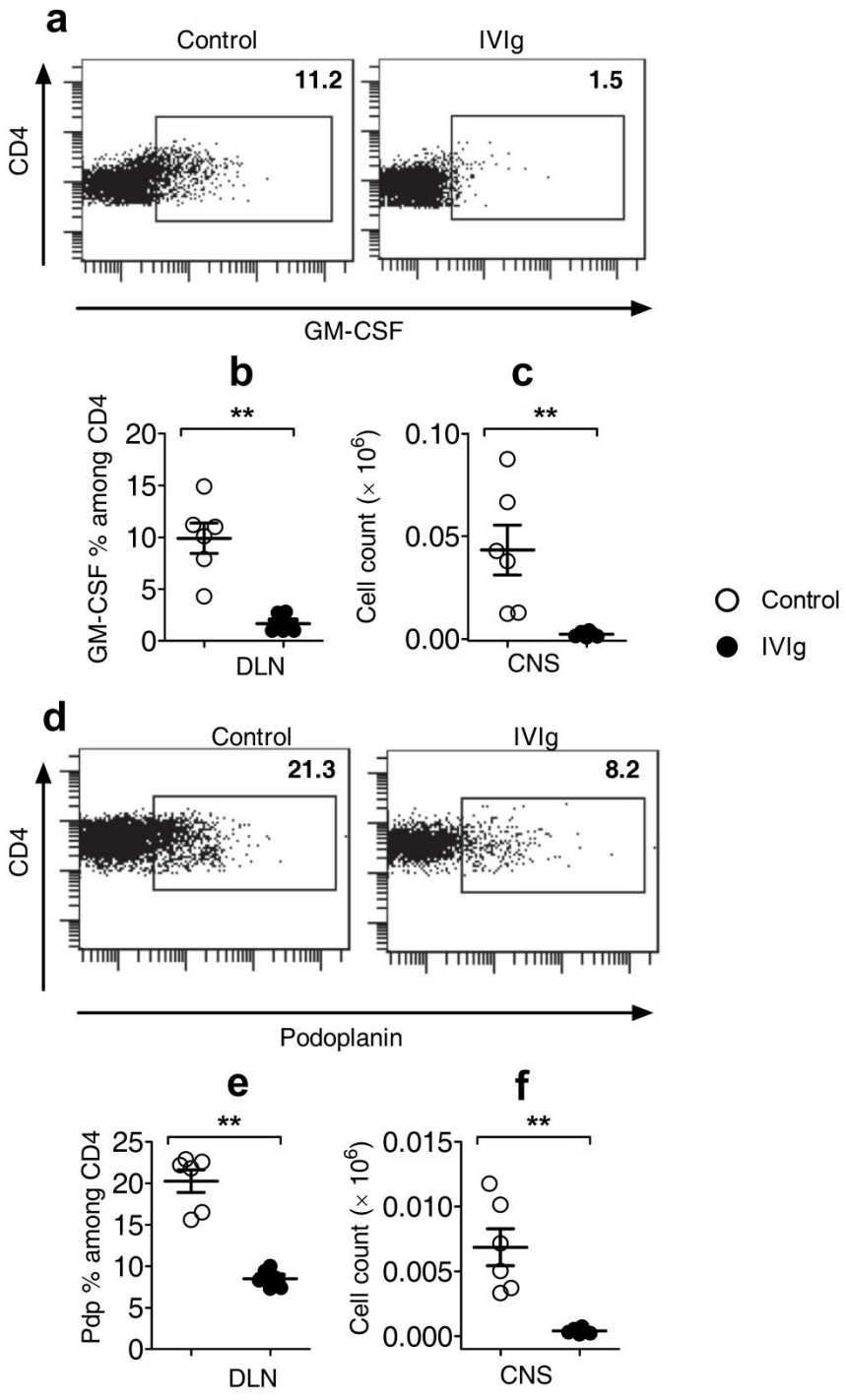


Figure 3 IVIg renders CD4 cells less pathogenic in EAE by down regulating encephalitogenic molecules like GM-CSF and podoplanin. EAE was induced in Wt C57BL/6J mice and IVIg was injected daily. Nine days after induction mice from each group were sacrificed and analyzed by Flow cytometry. Representative dot plot showing CD4 (Y-axis) and GM-CSF (X-axis) from control (left) and IVIg group (right). Number in each quadrant represents the percent among CD4 population. (a) Plot showing, GM-CSF$^+$ among CD4 in DLN from control (Open circles) and IVIg (filled circles) treated mice . (b) Absolute number of CD4$^+$ GM-CSF$^+$ in the CNS. (c) Representative dot plot showing podoplanin$^+$ gated for CD4$^+$ in control (Left) and IVIg (right) mice. (d) Percent of CD4 cells which are podoplanin$^+$ in DLN. (e) Absolute cell count of CD4$^+$GM-CSF$^+$ in the CNS. Mean value is depicted as a horizontal line among the symbols and error bars represent SEM (n=5), ***p*<0.01 determined by Mann Whitney U test.

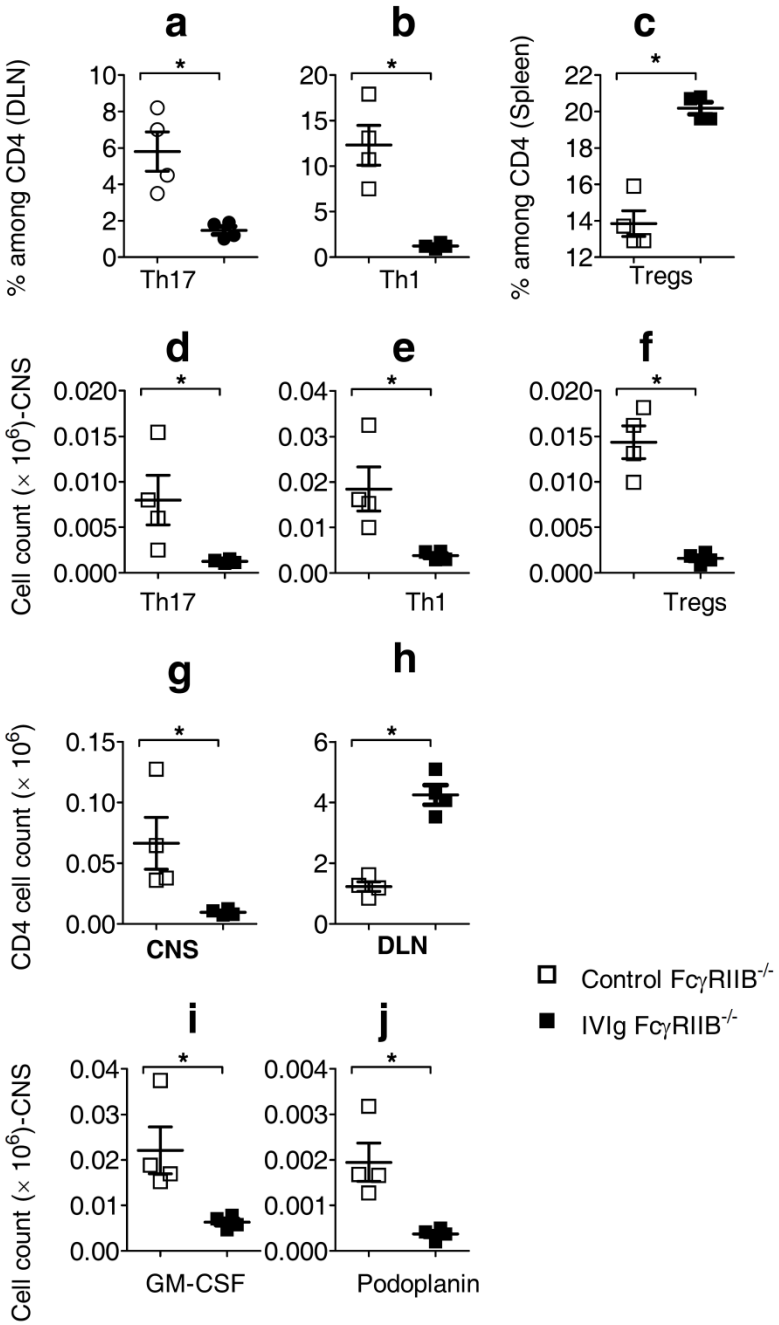


Figure 4 Inhibitory Fc receptor FcγRIIB is not required for IVIg- mediated reciprocal modulation of effector and regulatory CD4 subsets *in vivo*. Female FcγRIIB$^{-/-}$ mice under C57BL/6J background were immunized with 200µg of MOG_{35-55} peptide in CFA, this was followed by PTX injection as mentioned in experimental procedures. Control mice received 0.2M glycine (Open squares), treatment group received IVIg (Filled squares). 9 days after immunization mice were sacrificed and cells from DLN, spleen and CNS were evaluated by flow cytometry. Plots show frequency of Th17 (a) and Th1 (b) in DLN. (c) Foxp3^{+} Treg among CD4^{+} population in spleen. Absolute number of Th17 (d), Th1 (e) and Treg (f) cells infiltrating to CNS are shown. Cell counts of total CD4^{+} population in CNS (g) and DLN (h) at day 9 in control and IVIg groups are represented. CD4^{+} GM-CSF^{+} (i) and CD4^{+} podoplanin^{+} (j) in CNS are shown. Mean values are depicted as a horizontal lines among the symbols and error bars represent SEM (n=4), *p< 0.05 determined by Mann Whitney U test.

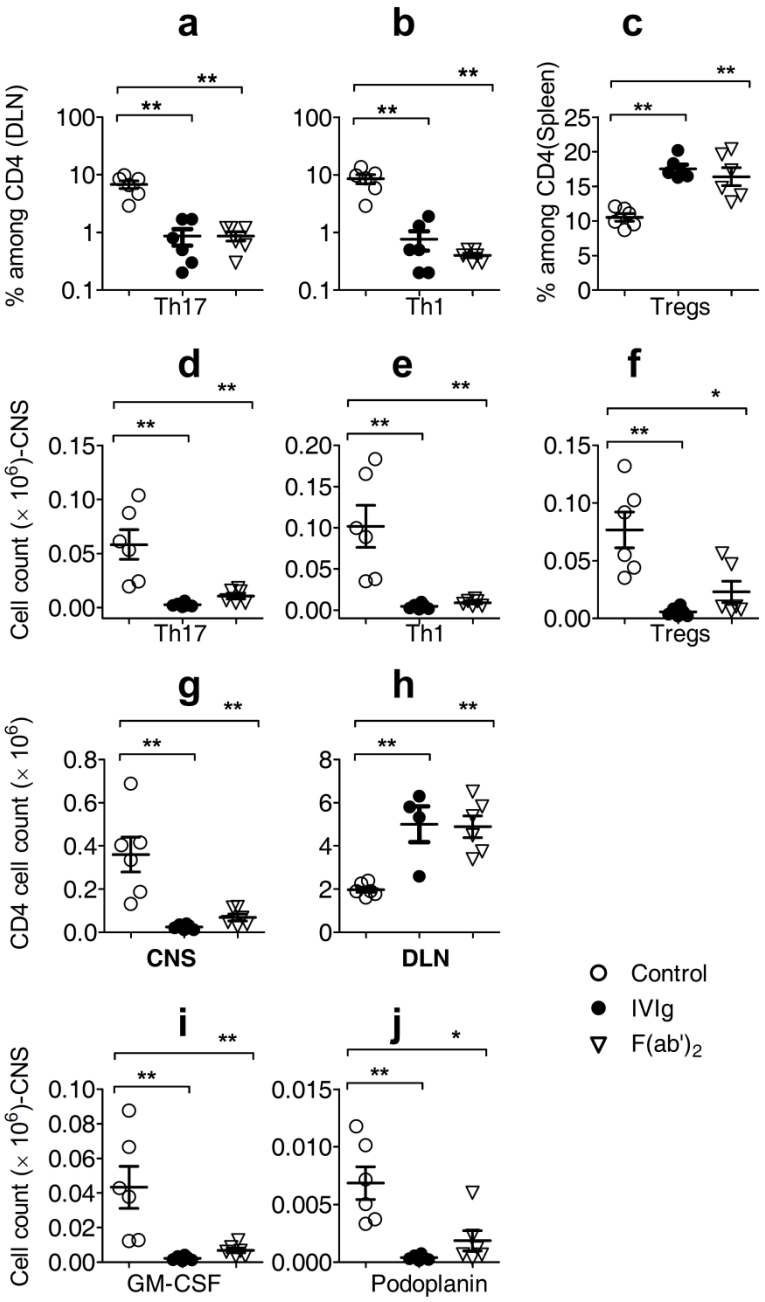


Figure 5 Fragment of antigen binding F(ab')$_2$ part of IVIg also retained capacity to reciprocally modulate effector and regulatory CD4 subsets *in vivo*. 10-12 week female WT C57BL/6J mice were immunized with 200µg of MOG_{35-55} peptide in CFA followed by PTX as mentioned in experimental procedures. Mice were treated with 0.2 M Glycine (Open circles) or IVIg (Filled circles) or Equimolar F(ab')$_2$ (open triangles)from day of immunization. Mice were sacrificed on day 9 and various cell types were evaluated by flow cytometry. Representation of percent of Th17 (a) and Th1 (b) cells in DLN. Foxp3$^+$ regulatory cells among CD4$^+$ population in spleen are plotted (c). CNS infiltrating cells were analyzed on the day of onset, cell counts were estimated by flow cytometry data, absolute number of Th17 (d), Th1 (e) and Treg (f) are plotted. Representation of absolute number of CD4 cells in CNS (g) and DLN (h) at the onset of EAE in control, IVIg and F(ab')$_2$ groups. Absolute number of CD4$^+$GM-CSF$^+$(i) and CD4$^+$podoplanin$^+$ (j) in CNS are shown. Mean values are depicted as a horizontal lines among the symbols and error bars represent SEM (n=5), **p*< 0.05, ***p*<0.01, ****p*< 0.001 determined by Mann Whitney U test.

