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The anti-inflammatory properties of intravenous immunoglobulin in a murine model of allergic airway disease
Effects on the development of regulatory T-cells

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Cette thèse intitulée:

The anti-inflammatory properties of intravenous immunoglobulin (IVIg) in a murine model of allergic airway disease
Effects on the development of regulatory T-cells

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Résumé

Les immunoglobulines intraveineuses (IVIg) constituent une préparation polyclonale d'IgG isolée et regroupée à partir du plasma sanguin de multiples donneurs. Initialement utilisé comme traitement de remplacement chez les patients souffrant d'immunodéficience primaire ou secondaire, les IVIg sont maintenant largement utilisées dans le traitement de plusieurs conditions auto-immunes, allergiques ou inflammatoires à une dose élevée, dite immuno-modulatrice. Différents mécanismes d'action ont été postulés au fil des années pour expliquer l'effet thérapeutique des IVIg dans les maladies auto-immunes et inflammatoires. Entre autre, un nombre grandissant de données issues de modèles expérimentaux chez l'animal et l'humain suggère que les IVIg induisent l'expansion et augmentent l'action suppressive des cellules T régulatrices (Tregs), par un mécanisme qui demeure encore inconnu. Également, les patients atteints de maladies auto-immunes ou inflammatoires présentent souvent un nombre abaissé de Tregs par rapport aux individus sains. Ainsi, une meilleure compréhension des mécanismes par lesquels les IVIg modulent les cellules T régulatrices est requise afin de permettre un usage plus rationnel de ce produit sanguin en tant qu'alternative thérapeutique dans le traitement des maladies auto-immunes et inflammatoires.

Par le biais d'un modèle expérimental d'allergie respiratoire induite par un allergène, nous avons démontré que les IVIg diminuaient significativement l'inflammation au niveau des voies aériennes ce, en association avec une différenciation des Tregs à partir des cellules T non régulatrices du tissu pulmonaire. Nous avons également démontré qu'au sein de notre modèle expérimental, l'effet anti-inflammatoire des IVIg était dépendant des cellules dendritiques $CD11c^{+}$ (CDs) pulmonaires, puisque cet effet pouvait être complètement reproduit par le

transfert adoptif de CDs provenant de souris préalablement traitées par les IVIg. À cet effet, il est déjà établi que les IVIg peuvent moduler l'activation et les propriétés des CDs pour favoriser la tolérance immunitaire et que ces cellules seraient cruciales pour l'induction périphérique des Tregs. C'est pourquoi, nous avons cherché à mieux comprendre comment les IVIg exercent leur effet sur ces cellules. Pour la première fois, nous avons démontré que la fraction d'IgG riche en acide sialique (SA-IVIg) (constituant 2-5% de l'ensemble des IgG des donneurs) interagit avec un récepteur dendritique inhibiteur de type lectine C (DCIR) et active une cascade de signalement intracellulaire initiée par la phosphorylation du motif ITIM qui est responsable des changements observés en faveur de la tolérance immunitaire auprès des cellules dendritiques et des Tregs. L'activité anti-inflammatoire de la composante SA-IVIg a déjà été décrite dans des études antérieures, mais encore une fois le mécanisme par lequel ce traitement modifie la fonction des CDs n'a pas été établi. Nous avons finalement démontré que le récepteur DCIR facilite l'internalisation des molécules d'IgG liées au récepteur et que cette étape est cruciale pour permettre l'induction périphérique des Tregs.

En tant que produit sanguin, les IVIg constitue un traitement précieux qui existe en quantité limitée. La caractérisation des mécanismes d'action des IVIg permettra une meilleure utilisation de ce traitement dans un vaste éventail de pathologies auto-immunes et inflammatoires.

Mots-clés : immunoglobulines intraveineuses, asthme, inflammation des voies respiratoires, récepteur lectine de type C, cellule dendritique, cellules T régulatrices, maladies auto-immunes et inflammatoires

Abstract

Intravenous immunoglobulin (IVIg) is a therapeutic preparation of normal human polyclonal IgG derived from pooled plasma from a large number of healthy donors. Initially used as replacement therapy for patients with primary and secondary immune deficiencies, IVIg is now also widely used for the treatment of a variety of autoimmune, allergic and systemic inflammatory disorders, at high immunomodulatory doses. The beneficial effect of IVIg in autoimmune and inflammatory diseases has been attributed to different mechanisms. Increasing evidence shows that IVIg induces expansion and enhances the suppressive function of regulatory T cells (Tregs) in different experimental animal models and human subjects, through an unknown mechanism. Human inflammatory and autoimmune diseases are known to be associated with Treg deficiency. Therefore, a more precise understanding of the mechanisms by which IVIg modulate Treg populations seems to be needed for more rational use of this compound as an alternative therapy in context of various inflammatory and autoimmune disorders.

Using a robust antigen-driven model of allergic airway disease, we have demonstrated that IVIg markedly attenuates airway inflammation and this effect is associated with the induction of Tregs from non-regulatory T cells in pulmonary tissues. We have also demonstrated that the anti-inflammatory actions of IVIg, in our model are dependent on a population of pulmonary $CD11c^+$ dendritic cells (DCs), as the action of IVIg could be completely replicated by adoptive transfer of $CD11c^+$ DCs from IVIg-treated mice. we have shown that tolerogenic DCs involve in the peripheral induction of Tregs. Given the requirement of DCs in the induction of Tregs, we explored the mechanism by which IVIg interacts and modulate these cells and for the first time demonstrated that the purified sialylated fraction of human IgG (SA-IVIg) (that consists 2-5% of

whole IgG) interacts with an inhibitory C-type lectin receptor on dendritic (DCIR) and this interaction triggers an ITIM intracellular signaling cascade. This subsequently results in rendering tolerogenic activities to DCs and peripheral induction of Tregs. The anti-inflammatory activity of SA-IVIg has been shown in previous studies, but the mechanism by which it modulates DCs functions is not well understood. We also demonstrated that DCIR facilitates the internalization of IgG molecules into DC and this internalization appears to be a crucial step for induction of Tregs.

IVIg is a costly therapeutic compound. Characterization of the mechanism of action of IVIg can lead to a better application of this plasma based therapy in a wide range of autoimmune and inflammatory diseases.