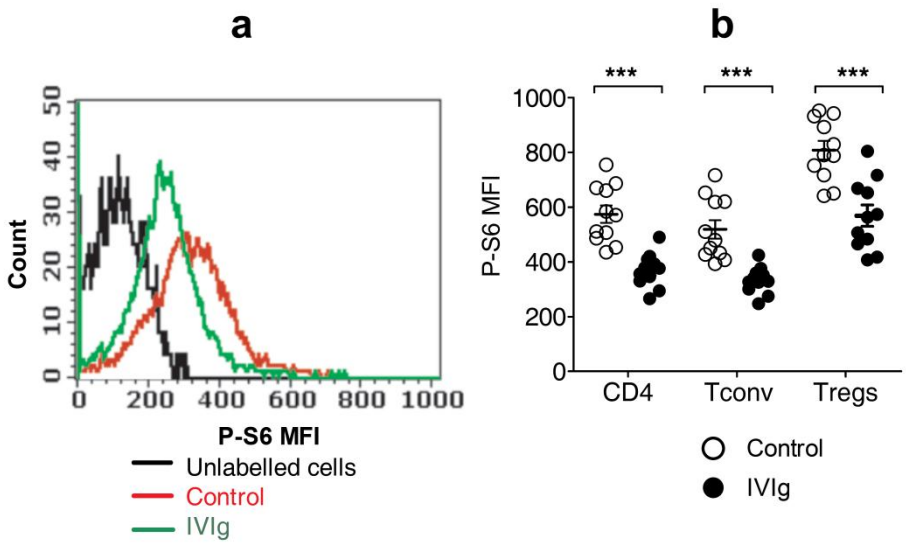
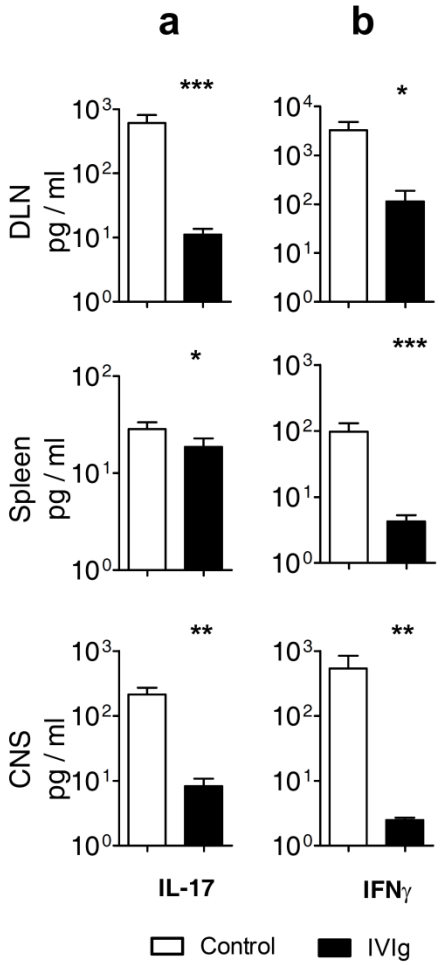
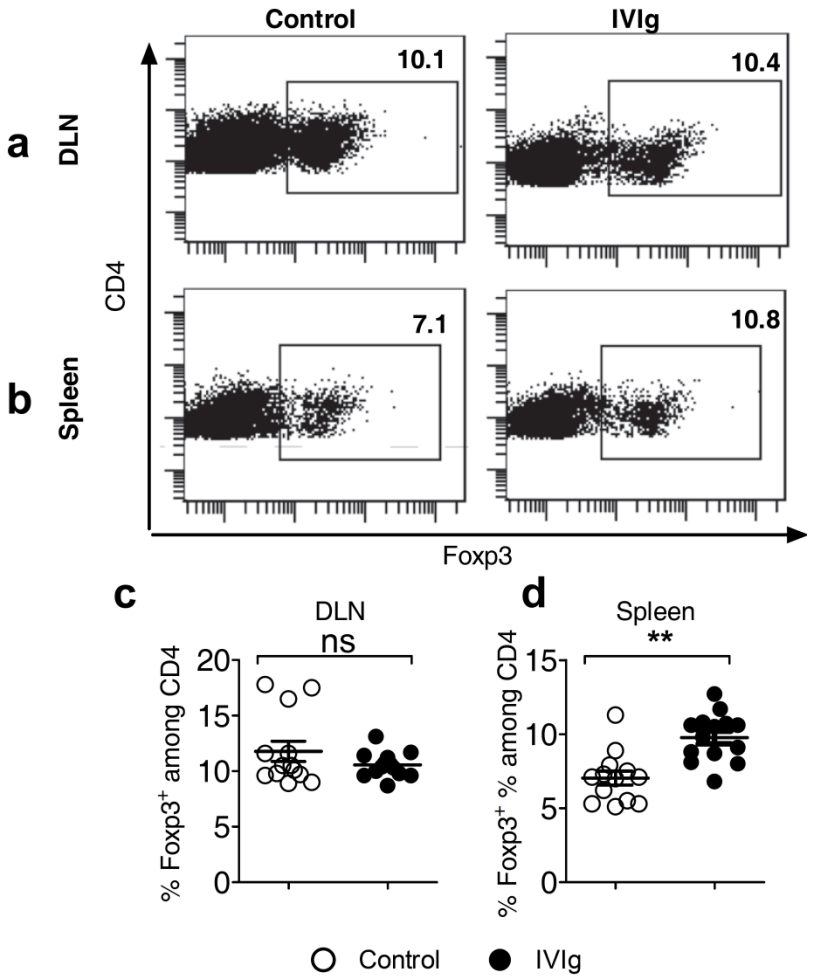


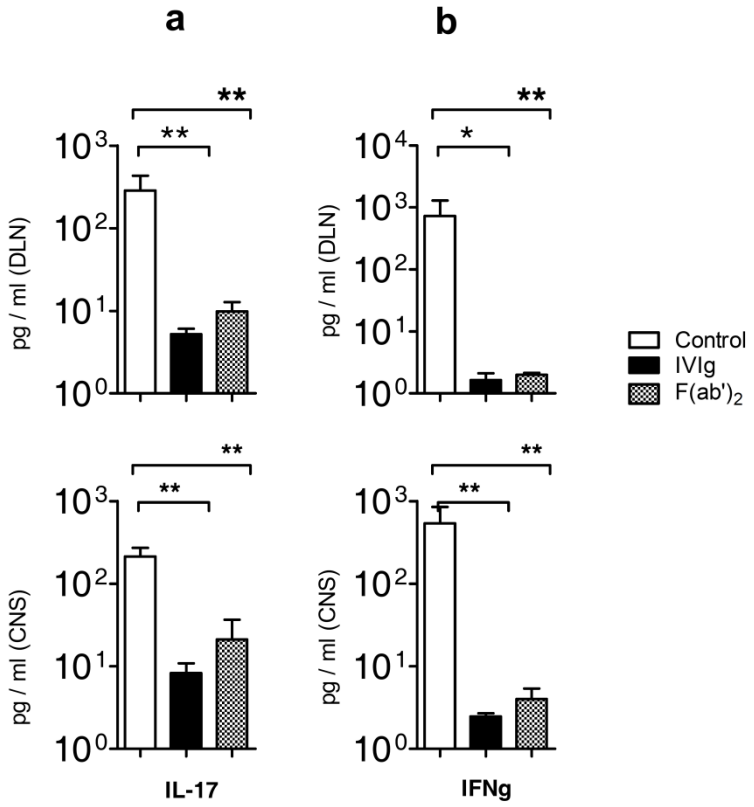
Figure 6 IVIg decreases activity of mTOR kinase in both effector and regulatory CD4 subsets in EAE. EAE was induced in 10-12 week female Wt C57BL/6J mice, 6 days after immunization mice were scarified to probe for mTOR activity in DLN. (a) Representative histogram showing MFI of phospho-S6 ribosomal protein (ser240/244) in CD4 cells from DLN of control (red) and IVIg (green) mice, auto fluorescence is shown as black line. (b) MFI of P-S6 protein in CD4 cells, conventional Th cells ($Foxp3^-$) and Treg ($Foxp3^+$). Control (Open circles) and IVIg (filled circles). Means are depicted as a horizontal lines among the symbols and error bars represent SEM (n>10, two independent experiments) ***p< 0.05 Two way ANOVA with Bonferroni's post t test.



Supplementary Figure 1 IVIg inhibits MOG_{35-55} specific secretion of IL-17 and IFNγ. Logarithmic scales are used to show the quantity of cytokine from control (open bars) and IVIg (filled bars) group (**n >10,** pooled from two independent experiments). EAE was induced in 10-12 week female Wt C57BL/6J mice. MOG_{35-55} specific cytokine secretion assay was performed by incubating cells of DLN, spleen and CNS with 10 µg/ml of MOG_{35-55} peptide for 24hrs. Supernatants were analyzed for cytokines IL-17A (a) and IFNγ (b). Error bars represent SEM, *p<0.05, **p<0.01 and ***p< 0.001 determined by Mann Whitney U test. Top panel (DLN) and middle panel (Spleen) represent data obtained from pre-onset of phase of EAE (at day 6), bottom panel (CNS) data is obtained at the day of onset (Day 9).



Supplementary Figure 2 In addition to inhibition of Th17 and Th1 cells, IVIg increases Treg in spleen. EAE was induced in Wt C57BL/6J mice and IVIg was injected daily. Flow cytometry was performed on day 9 to determine the frequency of $Foxp3^+$ cells. Representative dot plot showing CD4 (Y-axis) and Foxp3 (X-axis) from control (left) and IVIg group (right). Number in each quadrant represents the percent of $Foxp3^+$ cells among CD4 population. Data from DLN (panel a) and spleen (panel b) are represented. Data from two in independent experiments are shown. Plots show frequency of $CD4^+$ $foxp3^+$ in DLN (c) and Spleen (d). Open circles represent control mice and filled circle represent IVIg treated mice. Mean value is depicted as a horizontal line among the symbols and error bars represent SEM (n>12). ** p<0.01 and *** p< 0.001 determined by Mann Whitney U test.