Keywords: intravenous immunoglobulin, asthma, airway inflammation, C-type lectin receptor, dendritic cell, regulatory T cell, autoimmune and inflammatory disease

Contributions of Authors

Article I

Gabriel N. Kaufman performed the research resulted in the generation of figures 1a,b,c, 3 and 4 and Table 1 and made the main contribution in writing and editing the manuscript. Amir Massoud carried out all the experiments resulted in the generation of figures 1d-g, 2, 5, 6 ,7, and participated in writing and editing the manuscript. The coauthors: Andree-Anne Banville-Langelier, Yufa Wang, Julie Guay and Jonathan A. Garellek, were given authorship in acknowledgement for their roles in teaching and performing experiment in flow cytometry, ELISA and RT-PCR in this study. Severine Audusseau performed the proliferation assays. Drs. Christine McCusker, Walid Mourad and Ciriaco Piccirillo were integral for their mentorship and advises on this project and aided in editing process of this manuscript. Dr. Bruce Mazer was the principal investigator and responsible for generating the main hypotheses and coordinating this project. All authors reviewed the manuscript and had access to primary data.

Article II

Dr. B. Mazer and Dr. C. Piccirillo and Amir Massoud were responsible for generating the main hypotheses and designing the experimental work and had a direct supervision on some experimental works employed to examine these hypotheses. Amir Massoud had the major contribution in writing and editing the manuscript. Julie Guay, and Karim Shalaby and Yasaman Nouhi were acknowledged as a coauthor for their role in teaching and helping with RT-PCR, flow cytometry and FlexiVent. Aidan Ablona and Daniel Chan made contributions in making solutions and culturing cells. Eva Bjur aided with breeding and providing the Tg mice used in

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Article III

Amir Massoud and Dr. Bruce Mazer were responsible for generating the main hypotheses, designing the experimental work, had direct supervision on all the experiments employed to examine these hypotheses and played the main role in writing and editing the manuscript. Madelaine Yona took an active role in helping with some experiments and preparing some solutions and contributed in editing the manuscript. Drs. Walid Mourad and Ciriaco Piccirillo were integral for their mentorship and advices on this project as well as in the editing process of this manuscript. Dr. Bruce Mazer was the principal investigator and supervised and coordinated this project. All authors reviewed the manuscript and had access to primary data.

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General discussion

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List of Abbreviations

(E): Elastance

(R): Resistance

^{3}[H]thy: tritiated Thymidine

Ab: Antibody

ADCC: Antibody-dependent cytotoxicity

Ag: Antigen

AHR: Airway hyperresponsiveness

APC: Allophycocyanin

APCs: Antigen presenting cells

B6: C57BL/6 mice

BHR: Bronchial hyperresponsiveness

BM-DC: Bone marrow-derived dendritic cells

CD: Clusters of differentiation

CDR: Carbohydrate recognition domain

CFSE: Carboxyfluorescein succinimidyl ester

CHO: Chinese hamster ovary

CIDP: Chronic inflammatory demyelinating polyneuropathy

CLR: C-type lectin receptor

CLR: C-type lectin receptor

CNS**:** Central nervous system

CON A: Concanavalin A

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

DAB 1: Diaminobenzidine

DC: Dendritic cell

DCIR: Dendritic Cell Immunoreceptor

DEAE: Diethylaminoethanol

dLN: Draining lymph node

dsDNA: Double stranded DNA

EAE: Experimental autoimmune encephalomyelitis

ECL: Chemoluminescence

ELIZA: Enzyme-linked immunosorbent assay

Fab: Fragment antigen-binding

FBS: Fetal bovine serum

Fc: Fragment crystallizable

FcγR: Fc-gamma receptor

FEV1: Forced expiratory volume

FITC: Fluorescein isothiocyanate

Foxp3: Forkhead box protein 3

GBS: Guillain–Barré syndrome

GFP: Green fluorescent protein

GITR: Glucocorticoid-induced TNFR-related protein

GM-CSF: Granulocyte macrophage colony-stimulating factor

H&E: Hematoxylin and eosin

HIV: Human immunodeficiency virus

HLA: Human leukocyte antigen

HSA: human serum albumin

HSV: Herpes simplex virus

I.N: Intranasal

I.P: Intraperitoneal

I.T. Intratracheal

IFN: Interferon

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IHC: Immunohistochemistry

IL: Interleukin

IPEX: Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

ITAM: Immunoreceptor tyrosine-based activation motif

ITIM: Immunoreceptor tyrosine-based inhibition motif

ITP: Immune thrombocytopenic purpura

iTreg: induced regulatory T cells

IVIg: Intravenous immunoglobulin

IVIg: Intravenous immunoglobulin

KD: Kawasaki disease

KD: Knockdown

KD-DC: Knockdown dendritic cells

KO: Knockout

KO-DC: Knockout dendritic cells

LN: Lymph node

MCh: Methacholine

MHC: Major histocompatibility complex

MLR: Mixed leukocytes reactions

MS: Multiple sclerosis

MZ: Marginal zone

NTN: Nephrotoxic nephritis

nTreg: Natural occurring regulatory T cells

OVA: ovalbumin

PBS: Phosphate buffered saline

PBS: phosphate-buffered saline

pDC: Plasmacytoid dendritic cells

PE: Phycoerythrin

PEEP: Post-expiratory end pressure

PHA: Phytohaemagglutinin

PWM: Pokeweed mitogen

RT-PCR: Reverse transcription polymerase chain reaction

RW: Ragweed

SA-IVIg: Sialic-acid enriched IgG

siRNA: Small interfering RNA

SLE: Systemic lupus erythematosus

SOCS: Suppressor of cytokine signaling

TCR: T cell receptor

Teff: Effector T

TH: T helper

TNF: Tumor necrosis factor

TNF: Tumor necrosis factors

Treg: Regulatory T-lymphocytes

WT: Wild type

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Chapter I

Introduction

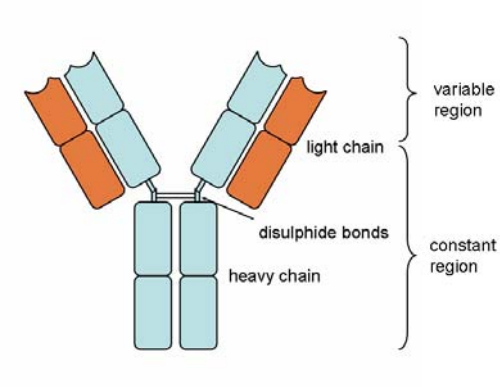
Review of the literature

1- A history of Intravenous Immunoglobulin and its clinical applications

Intravenous immunoglobulin (IVIg) is a therapeutic preparation of normal human polyclonal IgG antibodies obtained from pooled plasma samples of more than 10000 healthy blood donors.

Antibodies are glycoproteins belonging to the immunoglobulin superfamily. They are produced by B-lymphocytes and are typically made of basic structural units, each with two large heavy chains and two smaller light chains. Immunoglobulin is composed of two fragments, known as Fab and Fc (Fig.1 A). The Fab region is the variable part of the molecule, capable of recognizing specific antigens, and the Fc region is the constant region of immunoglobulin, with binding capacity to Fc-receptors on various immune cells. There are different isotypes of antibody heavy chains, and immunoglobulin molecules are grouped into these different isotypes based on which heavy chain they possess. Five different antibody isotypes are identified in mammals; immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin D (IgD), immunoglobulin G (IgG) and immunoglobulin E (IgE) (Fig.1 B).

A)



B)

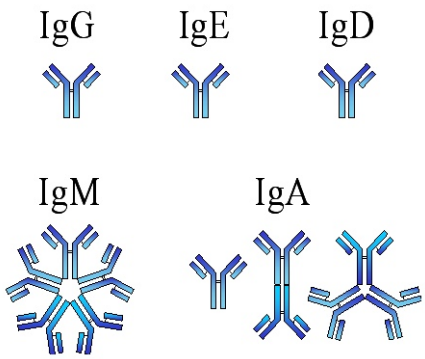


Figure 1. Schematic representations of IgG structure and five immunoglobulin isotypes. Adopted from: Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001.

A) Schematically representation of the structure of conventional IgG consisting of a light and heavy chain.

B) The five major isotypes of immunoglobulin structure. The basic functional unit of each antibody is a monomer immunoglobulin. Secreted antibodies can form dimeric structures as with IgA or pentameric structures with five immunoglobulin units such as IgM.

Commercially available IVIg consists of ≥96% IgG, representing the normal IgG subclass distribution of human serum (Burckhardt et al., 1989), which includes IgG1, IgG2, IgG3 and IgG4. Each subclass serves somewhat different functions in protecting the body against infectious agents. For example, the IgG1 and IgG3 subclasses are rich in antibodies against proteins such as the toxins produced by the diphtheria and tetanus bacteria, as well as antibodies against viral proteins. In contrast, antibodies against the polysaccharide (complex sugar) coating (capsule) of certain disease-producing bacteria (e.g. pneumococcus and Haemophilus influenzae) are predominantly of the IgG2 type in older children and adults. Some of the IgG subclasses can easily cross the placenta and enter the unborn infant's bloodstream, while others do not. IgG1 and IgG3 subclasses interact readily with the complement system, while others interact poorly, if at all, with complement proteins. Thus, an inability to produce antibodies of a specific class or subclass may render the individual susceptible to certain kinds of infections but not others (Boyle et al., 2006).

Immunoglobulin products from human plasma were first used in 1952 as a method of antibody replacement therapy for the treatment of primary immune deficiencies such as agammaglobulinemia or hypogammaglobulinemia, deficiencies in immunoglobulin production characterized by recurrent bacterial infections (Knezevic-Maramica and Kruskall, 2003). By providing antibodies to patients who have weakened immune systems, IVIg can help reduce the risk of infection. These first immunoglobulin transfusions were administered intramuscularly which limited the dosage that could be given.

Since the introduction of immunoglobulin by the intravenous routes, more aggressive dosing strategies have become possible. IVIg is increasingly being used as a method of treatment for a

wide range of autoimmune and inflammatory diseases, due to its immune-regulatory actions, when administered in high doses (generally 1-2 grams/kg body weight, as opposed to 200–400 mg/kg body weight in immune deficiency conditions). The immune-modulatory activity of IVIg in autoimmune disorders was initially discovered in 1981, by Imbach et al. (Imbach et al., 1981) who observed a rise in platelet counts after administrating IVIg to patients with congenital agammaglobulinemia who were also thrombocytopenic. In the same year, Imbach reported the successful treatment of immune thrombocytopenic purpura, an autoimmune condition resulting in low platelet counts, by administration of IVIg. Since then the efficacy of IVIg, as a therapeutic compound with immune-regulatory activities, has been reported in various autoimmune disorders, chronic inflammatory diseases (Emmi and Chiarini, 2002; Hemming, 2001; Kaveri et al., 1997; Shoenfeld and Katz, 2005), and neurologic pathologies (Dalakas, 1997).

IVIg is mainly used as treatment in three major categories:

a) Primary and secondary immune deficiencies

b) Autoimmune and inflammatory diseases

c) Acute infections

The US Food and Drug Administration has approved the use of IVIg for the following 8 conditions:

1. Primary immunodeficiency states
2. Chronic inflammatory demyelinating polyneuropathy (CIDP)
3. Immune thrombocytopenic purpura (ITP)
4. Secondary immunodeficiency in chronic lymphocytic leukemia

5. Pediatric human immunodeficiency virus (HIV) infection
6. Kawasaki Disease (KD)
7. Guillain-Barre Syndrome (GBS)
8. Prevention of graft vs. host disease in an adult bone marrow transplant recipients

Given the broad action of IVIg, it can also be used to treat a variety of other conditions, in which controlled trials establishing the safety and efficacy are still needed. Some of these off-label uses are:

1. Neurological diseases: Myasthenia gravis, Lambert-Eaton myasthenic syndrome, multifocal motor neuropathy and multiple sclerosis.
2. Dermatological diseases: Autoimmune blistering dermatoses, dermatomyositis, pemphigus, pemphigoid.
3. Rheumatological diseases: Rheumatoid arthritis (adult and juvenile), systemic lupus erythematosus, systemic vasculitides, dermatomyositis, polymyositis, inclusion-body myositis and wegeners granulomatosis.
4. Hematological diseases: Aplastic anemia, pure red cell aplasia, Blackfan-Diamond anemia, autoimmune hemolytic anemia, hemolytic disease of the newborn, acquired factor VIII inhibitors, acquired von Willebrand disease, immune mediated neutropenia and neonatal alloimmune/ autoimmune thrombocytopenia.
5. Infectious diseases: Acquired infectious diseases that could be deleterious in low birth weight baby (ie, less than 1500 g), extensive burns and HIV infection.
6. Respiratory disease: Asthma.