Supplementary Figure 3 F(ab')$_2$ also inhibits MOG$_{35-55}$ specific secretion of IL-17 and IFNγ. EAE was induced in 10-12 week female Wt C57BL/6J mice. Control mice received 0.2M glycine (Open bars), IVIg group received 0.8 g/kg BW Gamunex® (Filled bars) and mice in F(ab')$_2$ group received 0.5 g/kg pepsin treated IgG free fragment of antigen binding F(ab')$_2$ (Gray bars). MOG$_{35-55}$ specific cytokine secretion assay was performed by incubating cells of DLN (Top panel) and CNS (bottom panel) with 10 µg/ml of MOG$_{35-55}$ peptide for 24hrs. Supernatants were analyzed for cytokines IL-17A (a) and IFNγ (b). Logarithmic scales are used to show the quantity of cytokine. Error bars represent SEM (n=5), ns: not-significant, *p<0.05 and **p<0.01 determined by Mann Whitney U test.

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Discussion and perspectives

7 Discussion and Perspectives

Reciprocal modulation effector and regulatory T cells responses by IVIg

Activation of naïve CD4 T cells and their differentiation into various subsets is the crucial event in the initiation of an adoptive immune response. Dysregulated initiation of immune response leads to undesired pathogenic immune response might lead to self-antigens and inflammatory pathologies [179]. Several lines of evidence suggest that Th1 and Th17 play critical role in the development and progression of many autoimmune and inflammatory diseases. On the other hand, regulatory T cells expressing the transcription factor Foxp3 are involved in the suppression of auto-reactive T cells and regulation of immune response [127]. Several autoimmune diseases are associated with reduced number of Treg or defects in their functions [123-126]. Thus, balance between Treg and effector T cells including Th17 and Th1 determines the course of immune-mediated disorders [58, 73]. Th17 are the main pathogenic cells in immune-mediated tissue damages such as multiple sclerosis[107], chronic inflammatory bowel disease [180], psoriasis [181, 182], SLE [183], asthma, allergic contact dermatitis, dermatomyositis, pemphigus, allergic rhinitis, anti-neutrophil cytoplasmic antibody–associated vasculitis and rheumatoid arthritis [184-188]. Although IVIg is beneficial in the treatment of several of these complications, the precise mechanisms governing the Th cell polarization and the balance between Treg and Th17 cells by IVIg have not been identified. Therefore, I hypothesized that therapeutic efficacy of IVIg might implicate interference Th17 cells *in vivo.* I demonstrate that administration of IVIg to EAE mice significantly reduces the severity of disease by

inhibiting differentiation of Th17 and Th1 cells. I did not observe any changes in Th2 cell type. Further, *ex-vivo* stimulation of the lymphocytes with MOG_{35-55} showed decreased secretion of IL-17 and IFNγ but not IL-4 and IL-10. In consensus with the previous results, I observed an expansion of Treg [165]. Thus, the protective effect of IVIg in EAE underscores the reciprocal modulation of effector CD4 cells (Th1 and Th17) and Treg.

IVIg decreases pathogenicity of encephalitogenic T cells in EAE

Both Th17 and Th1 cells are involved in EAE, however Th17 cells have emerged as the main pathogenic mediators [44, 189]. Signature cytokines of Th17 cells like IL-17A, IL-17F, IL-21 and IL-22 are dispensable for the induction of EAE [190-194]. Encephalitogenic potential of Th17 cells in EAE has been attributed to molecules like GM-CSF [120] and podoplanin [195]. IVIg decreases the expression of GM-CSF and podoplanin in CD4 T cells. GM-CSF produced by Th17 cells potentiates neuroinflammation by attracting myeloid cells to the CNS and up-regulating their surface MHCII [193, 196]. Podoplanin expressed on Th17 cells is involved in the formation of deleterious ectopic lymphoid structures in CNS [195, 197-199]. By reducing the expression of these molecules in CD4 T cells, IVIg may thus inhibit the formation of inflammatory foci. The relevance of these findings in other autoimmune conditions and in patients treated with IVIg needs to be examined.

Modulation of CD4 T cell trafficking by IVIg

Presence of lymphocytic infiltrates in CNS has been well established as clinical feature of MS, EAE and many chronic inflammatory conditions [200-202]. Protective effect of IVIg treatment in EAE is associated with decreased number of lymphocytes

and absence of inflammatory foci in the CNS [165, 203]. It has been previously shown that IVIg interferes with leucocyte recruitment to the CNS in an α4-integrin-dependent manner [204]. However, Th17 and Th1 cells use different strategies to invade CNS [205, 206]. To investigate the effect of IVIg on trafficking of Th17 and Th1 in EAE, brain and spinal cords were analyzed on the day of onset (Day 9). IVIg inhibited infiltration of Th1, Th17 and Tregs into the CNS. IVIg inhibited infiltration of Th1, Th17 and Tregs to the CNS which is in consensus with previously published reports [165, 203, 204]. Surprisingly, I observed an increased accumulation of CD4 cells in the DLN and an overall decrease in absolute number of CD4 cells in the circulation. These data suggested that, T cell entry into DLN of IVIg-treated EAE mice was intact; however, their exit from DLN was affected. To further probe into the molecular mechanisms involved in sequestering of CD4 cells into the DLN, I investigated sphingosine 1 phosphate (S1P)–S1P receptor1 (S1P1) axis, which controls the trafficking and migration of lymphocytes [207, 208]. I show the mechanistic evidence for the interference of lymphocyte trafficking by IVIg. Lymphocytes use S1P–S1P1 pathway to exit from the lymph nodes. Differentiated T cells exit from the DLN by using a gradient of S1P across the DLN, lymph and blood by through S1P1 [207-209]. By down regulating S1P1 on CD4 cells, IVIg disrupts the lymphocyte egress machinery leading to sequestration of these cells in the DLN. However, whether IVIg affects the levels of circulating S1P blood needs to be verified. Thus, IVIg sequesters activated CD4 cells in DLN and prevents their migration to the target organ.

Reciprocal modulation of T cell responses by IVIg is independent of FcγRIIB and F(ab')$_2$ fragments of IVIg recapitulates the capacity to reciprocally modulate effector and regulatory CD4 subsets in EAE

Presence and up regulation of inhibitory receptor FcγRIIB is considered mandatory for the beneficial effect of IVIg in some animal models antibody mediated autoimmunity [210] [211]. The 2,6 sialylated Fc portion of IVIg is known to be responsible for the anti-inflammatory effect [142, 212, 213]. FcγRIIB in protection of EAE mice by IVIg is however unexplored. To further elucidate the molecular mechanisms of action of IVIg in EAE, I show that Th17 and Th1 inhibitory function of IVIg is intact in mice lacking FcγRIIB (FcγRIIB$^{-/-}$). Additionally, increase in Tregs in spleen, inhibition of GM-CSF and podoplanin and interfering of CD4 T cell trafficking by IVIg does not require FcγRIIB.

As Fc part of IVIg is implicated in the FcγRIIB-dependent functions of IVIg, my results on dispensable role of FcγRIIB on IVIg-mediated regulation of T cell populations raises a possibility that F(ab')$_2$ part of IgG molecule should be equally effective as that of intact IgG. To explore this, EAE mice were treated with IVIg or equimolar concentration of F(ab')$_2$ fragments. Mice were sacrificed on day 9 and analyzed for CD4 T cell populations by flow cytometry. Interestingly, F(ab')$_2$ also inhibited Th17 and Th1 cells, and enhanced the number of Treg similar to intact IVIg. MOG$_{35-55}$ specific cytokine secretion assay revealed decreased amounts of IL-17 and IFNγ from F(ab)'$_2$ or IVIg-treated mice. This is in consensus with previous reports from our *in vitro* results[17]. Similar to IVIg, F(ab')$_2$ retained the ability to inhibit infiltration of Th1, Th17 and Treg to the CNS . Modulation of lymphocyte trafficking and sequestration of CD4 cells in DLN

was also consistent in F(ab')$_2$-treated EAE mice. Further, F(ab')$_2$ treatment also decreased the expression of GM-CSF and podoplanin in CD4 cells. These results demonstrate that *in vivo* reciprocal modulation of CD4 subsets by IVIg is F(ab')$_2$-dependent. Hence I conclude that F(ab')2 retained the capacity to reciprocally regulate CD4 subsets similar to IVIg. However, exploring the role of SIGNR1 in this regulatory shift of CD4 subsets by IVIg is an interesting perspective.

mTOR kinase pathway is implicated in reciprocal regulation of T cell responses by IVIg

The complex process of T cell activation and differentiation *in vivo* is regulated by multiple cues in the microenvironment of secondary lymphoid organs. mTOR is a kinase that integrates these signals and plays a critical role in the T_{eff} and Treg responses[53,54]. In view of the fact that IVIg can directly interact with T cells[17] and influence the TCR signaling[55], I reasoned that reciprocal modulation of T cell responses by IVIg may involve mTOR signaling pathway. To investigate whether IVIg-mediated reciprocal modulation of effector and regulatory cells involves mTOR pathway, mice were sacrificed 6 days after immunization. I analyzed the activity of mTOR in CD4 cells from DLN by estimating the level of phospho-S6 ribosomal protein (ser240/244)[37]. CD4 cells from IVIg-treated mice displayed reduced phosphorylation of S-6 ribosomal protein as compared to the control. IVIg-treatment decreased the activity mTOR in both T_{eff} and Treg. These data suggest that by inhibiting activity of mTOR kinase in CD4 cells, IVIg can decrease the differentiation of naïve CD4 cells to effector subsets while simultaneously increasing the number of Treg. Whether Treg activating Tregitopes in

IVIg play any role in the reciprocal regulation of effector and Treg remains speculative[56]. Additionally, sequestering of CD4 cells in DLN by IVIg may be a consequence of affecting mTOR-pathway as S1P-mTOR axis is implicated in lymphocyte trafficking[57].

To conclude, pathogenesis of EAE involves activation and differentiation of naive neuroantigen-specific CD4 cells into Th17 and Th1 cells in the secondary lymphoid organs. These pathogenic T cells migrate to the CNS and potentiate axonal destruction by facilitating infiltration of other myeloid effector cells through GM-CSF and formation of ectopic lymphoid structures mediated by podoplanin. My results show that IVIg-mediated inhibition of EAE implicates multiple targets acting at different phases of immune response. Thus, IVIg can inhibit the initiation of pathogenic immune response by inhibiting the polarization of naïve T cell into Th17 and Th1 cells. This process is associated with a concomitant expansion Tregs *in vivo.* IVIg can circumvent neuronal degeneration by inhibiting the infiltration of CD4 T lymphocytes to the target organ by restraining their exit from the DLN through S1P-S1P1 pathway. Further, IVIg may decrease the tissue damaging potential of pathogenic T cells by down regulating key molecules such as GM-CSF and podoplanin. Together, my results provide a cellular and molecular basis underlying the beneficial effect of IVIg in certain T cell-dependent autoimmune and inflammatory conditions.

PERSPECTIVES

Understanding the role of IVIg-modulated DC in mediating reciprocal T cell responses

DCs are the professional antigen presenting cells (APC) which are specialized in antigen uptake, processing and presentation [1]. The maturation state of DC regulates immune response and tolerance. Immature and semi-mature DC presenting antigens are known to maintain tolerance by inducing Tregs while mature-DC induces strong immune response [5]. We have previously demonstrated that IVIg inhibits maturation and function of DC, also modulates the pattern of cytokines secreted by these cells [16]. IVIg impairs antigen presentation; in addition, IVIg-treated DC ameliorates ongoing autoimmune disease *in vivo* upon adoptive transfer [18]. [19]. In view of the fact that IVIg modulates function of DC and DC are involved in regulating T cell responses *in vivo*, I set out to explore the role or IVIg-DC in regulating the T cell responses. I studied the structural changes associated with IVIg treatment on DC. Transmission electron microscopy of DC differentiated in presence of IVIg for 5 days revealed a surprising increase in accumulation of lipid bodies as compared to the control **(Figure 1A, Annexes-I).** Lipid accumulation in DC generated in presence of IVIg was further confirmed by flow cytometry using Bodipy staining. Lipid content of IVIg treated DC was significantly higher than control **(Figure 1B, C Annexes-I**). Further, I observed a decreased antigen endocytosis capacity in IVIg-DC **(Figure 1E Annexes-I),** suggesting that IVIg-DC with high lipid content are defective in antigen uptake. Hence, DC in presence of IVIg take up more lipids, which is associated with decreased antigen engulfing capacity.