7. Miscellaneous: Acute idiopathic dysautonomia, acute disseminated encephalomyelitis, hemophagocytic syndrome, multiple myeloma, recurrent pregnancy loss and POEMS syndrome (or Crow–Fukase syndrome, a rare medical syndrome defined as the combination of a plasma-cell proliferative disorder typically myeloma and polyneuropathy, and effects many organs).

The half-life of IgG molecule is approximately 21-29 days, except in bone marrow transplant patients and febrile and septic patients who may have a hypermetabolic state which decreases the half-life of IgG to 10-14 days (Ramos-Medina et al., 2012). However, the effectiveness of IVIg therapy can last between 2 weeks and 3 months, suggesting potential alteration of immunoglobulin metabolism following infusion of IVIg.

Studies exploring the mechanisms of action of IVIg demonstrated the efficacy of IVIg therapy in various animal models of autoimmune and inflammatory diseases, which support the anti-inflammatory effect of this therapeutic compound in human trials. IVIg treatment has protective effects in murine models of severe coronary arthritis (a murine model of KD) (Duong et al., 2003). IVIg has also protective effects in murine model of ITP by rapidly raising the platelet counts (Tremblay et al., 2012). In experimental autoimmune encephalomyelitis (EAE) (the murine model of MS) infusion of IVIg is associated with reduced disease severity and improvement of underlying CNS pathology (Humle Jorgensen and Sorensen, 2005). IVIg therapy also results in reduction of anti-dsDNA antibody titers and improvement of renal pathology in NZB/W mice, (a mouse model for SLE), (Shoenfeld et al., 2002). In an allergen-driven murine models of allergic asthma, rabbit IgG also displayed anti-inflammatory activity, by decreasing airway inflammation and improving lung pathology (Yamamoto et al., 2010). The

protective effects of IVIg in murine models of inflammatory arthritis have also been elucidated (Kaneko et al., 2006).

IVIg has become the major plasma product on the global blood product market. The worldwide consumption nearly tripled between 1992 and 2003, from 19.4 to 52.6 tons per year (Buchacher and Iberer, 2006). The per capita use of IVIg in Canada increased by approximately 83% between 1998 and 2004 (and another 18% between 2004 and 2006), thus making Canada one of the highest per capita users of IVIg in the world. The demand is still increasing due to the expanding number of diseases that may be successfully treated by IVIg, and because IVIg treatment is well-tolerated. It is believed that the increased use of IVIg is, at least in part, attributed to the off-label use. However, due to the high cost of this plasma based therapy, as well as the increasing demand for human plasma, IVIg therapy requires careful study to ensure that it is used judiciously. In the next section we will describe the steps that are taken in the purification of IVIg from plasma.

2- Purification of IVIg from human plasma

IVIg is a product of immunoglobulin fractions isolated from the pooled plasma of human blood donors. Cohn ethanol fractionation is the dominant method for the isolation of IVIg; despite its low yield, it is easy to perform and also inactivates most human viruses including HIV (Pyne et al., 2002). In this method, the different fractions of plasma proteins are sequentially precipitated using increasing concentrations of ethanol. In general, the IgG yield of ethanol fractionation is between 3.5 and 4.2 g/L plasma as 40 to 50% of IgG are lost in the non-IgG supernatants or are co-precipitated with the impurities (Buchacher and Iberer, 2006).

To increase the efficiency of IgG recovery, an ion exchange chromatography step is added following the conventional ethanol fractionation. Ion exchange chromatography increases the purity of isolated IgG to approximately 97%. The IgG containing solution is first loaded to a DEAE sepharose column at pH 5.2. Next, the solution is transferred to a macropourous anion-exchange resin column at pH 6.5. Methods of virus inactivation (including pasteurization and treatment at low pH) are also integrated in the process (Hoppe et al., 1973).

Affinity chromatography using Protein A or G is another very efficient tool for the purification of different subclasses of IgG. However, for purification of large amounts of polyclonal IgG, this method becomes very costly (Schwart et al., 2001).

IVIg preparations contain monomeric and dimeric IgG molecules, which are in a dynamic equilibrium depending on concentration, pH, temperature, donor pool size, time and stabilizers added in order to keep the portion of dimeric IgG below a certain level (Bayry et al., 2004). Monomers represent the monomeric IgG (molecular weight approximately 150 KDa) and dimers represent a complex of 2 IgG molecules which can be oriented in various ways, including typical idiotype/anti-idiotype binding. Sugars, such as sorbitol, maltose, and sucrose, or amino acids are added to some formulations to prevent aggregate formation. The pH also affects stability of IVIg compound, and usually is adjusted to between 4.0-4.5 (Gelfand, 2006).

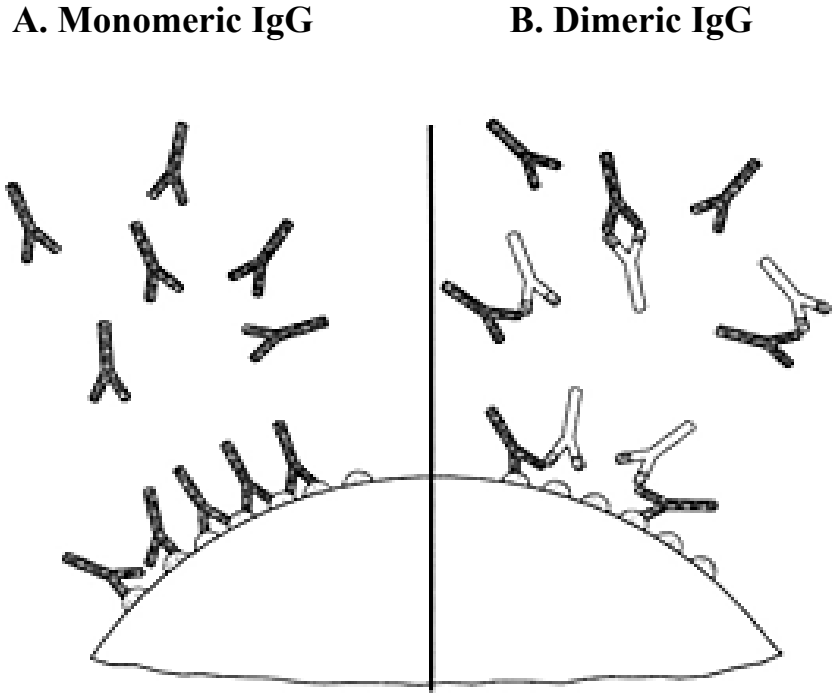


Figure 2. Representation of monomeric and dirmeric IgG

A. Monomeric IgG recognizes antigens on the cell surface of a cell by their variable regions.
B. The variable region of the anti-idiotypic antibodies in the immunoglobulin preparation binds to the circulating idiotypes and prevents them from reacting with the autoantigen.

Size exclusion chromatography is the final phase of the separation process and allows for the separation of different IgG fractions based on size. Monomeric and dimeric fractions are isolated by this method. In size exclusion chromatography the purification processes is followed by two more chromatography steps; the first using Q-Sepharose or CM-Sepharose columns and then on Sephacryl S-300 containing columns (Bayry et al., 2004). After these steps, sugars are added to the purified IgG to stabilize the preparation.

Commercially available IVIg can vary from one another depending on the procedures taken by manufacturers that might cause slight differences in the specific composition of the final product. These differences in composition can have clinical impact when used in therapy and hence need to be taken into account in evaluation of the clinical efficacy and safety of different IVIg products.

3- Side effects

IVIg therapy is generally a well-tolerated treatment with side effects occurring in less than 5% of patients (Levy and Pusey, 2000). Differences in manufacturing strategies may alter product efficacy and tolerability. The approaches used to minimize formation of IgG aggregates and stabilize preparations account for most of the variations in composition. Product features that are important to consider include sugar content, sodium concentration, volume load, osmolality, and amount of IgA (Carbone, 2007). The carbohydrate content of IVIg is particularly important in patients with diabetes mellitus; glucose-containing products may acutely affect glycemic control (Orbach et al., 2004). In addition, both sugar and sodium contents affect osmolality, and increased osmolality may be of concern in patients with a history of renal dysfunction, elevated blood pressure or heart failure (Ballow, 2007). Lyophilized products needing reconstitution also

tend to have higher osmolality than liquid formulations of IVIg. Patients who cannot tolerate increased osmolality may not tolerate a high volume load (Ballow, 2007).

Most adverse effects associated with IVIg administration are mild and transient and occur typically right after infusions. This may include chills, fever, headaches, fatigue, rigors, tremors, nausea, myalgia and malaise (Stangel and Pul, 2006). More serious adverse events occur less frequently and include anaphylaxis, aseptic meningitis, acute renal failure, and venous thromboembolism (Gelfand, 2006). IVIg preparations contain a small amount of IgA, and patients with IgA deficiency rarely may be at risk of anaphylaxis because of their production of IgE anti-IgA antibodies, particularly with prior exposure to blood products containing IgA. Anaphylaxis is rare, however, and most patients with IgA deficiency tolerate products with low IgA content (Gelfand, 2006). All patients receiving IVIg should have their vital signs checked at least every 15 minutes during the first hour of infusion and periodically thereafter (Murphy et al., 2005). Surveillance for tolerability and adverse events should be done judiciously with each infusion session. Pretreatment with acetaminophen, antihistamines, such as diphenhydramine, or corticosteroids may help prevent or minimize adverse events. Markers of renal function should be monitored before initiating IVIg therapy and periodically thereafter. (Sederholm, 2010).

4- Anti-inflammatory Mechanisms of IVIg

High dose IVIg is used to treat a wide spectrum of autoimmune and inflammatory diseases. Several mechanisms of action have been advanced, in different contexts, to explain the immune-regulatory actions of IVIg. The proposed mechanisms involve most of the components of the immune system and include; blockage of mononuclear phagocytic system (by saturating Fcγ-receptors), autoantibody neutralization by anti-idiotype antibodies, accelerated pathogenic

autoantibody clearance by saturation of the neonatal Fc receptor, modulation of cytokine networks, neutralization of complement components or other inflammatory mediators, and attenuation of antigen presenting cell (APCs), T or B-lymphocyte activation. In this section the most important accepted immune-regulatory explanations for the action of IVIg are summarized.

4-1- The importance of "natural antibodies" for the immune-regulatory actions of IVIg

IVIg preparations are composed of pooled antibodies from over 10,000 human blood donors, and thus, contain natural antibodies (antibodies that are produced without any previous infection, vaccination, foreign antigen exposure or passive immunization) originating from these donors. These natural antibodies may account for some of the beneficial therapeutic effects of IVIg.

In the absence of circulating IgG, such as in X-Linked Agammaglobulinemia (XLA) which is associated with a deficiency of mature B-cells, autoimmune pathologies are more prevalent (Etzioni, 2003), and interestingly, administration of IVIg may ameliorate this condition.http://bloodjournal.hematologylibrary.org/content/111/2/715.long - ref-33 Similarly, B-cell deficient mice, when induced with Experimental Auoimmune Encephalitis (EAE), fail to recover, unlike their WT counterparts (Dittel et al., 2000). These observations indicate a dysregulation of the immune system in the absence of natural antibodies. Thus, the protective effects of natural antibodies could reflect an important physiologic phenomenon.

The mechanism of action of natural antibodies is proposed to be dependent mainly on the $F(ab')^2$ fragment of IgG (Vani et al., 2008). IVIg contains different subsets of natural antibodies, including neutralizing antibodies that are able to interact with soluble and cell surface antigenic determinants. Neutralizing IgG in IVIg preparations can scavenge inflammatory molecules, such

as complement components and pro-inflammatory cytokines. Infusion of IVIg can result in rapid changes in the serum levels of pro- and anti-inflammatory cytokines (Gonzalez et al., 2004). IVIg has been shown to selectively trigger the production of interleukin-1 receptor antagonist, the natural antagonist of IL-1 (Aukrust et al., 1999) and also contains neutralizing antibodies to IL-1α. IVIg also has been shown to bind to a large number of foreign, cellular, and soluble antigens (reviewed by Crow and Lazarus and others) including the T-cell receptor beta chain, CD5, CD4, granulocyte-macrophage colony-stimulating factor, HLA class I molecule, glycolipids, superantigens, DNA, phospholipids, Fas and Fas-Ligand, T-cell receptors, IgE Fc receptors, galactose disaccharides, thyroglobulin, ferritin, red blood cells, CD40 and pathogenic organisms and their antigens (Hurez et al., 1994; Kaveri et al., 1996; Lazarus, 2010; Svenson et al., 1998; Vassilev et al., 1993; Viard et al., 1998).