There is an emerging notion that lipids in DC play a crucial role in antigen presentation function [20-22]. My preliminary observations can be extended to verify the hypothesis that IVIg modulates the antigen processing and presentation functions of DC by altering lipid accumulation and thereby regulating T cell responses. Further, whether IVIg injected *in vivo* also modifies the lipid content of DC needs to be examined. Characterizing the nature of these lipids in IVIg-DC is required to understand their role in modulation of antigen presentation.

To investigate whether IVIg interferes with cytokine networks involved in T cell polarization.

Local cytokine networks and complex signaling events at the immune-synapse between the antigen specific Th cell and APC determine the fate of these cells. For example, in presence of cytokines IL-12 and IFNγ naïve Th cells are polarized towards Th1, IL-4 skews them to Th2 phenotype, IL-6 and transforming growth factor-β (TGF-β) towards Th17 and TGF-β alone is involved in induction of Tregs [214]. Whether IVIg modulates the cytokine networks in T cell polarization needs to be addressed in EAE.

To decipher the molecular mechanisms of modulation of mTOR kinase pathway by IVIg

mTOR is a kinase involved in reciprocal regulation of effector and regulatory responses. mTORC1 is essential for Th1 and Th17 while mTORC2 is required for Th2 cell differentiation [215]. IVIg can decrease the differentiation of naïve CD4 cells to effector subsets while simultaneously increasing the number of Treg by inhibiting activity of mTOR kinase. Whether IVIg affects signaling of both complexes needs to be further explored. The molecular mechanisms of IVIg-mediated mTOR inhibition will open novel prospects on the role of normal immunoglobulins in the regulation of

complex signaling events at the immune-synapse between the antigen specific Th cell and APC. S1P-Akt-mTOR axis is implicated in Th1/Tregs regulation and lymphocyte trafficking, IVIg might be interacting with S1P in the circulation to affect this axis. The proposed studies further strengthen the claims that normal immunoglobulins have a determining role in immune homeostasis.

To understand whether IVIg shifts the activation threshold of T cells

Naïve T cells are activated at certain activation threshold which is sum of all activating and inhibitory stimulus in the micro-environment [216, 217]. This threshold is subject to dynamic tuning to ensure efficient response to pathogens and tolerance to self-antigens. Sub-optimal stimulation of naïve T cells leads to insufficient activation and failure to differentiate into effector subsets resulting in anergy [218, 219]. Interestingly, sub-optimal stimulation of Tregs is sufficient to activate and enhance their suppressive functions [220-222]. Interestingly, the pathways of anergy and iTregs converge at mTOR signaling pathway [223, 224]. As IVIg is known to interfere with early events of TCR stimulus *in vitro* [225] and from the results presented in my thesis that IVIg modulates activity of mTOR *in vivo*. It is tempting to speculate that IVIg, by shifting the threshold of activation of CD4 cells increases the window of sub-optimal stimulation, hence favoring Tregs but not the other effector T cells [221]. This hypothesis can be tested by either *in vitro* studies involving effect of IVIg on CD4 cells activated by a gradient of stimulation strength or *in vivo* by using intra-vital multi-photon microscopy to understand the effect of IVIg on the dynamics of interaction between APCs and T cells in lymph node.

To explore the clinical relevance of reciprocal modulation of Th cells by IVIg

Results presented in my thesis reveal mechanism of action of IVIg in an *in vivo* model of multiple sclerosis. By inhibiting development of Th17 and Th1 cells and expanding Tregs IVIg restores the balance towards homeostasis in EAE. This can be extended to decipher the relationship between Treg and Th17 cells in autoimmune patients following IVIg therapy and other experimental models of active autoimmunity.

To investigate the role of α2,6 sialylated IVIg in reciprocal modulation of T cell responses.

α2,6 sialylated on Fc portion of IgG (sial-IVIg) is claimed to be the active component in some animal models of antibody mediated autoimmunity. This sial-IVIg is known to interact with SIGNR1 on myeloid cells to exert its beneficial effects in K/B×N serum transfer arthritis model. However, recent results from animal model of ITP show contradictory trends and furthermore, $F(ab')_2$ is also sialylated [213, 226]. Studies involving role of sial-IVIg and SIGNR1 in IVIg-mediated protection of mice against EAE are warranted to test this hypothesis beyond K/B×N serum transfer arthritis model.

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Annexes

I. Role of IVIg-mediated accumulation of lipids in human dendritic cells: modulation of antigen presentation and T cell responses

Othy S, S. Lacroix-Desmazes, Bayry J and Kaveri S *(Manuscript in preparation)*

II. Intravenous immunoglobulin therapy for rheumatic diseases

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III. Immunomodulation by intravenous immunoglobulin: role of regulatory T Cells

Maddur MS, **Othy S**, Hegde P, Vani J, Lacroix-Desmazes S, Bayry J, and Kaveri SV (2010); *Journal of Clinical Immunology. 30 Suppl 1:S4-8.*

Annexe I

Role of IVIg-mediated accumulation of lipids in human dendritic cells: Modulation of antigen presentation and T cell responses

IVIg induces accumulation of lipids in human dendritic cells: Effect on antigen presentation and T cell responses

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Abstract

Initially used as replacement therapy in immune deficiencies, IVIg also widely used for the treatment of a number of autoimmune and systemic inflammatory diseases. IVIg exerts its beneficial effects by several mutually non-exclusive mechanisms. We have previously demonstrated that IVIg inhibits the maturation and function of dendritic cells (DC) and modulates their activation and survival. Cellular changes affecting the modulation of DC functions by IVIg however remain unexplored. Using electron microscopy and flow cytometry, we demonstrate that IVIg increases accumulation of lipids in DC by enhancing their uptake from medium. Further, increased accumulation of lipids by IVIg in DC is associated with a decrease in antigen uptake. These results provide novel insight

into the intracellular alterations in DC following treatment with IVIg, which might have repercussions on the immunoregulatory mechanisms of IVIg.

Introduction

DC are the professional antigen presenting cells (APC) that are specialized in antigen uptake, processing and presentation [1]. DC capture antigen in the periphery and migrates to secondary lymphoid organs. DCs interact with naive T cells and provide a combinatorial code of co-stimulatory molecules and cytokines in the secondary lymphoid tissues [2]. Decisions made at the synapse of DC and naive T cells shape the events that follow the fate of immune response [3]. Antigen-primed DC enter a developmentally programmed process of “maturation” which is characterized by up-regulation of various co-stimulatory molecules and secretion of cytokines depending on the context of antigen and other environmental cues of antigen encounter in the periphery [4]. The maturation state of DC regulates immune response and tolerance. Immature and semi-mature DC presenting antigens maintain tolerance by inducing Tregs while mature DC induces strong immune response [5]. Dysregulated DC functions are associated with many immune disorders and breaking of tolerance to the self [3, 6].

IVIg is a therapeutic preparation of normal human polyclonal IgG obtained from pools of plasma from a large number of healthy blood donors [7, 8]. Initially used as replacement therapy for patients with immune deficiencies, IVIg is now widely used for the treatment of a large number of autoimmune and systemic inflammatory diseases including neuromuscular and neuro-immunological diseases such as acute Guillain–Barré syndrome, myasthenia gravis, acute or chronic inflammatory demyelinating polyneuropathy, or stiff person syndrome [9-11]. IVIg exerts its beneficial effects by several mutually non-exclusive mechanisms [12-15]. IVIg inhibits maturation and function of DC, also modulates the pattern of cytokines secreted by these cells [16]. By down-regulating the interferon-α-mediated differentiation of DCs, and by inhibiting the uptake of nucleosomes, IVIg might exert an immunoregulatory effect in patients with lupus [17]. In addition, IVIg-treated DC ameliorates ongoing autoimmune disease *in vivo* upon adoptive transfer [18]. IVIg also modulates *in vivo* and *in vitro* T-cell responses by impairing antigen presentation [19]. Although IVIg is known to affect antigen presentation, the cellular mechanisms remain unexplored.

In the present study, we set out to understand cellular changes affecting modulation of DC functions by IVIg. Our preliminary results indicate an increased accumulation of lipids in DC-IVIg due to enhanced intake of fatty acids, which is associated with a decrease in antigen uptake.

Results and discussion

Transmission electron microscopy of DC differentiated in presence of IVIg for 5 days revealed a surprising increase in accumulation of lipid bodies as compared to the control **(Figure 1A).** Lipid accumulation in DC generated in presence of IVIg was further confirmed by flow cytometry using Bodipy staining. Lipid content of IVIg-treated DCs was significantly higher than in control **(Figure 1B, C**). All cells were positive for lipids; the mean MFI of Bodipy in IVIg-DC was higher than in the control indicating increased lipid content. To understand the mechanisms responsible for IVIg-meditated lipid accumulation in DC, we studied whether IVIg affects uptake of lipids by DC. Accordingly, we observed a two-fold increase in the uptake of Oleic acid conjugated to BSA (OA-BSA) or free fatty acid (Bodipy C_{16}) indicating increased uptake as a possible mechanism **(Figure 1D**). However, the effect of IVIg on *de novo* generation of lipid bodies in DC needs to be verified. Further we also observed a decreased antigen endocytosis in IVIg-DC **(Figure 1E),** suggesting that IVIg-DC with high lipid content are defective in antigen uptake. Indeed, there is an emerging body of evidence that lipids play crucial role in antigen presentation function of DC. DC with high lipid content has reduced capacity to process antigens and stimulate T cells. Lipid bodies in DC are involved in antigen cross-presentation; additionally DC functions can be reciprocally modulated by saturated and polyunsaturated fatty acids [20-22]. Thus, in view of the previous observations that IVIg inhibits maturation and function of DC and additionally IVIg-treated DC are potential inducers of tolerance *in vivo* [18, it is tempting to propose that IVIg modulates the antigen processing and presentation functions of DC by altering lipid accumulation and thereby reducing the T cell response. Our preliminary results suggest that, DC in presence of IVIg take up more lipids, which is associated with decreased antigen uptake capacity. Whether IVIg injected *in vivo* also modifies the lipid content of DC needs to be further examined. Characterizing the nature of these lipids in IVIg-DC may further help to understand their role in the modulation of antigen presentation.

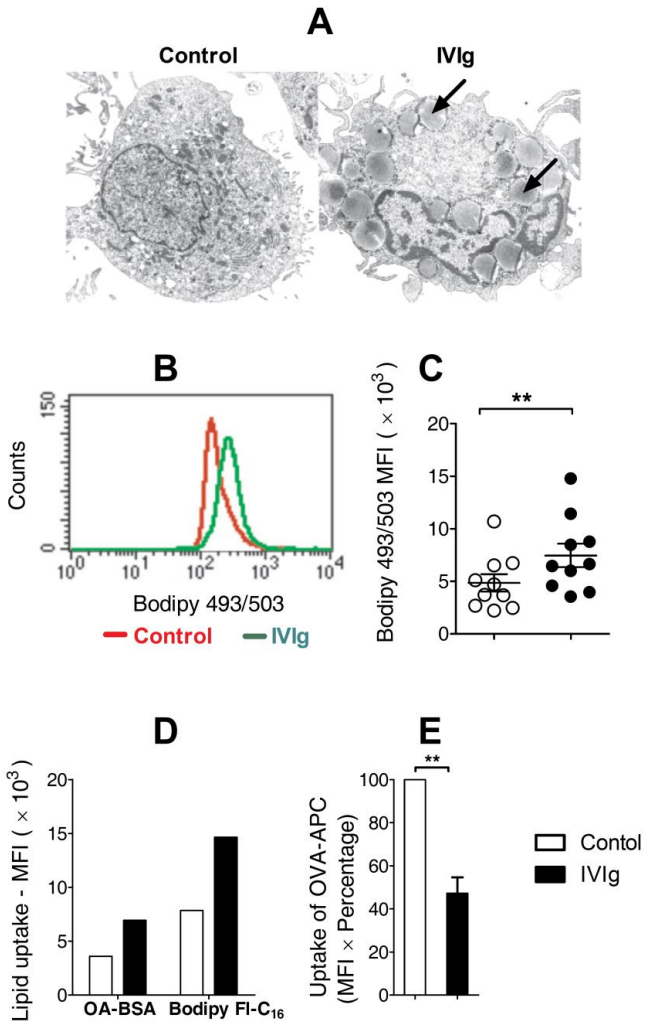

Figure 1: IVIg-mediated accumulation of lipids and decreased antigen uptake in human DC.