IgG molecules are known to bind to a variety of targets, and therefore, are capable of forming dimers and aggregates in the form of small immune complexes through binding to molecule(s) in the serum (Siragam et al., 2006). These dimers have been hypothesized to contribute to the beneficial effects of IVIg in a manner dependent on activating Fcγ receptors (Park-Min et al., 2007) which will be addressed later.

Each group of neutralizing antibodies has its unique biological function, which may contribute to the modulatory effects of IVIg. For example, the presence of autoantibodies against Fas in IVIg was responsible of inducing caspase-dependent cell death in Fas-sensitive leukocytes (Reipert et al., 2008). Other important subsets of natural antibodies within IVIg preparation are anti-glycan antibodies that can of bind to a broad range of carbohydrate structures. The majority of carbohydrate-specific IgG antibodies are from IgG2 subclasses (von Gunten et al., 2009). Some

glycans can elicit an immune response which results in the production of antibodies, particularly when glycans are associated with carrier proteins.

IVIg also contains significant amount of anti-idiotype antibodies (Fig. 2). The body normally produces small amounts of autoantibodies to protein sequences. In turn, anti-idiotype antibodies are produced to facilitate the inhibition and clearance of autoantibodies, and prevent initiation of immunologic responses to self-antigens. In normal serum, very small amounts of both auto/idiotype and anti-idiotype antibodies can be detected. IVIg may contain numerous versions of these natural antibodies, capable of reacting with many thousands of epitopes. Anti-idiotype antibodies present in the pool of IgG can manipulate the immune system by inhibiting the binding of autoantibodies to their cognate antigens, cellular receptors, cytokines and many other immune mediators (Vani et al., 2008), and therefore maintain immune homeostasis.

4-2- Modulatory effect of IVIg on adaptive immunity

The main components of the adaptive immune system are B-lymphocytes (which produce immunoglobulins) and T-lymphocytes (responsible for cell-mediated immunity). Adaptive immunity also relies on the capacity of Antigen Presenting Cells (APC) that present antigens to T-cells, bridging innate immunity to the adaptive arm. Adaptive immune responses are initiated after T-cell recognition of an epitope within the context of major histocompatibility complex (MHC) molecules expressed on the surface of APC. This antigen-mediated T-cell activation triggers the differentiation of naive T cells into effector T cells, the expansion of the effector T cell repertoire and the subsequent activation of other immune cells. $CD4^+$ T cells are key effectors of the adaptive immune system, driving maturation and development of other cells, thus giving them the title of helper T cells (Th cells). Upon activation, naïve $CD4^+$ T cells

differentiate into different subsets of effector T cells with distinct functions. This differentiation is also governed by the cytokine milieu, and is regulated by activation or production of lineage-specific transcription factors within T cells (Reiner, 2009). The major subsets of helper T cells include Th1, Th2, regulatory T cells (Treg) and Th17 cells (Reiner, 2009).

4-2-1- Modulatory effects of IVIg on helper T-cell subsets

IVIg may directly interact with different subsets of T cells, attenuating production of pro-inflammatory cytokines, such as IL-2, IFN-γ and TNF-α, while enhancing the level of inhibitory cytokine production, such as IL-10 (Pashov et al., 1997; Pashov et al., 1998).

In addition, IVIg modulates activation and proliferation of T cells. Amran et al. (Amran et al., 1994) showed that addition of IVIg to anti-CD3 and tetanus-activated T cells inhibited the proliferation of these cells in a dose-dependent manner. Addition of exogenous IL-2 or IL-4 to these activated T cell cultures reversed the inhibitory effect of IVIg. This suggested that IVIg might interfere with cytokines, leading to attenuation of T cell proliferation. MacMillan et al. (MacMillan et al., 2009) showed that $F(ab')^2$ fragments of IVIg were sufficient to inhibit T-cell proliferation, following stimulation with super antigens; the presence of APC was not required for this inhibition. This suggested that IVIg reduced polyclonal T-cell activation by directly affecting T cells. IVIg can also attenuate APC activation and thus indirectly arrest T cell responses.

IVIg contains antibodies that recognize T-cell recognition and activation molecules, including CD4, CD5, and the T cell receptor (TCR) (Hurez et al., 1994; Lake et al., 1995). Anti-CD4 antibodies isolated from IVIg by affinity-chromatography were able to bind human $CD4^+$ T cells

and inhibit their activation (Hurez et al., 1994). These anti-CD4 antibodies inhibited proliferative responses in mixed leukocytes reactions and in proliferation following in vitro HIV-infection of human $CD4^+$ T cells (Hurez et al., 1994). Therefore, the presence of anti-CD4 antibodies in IVIg may be relevant to its immune-regulatory actions. IVIg has also been found to contain anti-CD5 antibodies, and therapeutic concentrations of IVIg inhibited the binding of CD5 monoclonal antibodies to CD5-expressing B and T cells in culture (Vassilev et al., 1993). The anti-CD5 antibodies present in IVIg may be important in modulating autoimmune responses, for example, by inhibiting cytotoxic T cells or autoantibody-producing B cells. Using a biosensor analysis system, it was demonstrated that a subset of antibodies present in therapeutic IVIg can bind to nonpolymorphic, highly conserved residues on HLA-B7 class I molecules (Kaveri et al., 1996). This sequence is likely to be involved in the interaction of class I molecules with the cytotoxic T-cell receptors and this interaction may be essential in the maturation of $CD8^+$ T cells (Kaveri et al., 1996). IVIg also contains trace amounts of soluble CD4, CD8, and HLA antigens, which may act as decoy ligands, interfering with antigen-mediated T cell activation and contributing to immunosuppression (Blasczyk et al., 1993; Lam et al., 1993). In addition, a number of other studies have demonstrated the inhibitory effect of IVIg in expression of activation markers by T cells, such as CD25 or CD69 (Heidt et al., 2009; Klaesson et al., 1993; Kondo et al., 1991; MacMillan et al., 2009; Stohl, 1985; Tawfik et al., 2012; Tha-In et al., 2006; Toyoda et al., 1994).

IVIg may contribute to the suppression of T cell differentiation to pro-inflammatory subsets. Maddur et al. (Maddur et al., 2011) demonstrated that IVIg inhibited differentiation of naïve $CD4^+$ T cells, stimulated to differentiate to Th17 cells, and suppressed the lineage-specific transcription factor ROR-gamma production within stimulated cells (Maddur et al., 2011). This

was mediated by the $F(ab')^2$ fragment of IVIg. Interestingly, in this study pure $CD4^+$ T cells were used in the absence of other accessory immune cells (i.e. APC), suggesting that the inhibition of Th17 differentiation was achieved by specific interaction of $F(ab')^2$ fragments to T-cells. This suggests a protective effect of IVIg in Th17 cell-mediated autoimmune conditions.

4-2-2- Modulatory effects of IVIg on B cells

The immune-regulatory activities of IVIg might involve inhibition of humoral immunity and immunoglobulin production by B cells, through various proposed mechanisms.

B cells, which represent 5-15% of the circulating lymphoid pool, are responsible for antibody production, which acts against extracellular pathogens. B cells are activated in response to a variety of stimuli and ultimately differentiate to memory B cells and plasma cells. Plasma cells are usually restricted to the secondary lymphoid organs, comprising less than 0.1% of the lymphocytes in circulation. Autoreactive B cells (B-cells producing auto-antibodies) may be stimulated by either autoantigens or through non-specific polyclonal activation. Soluble immunoglobulins, produced by plasma cells against autoantigens, are responsible for the majority of clinical features in antibody mediated autoimmune diseases (Jacob and Rajabally, 2009).

The interaction of anti-idiotypic antibodies, present in the IVIg pool, to auto-idiopathic or B cell receptors (BCR) contributes to modulation of humoral immunity in autoimmunity (Durandy et al., 2009). Dussault et al. (Dussault et al., 2008) have further demonstrated that interaction of IgG (in dimeric forms) with B cells receptors (BCR) results in cross-linking and phosphorylation of ERK1/2 (which is downstream of the BCR) and subsequently represses B cell activation and

immunoglobulin production. Stohl et al. (Stohl, 1985) demonstrated that monomeric IgGs within IVIg can inhibit polyclonal immunoglobulin production by normal peripheral blood mononuclear cells (PBMCs) stimulated with pokeweed-mitogen (PWM), which might contribute to the prevention of pathogenic autoantibody production by B cells.

Other immune-regulatory effects of IVIg that can affect humoral immunity include inhibition of B cell differentiation (Kondo et al., 1994; Stohl and Elliot, 1996), inhibition of interleukin-6 and TNF-α production (two crucial mediators in activation of humoral immunity) (Sundblad et al., 1994), induction of B cell apoptosis via binding with FAS-L (Toyoda et al., 2003) and down-regulation of specific autoreactive B cells (Sigman et al., 1998; Vassilev et al., 1999; Zhuang et al., 2002).

A recent study by Proulx et al. (Proulx et al., 2009) demonstrated that culturing purified human B cells in the presence of therapeutic concentrations of IVIg resulted in internalization of different subsets of IgG into the cells, that subsequently interact with a series of intracellular proteins involved in antigen processing and presentation, such as MHC I and II molecules. This internalization has been shown to be independent of BCR and/or Fcγ receptors (Proulx et al., 2009). This may lead to alteration in antigen processing and presentation, leading to attenuation of the subsequent T cell activation by the affected cells.

Despite the evidence of inhibitory effects of IVIg on B and T lymphocytes via direct interaction with surface molecules or inhibition of pro-inflammatory mediators secreted by those cells, newer data may require a reevaluation of these concepts. Padet et al. (Padet et al., 2011) have demonstrated that IVIg preparations contain subsets of IgG that are specific for phytohaemagglutinin (PHA), Concanavalin A (Con A) and PWM (pokweed mitogen), which are

frequently used as polyclonal stimulators in *in vitro* studies, and whose effects are neutralized by addition of IVIg to culture. Furthermore, they have shown that removal of PHA- or Con A-reactive IgG from IVIg results in a loss of inhibitory activity of IVIg on T cells *in vitro*. This finding challenges the previously accepted notion that IVIg exerts its anti-inflammatory effects by acting directly on T cells and suggests that effects of IVIg observed in T and B-cell inhibition *in vitro* is rather a consequence of the interaction of IVIg with polyclonal stimulators.

4-2-3- IVIg-mediated regulation of Antigen-Presenting Cells

IVIg may exert its immune-regulatory effects on antigen-presenting cells (APCs), particularly dendritic cells (DC). An essential step in the immunopathology of autoimmune disease involves APCs presenting antigen to autoantigen-specific $CD4^+$ T helper cells. Thus, it is of interest to examine IVIg-mediated modulatory effects on APCs.

IVIg treatment of DC *in vitro* inhibits the differentiation and maturation of DC and reduces the capacity of mature DC to secrete pro-inflammatory cytokines upon stimulation, while enhancing the production of IL-10 (Bayry et al., 2003a) (an inhibitory cytokine which is crucial for maintenance of peripheral tolerance).

While the direct effect of IVIg on T cells has been demonstrated to be dependent on the $F(ab')^2$ fragment of IgG (as there are no Fc-receptors on T-cells), the effect of IVIg on APCs is mainly attributed to the Fc fragments, although some studies have implicated the $F(ab')^2$ fragment (Fig. 3-2) (Bayry et al., 2003a).

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Natural antibodies in the serum of healthy individuals (and therefore in the IVIg pool) contain self-reactive antibodies that target soluble molecules or cell surfaces antigens. Bayry et al. (Bayry et al., 2004) identified natural antibodies reactive with the CD40 molecule as an important participant in differentiation of DC from monocytes (Fig. 3-1). Treating DC with anti-CD40 monoclonal antibodies results in enhancing their activation and maturation whereas, Bayry et al. have shown that maturation of DC in the presence of CD40-reactive natural antibodies derived from IVIg decreased, accompanied by increased production of IL-10 and decreased IL-12 (Fig. 3-2). They also demonstrated that activation of cyclic adenosine monophosphate (cAMP)-response element-binding protein (CREB-1), involved in the initiation of transcriptional activation of the IL-10 gene, increased in IVIg-treated DC.