A. Healthy human donor $CD14^+$ monocytes were differentiated into DC by in the presence of 500 IU / mL rhIL-4 and 1000 IU / mL rhGM-CSF in RPMI/10% FCS for 6 days. [1](Bayry et al., 2003a)[1]Transmission electron micrographs showing accumulation of lipid bodies (Black arrows) in DC generated in presentence of 0.15 mM IVIg (IVIg-DC).

B. Representative histogram showing flow cytometric analysis of 6 day old monocyte derived DC after staining with Bodipy 493/503® in PBS for 15 min at 20 °C. Control in red and IVIg-DC in green.

C. Mean fluorescence intensity (MFI) of Bodipy 493/503® in control (open circles) and IVIg-DC (filled circles) from ten independent experiments is represented. Mean value is depicted as a horizontal line among the symbols and error bars represent SEM, $^{**}p<0.01$ determined by non-parametric Wilcoxon signed rank test.

D. Five-day old DCs, generated in presence (filled bars) or absence (open bars) of IVIg were assessed for uptake of fatty acids from the medium. MFI of Bodipy staining after adding 300 µM Oleic acid-Bovine serum albumin (OA-BSA) (Sigma Aldrich) for 24 hour (Left) or 3 µM BODIPY® FL C_{16} (Life technologies) uptake assay performed a 37^{o} C for 15 min (Right).

E. DCs were loaded with 25 mg/ml of Ovalbumin-APC (Life technologies) for 15 min at 37^{o} C. Antigen uptake was obtained by multiplying MFI with percent of positive cells for OVA-APC. Data from three independent experiments is shown. IVIg-DCs (filled bars) as percent change from controls (normalized to 100% - open bars), error bars represent SEM, $^{**}p<0.01$ determined by paired student t test.

Material and Methods:

For generation of human DC: $CD14^+$ monocytes were purified from healthy donor peripheral blood mononuclear cells. $CD14^+$ cells were differentiated into DC by culturing in presence of 500 IU/mL rhIL-4 and 1000 IU/mL rhGM-CSF for 5-6 days in RPMI medium supplemented with 100 IU/ml Penicillin, 100 ug/ml Streptomycin (Gibco) and 10% fetal calf serum (FCS). IVIg (Gamunex ® Talecris Biotherapeutics USA) was dialyzed extensively against RPMI and added to the culture medium at 0.15 mM during the differentiation.

Analysis of structural changes and lipid assays: DC generated were subjected electron microscopy to elucidate structural changes in cellular architecture. To stain lipids cells were washed with PBS at stained with BODIPY 493/503 at 2 µg ml−1 in PBS for 15 min at 20 °C. Cells in all experiments were acquired and analyzed using BD LSR II and FACS Diva software. Assays for lipid and antigen uptake were performed using BODIPY® FL C_{16} and OVA-APC respectively.

Statistical analysis: Graph pad prism is used for statistical analysis and plot the data. P values mentioned in the figure legends were calculated using Wilcoxon signed rank and paired student t test according to the data.

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Leading Article

Intravenous Immunoglobulin Therapy for Rheumatic Diseases

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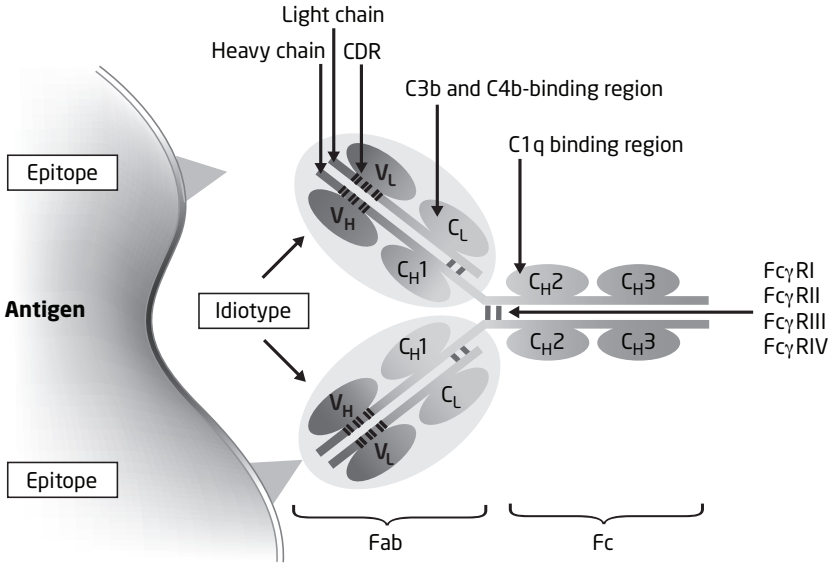
The immunoglobulins (Igs) are one of the major protein families found circulating in the blood. Igs are glycoproteins and are composed of two heavy and two light chains. Both chains are made up of variable and constant domains (**Figure 1**). The Igs recognize specific antigens through the fragment antigen-binding (Fab) region, whereas the fragment crystallizable (Fc) region interacts with Fc receptors and Fc-binding proteins such as complement. Igs are classified into five major isotypes based on their heavy chain constant domains: IgG, IgA, IgM, IgD, and IgE. Each Ig is made up of an identical pair of one of the two types of light chains: κ or λ. IgG is the most abundant Ig in the circulation and mediates the majority of antibody-based immunity against foreign antigens. IgG has a molecular weight of 150 kDa and exists in four different subclasses: IgG1, IgG2, IgG3, and IgG4.

Intravenous Ig

Intravenous Ig (ivIg) is a therapeutic preparation of IgG obtained from pooled plasma from several thousand healthy donors. Initially used in primary and secondary immune deficiencies, ivIg is now increasingly being used for the treatment of diverse autoimmune and systemic inflammatory diseases [1–4]. The list of disorders in which sufferers have reportedly responded to ivIg treatment includes a wide spectrum of diseases mediated by autoantibodies or believed to depend primarily on the activity of auto-aggressive T cells. In fact, ivIg has been used to treat >100 different pathologies. However, the beneficial effect of ivIg has been established by prospective randomized trials in only a few of these diseases, including Guillain–Barré syndrome, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis, dermatomyositis, and Kawasaki disease (KD) [2–6]. Nonetheless, in many other conditions ivIg has been shown to be an effective therapeutic option in uncontrolled studies and continues to be investigated; among these conditions are anti-factor VIII autoimmune disease, the antiphospholipid syndrome (APS), polymyositis, systemic lupus erythematosus (SLE), and Crohn's disease (**Table 1**).

Figure 1. The structure of IgG. The site of interactions between IgG and antigen (epitope) is shown, as are the binding sites for the complement components C1q and activated C3b and C4b, and the sites of interaction between the heavy chains of IgG and FcγR.



CDR: complementarity determining region; C_H: constant heavy domain; C_L: constant light domain; Fab: fragment antigen binding; Fc: fragment crystallizable; FcγR: Fcγ receptor; IgG: immunoglobulin G; V_H: variable heavy domain; V_L: variable light domain.
The figure is redrawn from [3], with permission from Nature Publishing Group.

The available clinical and experimental data indicate that a wide spectrum of rheumatic conditions could benefit from ivIg therapy [1–4].

All commercial preparations of ivIg consist of intact IgG molecules with a distribution of IgG subclasses corresponding to that found in normal (healthy) human serum. Most preparations contain traces of IgA, which can sensitize IgA-deficient patients during long-term treatment. ivIg also contains trace amounts of soluble CD4, CD8, and human leukocyte antigen molecules, as well as certain cytokines [1]. As ivIg is prepared from pools of plasma from thousands of healthy blood donors, it can be assumed that the preparation contains samples of the entire repertoire of antigen-binding variable regions of IgG that would be present in normal serum. Therefore, ivIg comprises antibodies directed to a broad range of pathogens and foreign antigens that are critical for replacement therapy in patients with primary and secondary immunodeficiencies such as X-linked agammaglobulinemia and common variable immunodeficiency. ivIg also comprises antibodies to a number of self-antigens as well as anti-idiotype antibodies, which are thought to be essential for the immunoregulatory effects of ivIg in autoimmune and inflammatory disorders. The half-life of infused ivIg is approximately 3 weeks.

Kawasaki disease

KD is an acute childhood illness that involves the skin, mouth, and lymph nodes and is characterized by vasculitis, high fever, skin rashes, cervical lymphadenopathy, conjunctivitis, and oral enanthema. The beneficial effect of ivIg treatment in children with acute KD has been demonstrated in a multicenter, randomized, controlled trial [7]. Patients were randomized to receive ivIg either as a single infusion of 2 g/kg body weight over 10 h or as four daily

Table 1. Rheumatic diseases for which the beneficial effect of ivIg has been reported.

Polymyositis
Dermatomyositis*
Inclusion body myositis*
Kawasaki disease*
Antineutrophil cytoplasmic antibody-positive systemic vasculitis*
Antiphospholipid syndrome
Rheumatoid arthritis and Felty's syndrome
Juvenile rheumatoid arthritis
Systemic lupus erythematosus
Systemic sclerosis
Stiff-person syndrome
Sjogren's syndrome

*Indicates diseases for which evidence for the effect of ivIg has been obtained in controlled trials.

ivIg: intravenous immunoglobulin.

infusions of 400 mg/kg. Patients treated with the single infusion had lower mean temperatures as well as a shorter mean duration of fever. Furthermore, high-dose ivIg therapy led to a significant decline in inflammatory mediators and coronary lesions. The investigators concluded that in children with acute KD, a single, large dose of ivIg is more effective than a regimen of four daily infusions of low-dose ivIg, which underlined the need to identify the optimal dose regimen for ivIg therapy. A meta-analysis confirmed that children fulfilling the diagnostic criteria for KD should be treated with single-dose ivIg 2 g/kg within 10 days of onset of symptoms of fever [8]. However, approximately 20–30% of patients treated with ivIg may show resistance to the therapy [9–11].

Following ivIg therapy in KD patients, the neutrophil expression of CD11b (a molecule that is implicated in the adhesion of neutrophils to endothelial cells), the levels of inflammatory cytokines (such as interleukin-6 [IL-6] and granulocyte colony-stimulating factor), and the levels of C-reactive protein (an acute-phase protein) are substantially reduced [12,13]. This inhibition of inflammatory mediators is reciprocally associated with a substantial increase in levels of the anti-inflammatory molecule IL-1Ra. A comparative analysis of the gene expression profiles of peripheral blood mononuclear cells and monocytes obtained from patients with acute KD before and after ivIg therapy demonstrated that ivIg suppresses the functions and inflammatory phenotype of monocytes and macrophages [14]. In particular, ivIg inhibited the expression of the activatory Fcγ receptors FcγR1 and FcγRIII; the chemokine receptor CCR2, which is a receptor for monocyte chemoattractant protein-1 – a chemokine that specifically mediates monocyte chemotaxis; and the monocyte-derived inflammatory mediators adrenomedullin, S100A8, S100A9, and S100A12. Interestingly, the transcripts of inhibitory FcγRIIB were not modified following ivIg therapy, indicating that ivIg regulates the balance of activatory and inhibitory Fcγ receptors not by enhancing the expression of inhibitory Fcγ RIIB, as shown in experimental models, but rather by downregulating the expression of the activatory Fcγ receptors.

Inflammatory myopathies

There are three major subsets of the inflammatory myopathies: polymyositis, dermatomyositis, and inclusion body myositis, which are characterized by proximal and often symmetrical muscle weakness [15]. An initial open-label study on ivIg in a small number of patients with chronic refractory polymyositis or dermatomyositis demonstrated significant clinical improvement, as assessed by the measurement of proximal muscle power and biochemical studies [16]. During ivIg therapy, steroid doses were significantly reduced, thus indicating that ivIg has a steroid-sparing effect. The investigators concluded that ivIg is an efficacious therapy for polymyositis and dermatomyositis and should play a role in the treatment of these diseases, replacing or reducing steroid and immunosuppressive medications [16,17].

These results were confirmed in a double-blind, placebo-controlled study of patients

with biopsy-proven, treatment-resistant dermatomyositis [18]. Patients treated with a single infusion of ivIg 2 g/kg body weight each month for 3 months showed a significant improvement in scores for muscle strength and neuromuscular symptoms compared with patients given placebo. Repeated biopsies of the muscles of patients whose strength improved to almost normal levels following ivIg therapy showed an increase in muscle fiber diameter, an increase in the number and a reduction in the diameter of capillaries, resolution of complement deposits on capillaries, and a reduction in the expression of intercellular adhesion molecule 1 (ICAM-1) and major histocompatibility complex class I (MHC I) antigens [18,19].

Recently, a study was conducted to determine the molecular mechanisms of high-dose ivIg therapy in inflammatory myopathies [20]. Thirteen treatment-resistant patients – six with polymyositis, four with dermatomyositis, two with inclusion body myositis, and one with juvenile dermatomyositis – were treated with three courses of ivIg 2 g/kg at monthly intervals. The effects of ivIg on muscle function and immunological molecules in the skeletal muscle of these patients were investigated. Following ivIg therapy, improved muscle function was observed in three patients (one patient with polymyositis, one with dermatomyositis, and one with inclusion body myositis) and serum creatinine kinase levels were reduced in five patients. The number of T cells and macrophages, the expression of MHC I antigens on muscle fibers, and the levels of ICAM-1, vascular cell adhesion molecule-1, and membranolytic attack complex deposits on capillaries were not significantly altered following ivIg treatment. The investigators concluded that these immunological parameters are not indicators of clinical response to ivIg therapy in patients with myopathies, and suggested that the immunological changes described in patients following ivIg therapy cannot be generalized [20].

Although the beneficial effect of ivIg in dermatomyositis patients has been demonstrated in randomized clinical trials, the therapeutic effects of ivIg in inclusion body myositis patients were marginal [2,21,22]. Although a few inclusion body myositis patients had a definite clinical improvement, their total gains in muscle strength did not reach statistical significance compared with the placebo-treated group. Similarly, uncontrolled trials on ivIg therapy in polymyositis patients have shown improvements in muscle strength [2,16,23], but confirmatory results from randomized clinical trials are required to fully establish the spectrum of efficacy.

Systemic lupus erythematosus

SLE is an autoimmune disease that is characterized by the involvement of multiple organs, including the skin, kidneys, and the central nervous system, and the presence of high titers of autoantibodies that are predominantly specific for DNA and nucleosomes. Several case reports and open-label trials have shown that ivIg (2 g/kg over a 5-day period) therapy is a beneficial and safe adjunct therapeutic agent for >20 manifestations in patients with SLE, including neurological, cutaneous, renal, and cardiovascular [24–27].

In one study, a beneficial clinical response following ivIg treatment in SLE patients was associated with a decline in Systemic Lupus Activity Measure scores, normalization of complement activation, and a reduction in the levels of antinuclear antibodies [28]. The clinical manifestations that responded most to treatment were arthritis, fever, thrombocytopenia, and neuropsychiatric lupus. Furthermore, ivIg has a high response rate among pregnant patients with SLE-associated recurrent spontaneous abortion and may be considered a safe and effective therapy in this population [29].

ivIg has been shown to inhibit type I interferon-mediated differentiation of dendritic cells – the sentinels of the immune system that initiate pathogenic autoimmune responses – and to block the endocytosis of nucleosomes by these cells [30]. Anti-idiotype antibodies found in ivIg preparations that

are directed against double-stranded DNA, phosphorylcholine, and phospholipids have been shown to be effective in experimental models of lupus [31]. The *in vitro* and *in vivo* exposure of B lymphocytes from lupus-prone mice to ivIg results in an increased expression of their surface inhibitory FcγRIIB receptors [32]. $F(ab')_2$ fragments of ivIg had similar activity compared with the intact preparation, whereas Fc fragments had no effect.

Despite these encouraging reports on the efficacy of ivIg therapy in SLE, the clinical value of the treatment has not been established by placebo-controlled clinical trials. At present, ivIg is indicated in severe cases of SLE that are non-responsive to other therapeutics, or as a steroid-sparing agent when patients are being treated with high-dose steroids. In addition, the appropriate indications for its use and the optimal dosage and duration of therapy are yet to be established for SLE [33]. In a recent study, although low-dose ivIg (approximately 0.5 g/kg body weight) was associated with clinical improvement in many specific disease manifestations, along with a continuous decrease over time in SLE Disease Activity Index scores, the symptoms of thrombocytopenia, alopecia, and vasculitis did not improve following low-dose ivIg therapy, indicating that high-dose therapy might be more beneficial in lupus patients [34]. Furthermore, observations from case reports have indicated that a combination of ivIg with other immunotherapeutics, such as anti-tumor necrosis factor-α (anti-TNF-α), is an effective therapeutic strategy for pregnant patients with severe lupus nephritis [35].

Systemic vasculitis

ivIg is an effective treatment for several vasculitides, including systemic and organ-specific diseases such as Wegener's granulomatosis, microscopic polyangiitis, and Churg–Strauss vasculitis, and this therapy is less toxic than conventional immunosuppressive agents [36–38]. Levels of anti-myeloperoxidase antibodies and antineutrophil cytoplasmic antibodies (ANCA) decreased concomitantly with clinical improvement in the patients with Churg–Strauss vasculitis and Wegener's granulomatosis, respectively.

A randomized, placebo-controlled trial investigating the efficacy of a single course of ivIg 2 g/kg body weight in patients with previously treated ANCA-associated systemic vasculitis (AASV) with persistent disease activity demonstrated that this treatment regimen is efficient in reducing disease activity in persistent AASV; however, this effect was not maintained beyond 3 months [39]. Furthermore, an uncontrolled study of ivIg treatment in ANCA patients found that complete remission of disease activity could not be achieved despite using repeated courses of ivIg therapy [40]. In contrast to these results, a recent open-label study demonstrated that ivIg therapy (0.5 g/kg/day for 4 days per month for 6 months) induced complete remissions of relapsed ANCA-associated vasculitides (Wegener's granulomatosis or microscopic polyangiitis) in 13 of 22 patients at 9 months after treatment initiation [41]. Therefore, the dosage, appropriate therapeutic window, and duration of ivIg therapy need to be established in order to achieve sustained therapeutic benefits of ivIg in AASV.

Antiphospholipid syndrome

APS is a rapidly progressive, life-threatening disease characterized by the presence of antiphospholipid antibodies (aPL), which cause multi-organ vascular thromboses and dysfunction as well as pregnancy morbidity. Both experimental and clinical data provide evidence for beneficial effects of ivIg in the treatment of APS [42–45]. Treatment with ivIg inhibited the thrombogenic effects of aPL *in vivo* and reduced circulating levels of anticardiolipin antibodies via an FcγR-independent mechanism [46]. Several studies have also demonstrated the therapeutic efficacy of ivIg in the majority of APS patients with recurrent abortions [42,43]. However, the efficacy of ivIg therapy was inferior to that of low-molecular-weight heparin plus low-dose aspirin therapy, which resulted in a higher live birth rate than ivIg in the treatment of APS in women with

recurrent abortion [43,47]. Therefore, instead of using ivIg therapy alone, combination of ivIg with aspirin and/or heparin might be beneficial to APS women with recurrent abortion; this strategy requires further clinical investigation. In addition, ivIg has been found to be beneficial in aPL-positive patients undergoing *in vitro* fertilization [48].

Results from experimental models have indicated that anti-idiotypic activity of ivIg against pathogenic aPL is the most important mechanism of action of ivIg in the treatment of APS. Anti-β_2-glycoprotein-I anti-idiotypic antibodies that were contained in ivIg preparations inhibited human trophoblast cell invasion *in vitro* and significantly improved the pregnancy outcome in experimental BALB/c mice with APS [49]. In addition, in another experimental APS murine model, ivIg induced oral tolerance mediated by transforming growth factor-β (TGF-β) and IL-10-secreting CD8α^+ cells [50].

Systemic sclerosis

Systemic sclerosis (SSc) is a heterogeneous, systemic ailment that affects the connective tissues of the skin, internal organs, and the vascular walls. The disease is characterized by microvasculature modifications, vast deposits of collagen and other matrix substances in the connective tissue, and a perturbed immune system [51]. Several open-label and case studies have indicated benefits of ivIg therapy in SSc [52–54]. Treatment with ivIg 2 g/kg body weight for 4 days per month for 6 consecutive months reduced joint pain and tenderness, with significant recovery of joint function in SSc patients with severe and refractory joint involvement [52]. In addition, ivIg has been found to modulate cutaneous involvement in SSc patients and to reduce the degree of skin fibrosis [53,55]. Randomized clinical trials are required to confirm these observations.

Other rheumatic conditions

Patients with several other rheumatic diseases such as juvenile rheumatoid arthritis, stiff-person syndrome, and Sjogren's syndrome might benefit from ivIg therapy [2,56–58], but sufficient data and controlled trials are lacking.

Mechanisms of action of ivIg

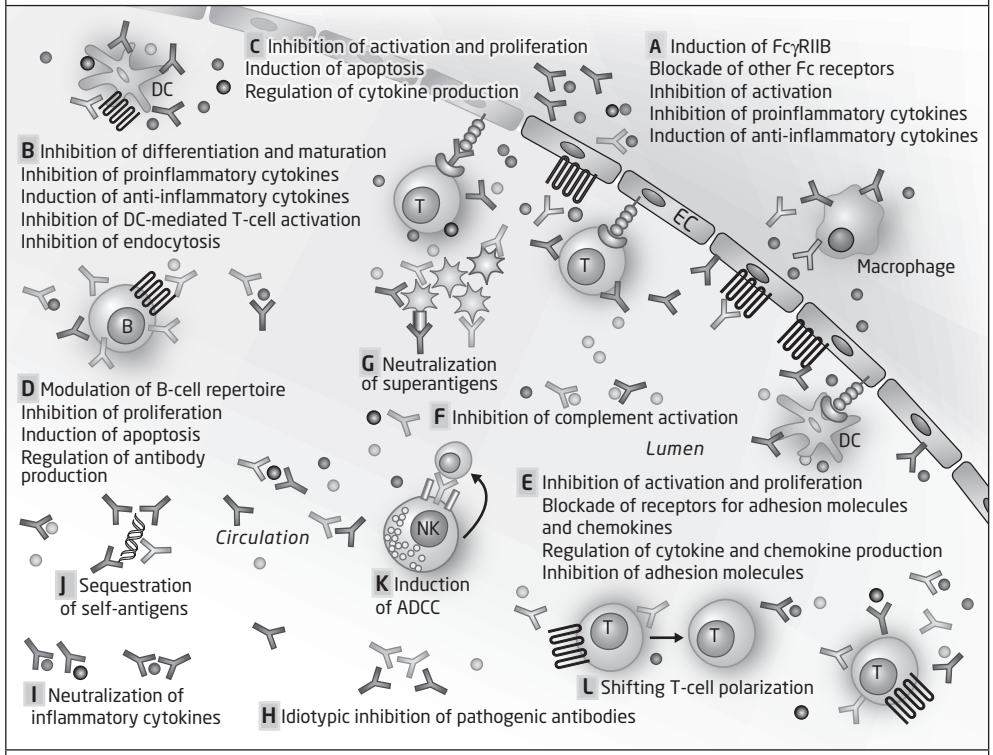
The mechanisms of action of ivIg are multiple and may differ between diseases and even between subgroups of patients within a similar disease spectrum. Each mechanism presented in **Figure 2** may be involved, to a certain extent, in the beneficial effects of ivIg in different diseases. Some mechanisms depend on the interaction between the Fc portion of infused ivIg antibodies and the FcγRs on target cells. Others may rely on the variable regions of antibodies in ivIg preparations. The distinction between Fc-dependent and variable-region-dependent mechanisms is, however, artificial, as several effects of ivIg are amplified or, indeed, made possible by the binding of Fc to cells targeted by variable regions. In addition, the integrity of the IgG molecule is important to the stability and to the half-life of infused ivIg *in vivo* [1–4].

Adverse reactions to ivIg

Generally, ivIg is a safe therapeutic preparation with minimal side-effects. Mild side-effects such as headache, nausea, low-grade fever, and increased blood pressure are common, but can be relieved with pre-treatment medications or by temporarily stopping the ivIg infusion [59]. However, patients with IgA deficiency should be treated with caution, as contaminant IgA molecules in ivIg may cause anaphylactic reactions. Such patients should be treated with IgA-depleted ivIg preparations. The high sugar content in ivIg preparations, which is used as a stabilizing agent, may worsen pre-existing kidney diseases. However, several new-generation ivIg preparations lack sugar and are hence safe for use in such settings [60]. ivIg may also cause aseptic meningitis in some patients, possibly owing to antibodies that mimic ANCAs and activate neutrophils in a TNF-α-dependent manner [61].

Variations in ivIg preparation methods, the purity of IgG content and contaminant molecules (such as traces of IgA, IgM, TGF-β, CD4, and CD8 molecules), the stabilizing agents (D-sorbitol, L-proline,

Figure 2. A schematic representation of the proposed mechanisms of action of ivIg in rheumatic diseases. The mechanisms that underlie the beneficial effect of ivIg involve its direct interaction with various cellular and soluble components of the immune system. ivIg stimulates the expression of FcγRIIB on a subset of macrophages, while blocking the expression of FcγRIIA. ivIg also modulates cytokine secretion, blocks the Fc receptors, and inhibits the activation of macrophages (**A**) and DCs (**B**). In addition to the inhibition of activation and production of pro-inflammatory cytokines by T cells (**C**), ivIg downregulates DC-mediated T-cell proliferation. At the B-cell level (**D**), ivIg modulates the antibody synthesis and B-cell repertoire, inhibits B-cell proliferation, and induces B-cell apoptosis. In endothelial cells (**E**), ivIg blocks the proinflammatory cytokines, chemokines, and adhesion molecules. Other mechanisms of ivIg include interference with complement activation (**F**); neutralization of superantigens (**G**), pathogenic autoantibodies (**H**), and cytokines (**I**); sequestration of self-antigens (**J**); induction of ADCC (**K**); and shifting the balance between T-helper cell subsets (**L**). The area encompassed by ECs represents the vascular lumen. Adhesion molecules on ECs are depicted. ivIg is depicted in the form of antibody structures with different colors to highlight the fact that it is a polyclonal IgG obtained from pools of plasma from a large number of healthy blood donors.



ADCC: antibody-dependent cell-mediated cytotoxicity; B: B cell; DC: dendritic cell; EC: endothelial cell; FcγR: Fcγ receptor; IgG: immunoglobulin G; ivIg: intravenous Ig; NK: natural killer cell; T: T cell.

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sucrose, glycine, glucose, and poly[ethylene glycol]), the route and rate of administration, and the osmolarity of the various preparations are the major parameters that influence the therapeutic efficacy and adverse reactions in patients following ivIg infusion [60]. Therefore, caution should be exercised before generalizing any events in patients who are treated with ivIg.

Perspectives

ivIg is effective in the treatment of several rheumatic diseases, but the appropriate window for treatment, the dosage, and the duration, especially as first-line therapy, are not fully established for many disorders. Further controlled studies of ivIg, combined with dose-finding and quality-of-life assessments are warranted to improve the

evidence base for clinical practice. The pharmacoeconomics of ivIg therapy are not well known for the majority of diseases, but this knowledge is critical as ivIg is a relatively expensive therapy. A study of Guillain–Barré syndrome has shown that the cost of ivIg therapy is comparable with that of plasma exchange therapy (approximately US$10 000 or €7800 for each patient without complications), but ivIg was associated with statistically significant cost reductions owing to shortened hospital stays, lower costs of procedures and hospitalization, fewer complications, and fewer patients using assisted ventilators compared with plasma exchange therapy [62]. Therefore, a systematic study of the pharmacoeconomics of ivIg therapy in diverse rheumatic diseases may help to decide whether to use ivIg for these conditions, and may help to find a solution for the problem of ivIg preparation shortage [63]. In addition, when considering the efficacy of ivIg therapy, the variability of patients and their past histories (i.e. previous therapies and whether the immune system has been altered by these previous therapies) also need to be considered. These factors are crucial, as ivIg is given in most of these patients as a last therapeutic resort rather than as a primary therapeutic agent.

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Immunomodulation by Intravenous Immunoglobulin: Role of Regulatory T Cells

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Abstract An altered immune homeostasis as a result of deficiency or defective function of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) is common in several autoimmune diseases. Hence, therapeutic strategies to render Tregs functionally competent are being investigated. Intravenous immunoglobulin (IVIG) is being increasingly used for the treatment of a wide range of autoimmune and inflammatory diseases. Recent studies have demonstrated that IVIG induces the expansion of Tregs and enhances their suppressive functions. These effects of IVIG on Tregs correlate with the beneficial effects of IVIG in patients with autoimmune diseases. Thus, modulation of Tregs by IVIG represents a novel mode of action that explains the therapeutic effects of IVIG in T cell-mediated autoimmune diseases. However, the molecular mechanisms involved in IVIG-mediated modulation of Tregs are unclear and need further investigation.

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Keywords IVIG · intravenous immunoglobulin · regulatory T cells · autoimmune diseases · inflammation · immunomodulation

Introduction

Intravenous immunoglobulin (IVIG) is an established therapeutic for a wide range of autoimmune and immune-mediated inflammatory diseases [1, 2]. IVIG is a polyspecific immunoglobulin preparation obtained from pooled plasma of several thousand healthy donors [3, 4]. The beneficial effects of IVIG are attributed to multiple, mutually nonexclusive mechanisms that include modulation of Fc receptor expression and function, interference with activation of complement and the cytokine network, regulation of cell growth, and the effects on the activation and effector functions of dendritic cells, macrophages, natural killer (NK) cells, and T and B cells [1, 4].

Interestingly, recent reports have demonstrated a prominent role of $CD4^+CD25^+$ regulatory T cells (Tregs) in IVIG-mediated beneficial effects in autoimmune diseases [5]. Tregs play a critical role in the maintenance of immunological unresponsiveness to self-antigens and in the prevention of immune aggression and autoimmune diseases [6, 7]. Thus, the expansion of Tregs with an enhanced suppressive function represents a novel therapeutic approach in the treatment of autoimmune pathologies [8]. Recent studies have demonstrated that IVIG induces the expansion of Tregs and enhances their suppressive functions. Interestingly, these effects also correlate with the beneficial effects of IVIG in patients with autoimmune diseases. Thus, modulation of Tregs by IVIG represents a novel mode of action that explains the therapeutic effects of IVIG in T cell-mediated autoimmune diseases.

Phenotypic and Functional Features of Tregs

Tregs constitute a key player in the maintenance of self-tolerance in the peripheral tissues and in the prevention of deleterious immune responses. Recent evidence from experimental and clinical studies have clearly demonstrated that the deficiency of Tregs due to either genetic consequences or deliberate depletion results in exaggerated immune responses that lead to autoimmune diseases and inflammation [7].

Tregs are identified as either natural Tregs (nTregs) that emerge from the thymus or adaptive or induced Tregs (iTregs) that develop in the periphery from naïve $CD4^+$ T cells [8]. Tregs express characteristic markers such as CD25 (IL-2Rα), cytotoxic lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis receptor (GITR), chemokine receptor 4 (CCR4), lymph node homing receptor (CD62L), and forkhead box protein (FoxP3), the lineage-specific transcription factor [9]. Continuous expression of FoxP3 is critical for the suppressive function of Tregs. Interestingly, nTregs and iTregs share similar phenotypic markers, although iTregs are functionally unstable and distinct from nTregs. nTregs display a diverse T-cell receptor (TCR) specific for self-antigens. However, IL-2 and transforming growth factor-β (TGF-β) are required for the maintenance, survival and functioning of both nTregs and iTregs [10].

The cellular targets of Treg-mediated suppressor functions include $CD4^+$, $CD8^+$ T cells, dendritic cells (DCs), B cells, macrophages, monocytes, mast cells, natural killer (NK) cells, and NKT cells [9, 11]. Several mutually nonexclusive mechanisms have been proposed to explain the Treg suppressive function on these cells, which are mediated by both soluble factors and cell-associated molecules [7]. Although Tregs are antigen-specific, they can suppress responder T cells upon activation, irrespective of their antigen specificity [8]. Tregs can directly suppress responder T cells by secreting suppressor cytokines (TGF-β, IL-10, and IL-35), by depriving IL-2, and by causing granzyme- and perforin-mediated cytolysis leading to cell cycle arrest and apoptosis [6, 7, 9]. Furthermore, Treg-induced intracellular cyclic adenosine monophosphate (AMP) by CD39 and CD73 leads to inhibition of T cell proliferation and IL-2 production [9]. Tregs also inhibit the interaction of effector T cells with DCs, thereby interfering with T cell activation [12]. Thus, Tregs suppress the proliferation of naïve T cells and their differentiation from effector cells. In addition, the development of Tregs from naïve T cells is linked to Th17 differentiation. Under a steady state, Tregs can block the development of Th17 cells, which is mediated by inhibition of RORC expression by FoxP3 in a STAT3-dependent manner [13].

Treg interaction with DCs mediated by CTLA-4, LAG-3, and suppressor cytokines has been shown to downregulate the expression of co-stimulatory molecules CD80 (B7-1), CD86 (B7-2), and CD40 and the MHC–peptide complexes while upregulating the inhibitory B7-H3 molecules that lead to an impaired T cell stimulatory function of DCs [14–16]. Treg-modulated DCs also produce significantly lower levels of inflammatory cytokines IL-12, IL-1β, IL-6, and IL-8 and higher amounts of anti-inflammatory cytokine IL-10. Furthermore, CTLA-4-induced indoleamine 2,3-dioxygenase in DCs converts tryptophan into kynurenines, which act as potent immunosuppressive metabolites and can also induce de novo generation of Tregs. By a granzyme/perforin pathway, Tregs exert CD18/CD54 interaction-dependent cytotoxicity against both immature and mature DCs. Treg-expressed CD39 degrades adenosine triphosphate (ATP) to AMP and blocks the ATP-mediated activation of DCs [6, 7, 9, 11]. In addition, Tregs modulate the cross-talk between DCs and NK cells by controlling the mature NK cell number in the lymphoid organs [11].

In line with DCs, Tregs exert direct suppressive effects on monocytes and macrophages by downregulating the expression of MHC class II, CD40, CD80, and CD86 and the secretion of inflammatory cytokines (IL-1β, IL-6, IL-8, tumor necrosis factor [TNF], and macrophage inflammatory protein 1α [MIP-1α]), while favoring high expression of B7-H4 and anti-inflammatory cytokine IL-10. Thus, Treg-modulated monocytes and macrophages are poor stimulators of T cells. Tregs also enhance Fas/FasL-mediated apoptosis of lipopolysaccharide-treated monocytes [11].

Tregs also modulate the functions of B cells in several ways. Tregs reduce autoantibody production, inhibit T cell-dependent B cell responses by cell surface TGF-β1, and induce apoptosis of antigen-specific B cells via perforin and granyzymes.

Despite an established role for Tregs in the maintenance of self-tolerance and prevention of immune-mediated pathologies, Tregs fail to control persistent and chronic inflammation. Therefore, therapeutic strategies aimed at expanding Tregs and rendering them functionally competent are being explored [11]. In this context, IVIG is considered to be a promising therapeutic that can induce functionally competent Tregs in autoimmune and inflammatory conditions.

Modulation of Tregs by IVIG: a Novel Mechanism of Action

The established therapeutic efficacy of IVIG in T cell-mediated diseases such as Guillain–Barré syndrome, chronic inflammatory demyelinating polyneuropathy, and relapsing–

remitting multiple sclerosis (MS) was supported by recent findings of the expansion and enhanced suppressive function of human and murine Tregs following IVIG treatment. Thus, immunomodulation by IVIG through Tregs represents a novel mechanism of action.

Expansion of Tregs by IVIG

Using murine experimental autoimmune encephalomyelitis (EAE), an accepted model for MS, we have demonstrated the critical involvement of Tregs in IVIG-mediated protection against this disease. Interestingly, IVIG-induced protection was associated with an early and sustained peripheral expansion of antigen-specific $CD4^+CD25^+FoxP3^+$ Tregs in spleen and lymph nodes. Furthermore, depletion of Tregs using monoclonal antibody PC61 (anti-CD25) prior to EAE induction and treatment abolished the protective effects of IVIG [17]. Thus, it can be concluded that the beneficial effect of IVIG in relapsing–remitting MS might be related to the reestablishment of the Treg compartment.

The mechanism of IVIG-mediated enhancement of Treg numbers in lymphoid organs was investigated by employing adoptive transfer of TCR transgenic T cells specific for influenza hemagglutinin. We found that an increase in Treg numbers in the spleen following IVIG treatment was due to an expansion of the existing Treg population rather than their de novo generation [17]. Furthermore, in wild-type mice protected from EAE, IVIG did not enhance the secretion of TGF-β, a cytokine that favors the differentiation of $CD4^+$ T cells into iTregs.

Analogous to our in vivo results, De Groot and colleagues [18] demonstrated the expansion of iTregs in human peripheral blood mononuclear cells (PBMCs) by Treg-activating regions (referred to as Tregitopes) derived from the Fc portion of IgG molecules. Thus, co-incubation of PBMCs with antigens and Tregitopes enhanced the expression of cell surface markers such as $CD25^{high}$, CTLA-4, and GITR. Furthermore, they supported their observation with in vivo studies by showing that the administration of the murine homologue of the Fc region Tregitope resulted in the suppression of an immune response to a known immunogen. The authors hypothesized that the tolerizing effects of IVIG is related to Tregitope-mediated activation of Tregs [18].

In consensus with the experimental evidence, IVIG therapy enhanced the number of peripheral Tregs in patients with acute-stage Guillain–Barré syndrome and Kawasaki disease. The increased Treg numbers from IVIG was also correlated with an improvement of clinical parameters and symptoms [19, 20]. Similarly, following IVIG therapy in patients with systemic lupus erythematosus, an increase in $CD4^+CD25^+CD45RO^+$ T cell frequency was observed with progressive clinical improvement [21].

Enhancement of the Suppressive Function of Tregs by IVIG

Accumulating evidence from recent in vivo and in vitro studies clearly support the significance of enhanced suppressive function of Tregs in the therapeutic benefits of IVIG. In an EAE model, we demonstrated that adoptive transfer of Tregs from IVIG-treated mice to naïve mice followed by immunization with myelin oligodendrocyte protein (MOG) resulted in milder EAE compared with Tregs from untreated mice. Furthermore, Tregs from IVIG-treated mice were more efficient in suppressing the in vitro proliferation of TCR-stimulated $CD4^+FoxP3^-$ T cells compared with Tregs from untreated mice. In addition, IVIG-modulated Tregs efficiently prevented CNS damage in MOG-immunized mice by restricting encephalitogenic T cell infiltration and reducing the IFN-γ secretion. Thus, IVIG-expanded Tregs inhibit effector T cell development in the peripheral lymphoid organs, instead of targeting their function in the intended organ [17].

Analogous to the in vivo experimental model, IVIG treatment also enhances the suppressive function of human Tregs. Kessel and colleagues [22] demonstrated an increase in the expression of TGF-β, IL-10, and FoxP3 in $CD4^+CD25^{high}$Tregs following IVIG exposure. Furthermore, IVIG-modulated Tregs efficiently decreased the TNF-α production by $CD4^+CD25^-$ effector T cells [22]. Similarly, an activation of nTregs with a twofold increase in FoxP3 expression was observed following in vitro culture of PBMCs in the presence of Tregitopes derived from IgG [18]. Interestingly, Tregitope-induced Tregs significantly reduced the IL-5 production and converted Th2 cells to iTregs in the cultures of PBMCs obtained from donors allergic to birch pollen [18]. Thus, IVIG-expanded Tregs are functionally efficient in controlling the exaggerated immune responses.

Mechanisms of Modulation of Tregs by IVIG

The possible mechanisms involved in the IVIG-mediated modulation of Treg functions are depicted in Fig. 1. IVIG has been demonstrated to interact directly with the $CD4^+CD25^+$Tregs and conventional $CD4^+CD25^-$T cell surface molecules in mice. However, binding of IVIG to Tregs was higher than conventional T cells. Interestingly, IVIG enhanced the proliferation of murine Tregs in vitro without inducing TGF-β and IL-10 [17]. Furthermore, IVIG contains natural autoantibodies reactive to self molecules and might influence the Treg activation and expansion via direct interaction of natural autoantibodies with cell surface molecules such as CD4, CD5, CD95, TCR, and MHC [1]. However, in view of the multiple factors that influence Treg development and function, and

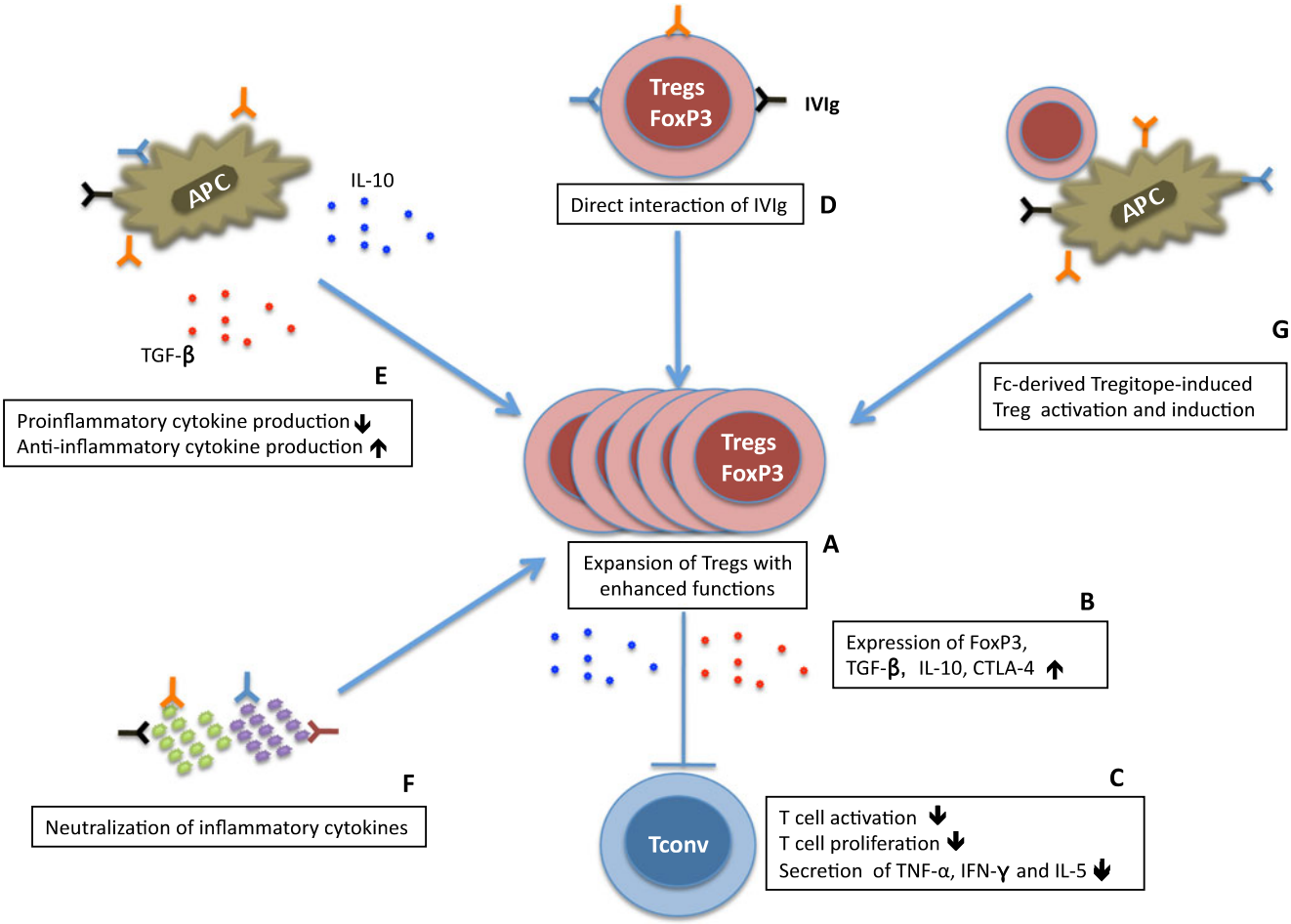

Fig. 1 The proposed mechanisms involved in the modulation of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) by IVIG. Exposure of Tregs to IVIG leads to expansion and enhanced suppressive function (**a**). These IVIG-modulated Tregs exhibit an increased expression of FoxP3, TGF-β, and IL-10, which are the mediators of suppressive functions of Tregs (**b**). IVIG-modulated Tregs are efficient inhibitors of conventional T cell (Tconv) activation, proliferation, and cytokine secretion (**c**). These effects of IVIG on Tregs might be mediated by direct interaction of self-reactive natural autoantibodies with T cell surface molecules (**d**). In addition, IVIG-mediated modulation of cytokine network as a result of altered cytokine production from antigen-presenting cells (*APC*) (**e**) and neutralization of inflammatory cytokines (**f**) can create a microenvironment favorable for Treg expansion that also enhances their suppressive function. Finally, the conserved T cell epitopes derived from the Fc region (Tregitopes) can induce and activate Tregs (**g**)

the diverse targets of IVIG, involvement of other cellular compartment and their cytokines in the modulation of Tregs by IVIG cannot be excluded [1, 8]. Accordingly, De Groot and colleagues [18] proposed a model where IgG-derived Tregitopes presented on MHC II+ Ag-presenting cells activate Tregs, leading to downregulation of effector cell activation and function via regulatory cytokine or contact-dependent signalling, or both. However, the model failed to explain the antigen dependence for activation/expansion of antigen-specific Tregs.

IVIG interacts with different innate immune cells like macrophages, DCs, NK cells, monocytes, and neutrophils to inhibit the production of proinflammatory cytokines (IL-1β, IL-6, IL-12, TNF-α), while favoring anti-inflammatory cytokines (IL-1RA, TGF-β, IL-10) [5]. IVIG may also contain anti-inflammatory cytokines like TGF-β, which can favor Treg induction. In addition, IVIG also contains neutralizing antibodies to several inflammatory cytokines [1]. Recently, monoclonal antibodies to inflammatory cytokines such as TNF-α and IL-15 have been successfully used in the treatment of several autoimmune diseases. Such therapy was associated with the induction of Tregs and the restoration of its functions [23]. Therefore, by modulating the inflammatory environment, IVIG may facilitate the expansion and enhanced functioning of Tregs.

The distinction between effects on nTregs versus iTregs (in humans, $CD4^+CD25^{high}$ cells are a mixture of both) and between the expansion of pre-existing $FoxP3^+$ cells versus their de novo conversion from conventional T cells is not always clear due to limitations of the experimental setup and the complexities of the human system. It is speculated that the interaction of IVIG with iTregs is also important in

humans in view of their potential role in regulating the pathogenesis of autoimmune diseases [24]. In the EAE model, $F(ab)_2$ and Fc preparations of IVIG did not differ in their protective effect and Treg induction [17]. In contrast, De Groot and colleagues [18] implicated Fc-derived Tregitopes in the activation and expansion of Tregs. Thus, the mechanisms underlying the IVIG-mediated modulation of Tregs might implicate multiple mechanisms depending on the pathology.

Conclusion

Tregs play an indispensable role in the maintenance of immune homeostasis and in the prevention of an autoimmune disease. Hence, the deficiency of Tregs or their functions lead to deleterious immune aggression that results in autoimmune and inflammatory diseases. IVIG, a widely used therapeutic preparation in several immune-mediated diseases, exerts immunomodulatory effects by targeting various soluble and cellular compartments of the immune system. Emerging research evidence has revealed the role of modulation of Tregs in the therapeutic effects of IVIG and represents a novel mechanism of action in T cell-mediated diseases. In view of rational therapeutic strategies that aim to enhance or restore Treg functions in the treatment of autoimmune diseases, IVIG proves to be a promising tool. However, despite the demonstration of expansion and enhanced suppressive functions of Tregs by IVIG, the underlying mechanisms are unclear. Thus, deciphering the active components of IVIG that mediate the interaction between Tregs and the molecular events involved is a priority in understanding the mechanisms of action of IVIG.

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