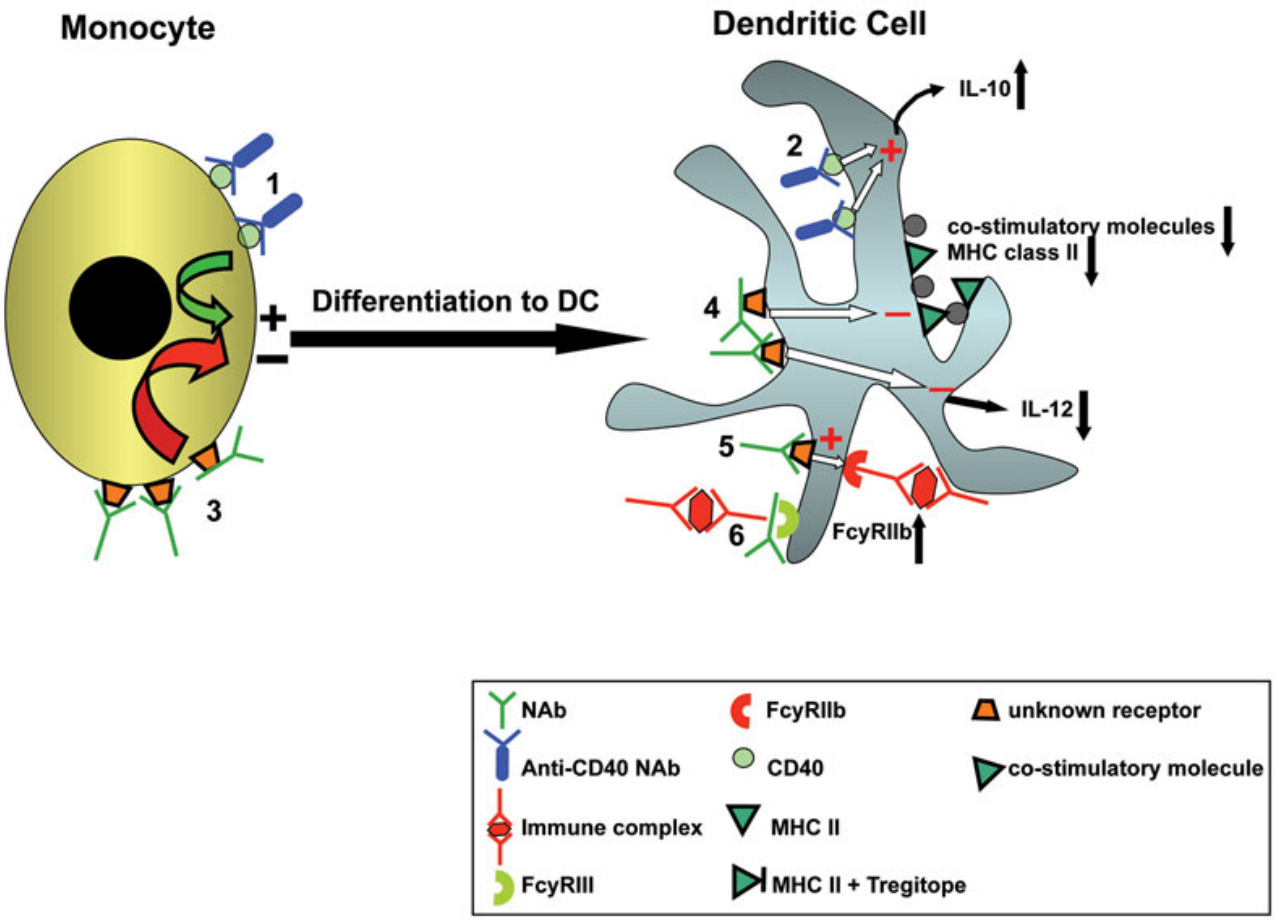

Figure 3. Effects of IVIg on differentiation and functions of dendritic cells. Adopted from (Kwekkeboom, 2012)

1) Anti-CD40 Abs stimulate the differentiation of monocytes to DC. 2) Anti-CD40 Abs stimulate production of anti-inflammatory IL-10 by DC. 3) Other Abs inhibit differentiation of monocytes to DC by binding to (an) unknown receptor(s). Abs may bind either via their $F(ab')^2$ part (by specific recognition of the receptor as an antigen) or via their Fc-part to such receptor(s). 4) Abs inhibit upregulation of MHC class II molecules and co-stimulatory molecules on DC, and suppress production of pro-inflammatory IL-12 by binding to (an) unknown receptor(s), resulting in decreased T cell stimulatory capacity. 5) Abs upregulate expression of inhibitory Fcγ receptor IIb on DC, resulting in inhibition of DC-function upon binding of immune complexes. 6) Abs inhibit binding of immune complexes to activating FcγRIII, thereby suppressing presentation of auto-antigen associated with immune complexes to T cells.

How IVIg-primed DC exert their regulatory activities and effect other components of the immune system in not known. It has been hypothesized that IVIg-exposed DCs are impaired in the priming and activation of T cells, however, Crow et al. (Crow and Lazarus, 2008) demonstrated the protective effects of both IVIg and IVIg-primed DC in SCID mice with ITP (which lack functional B and T cells). This led to the conclusion that modulation of DC by IVIg might suppress autoimmunity independent of the adaptive immune system.

The immunologically relevant molecule(s) on APCs that are targeted by IVIg have not been completely identified. Determining the surface molecules and signaling events that participate in the modulation of DC by IVIg will further contribute to elucidation of the mechanism underlying the complex immunoregulatory effects of IVIg.

4-2-3-1- Fcγ receptor-dependent regulatory actions of IVIg

The family of Fcγ receptors (FcγRs) recognize the Fc portion of IgG antibodies (Nimmerjahn and Ravetch, 2008). FcγRs differ in function, cellular distribution, and affinity for the Fc portion of Ig. In humans, five activating FcγRs have been identified: the high-affinity receptor FcγRI, which can bind monomeric IgG, and four low-affinity receptors (FcγRIIA, FcγRIIC, FcγRIIIA and FcγRIIIB), which only bind multimeric IgG in the form of immune-complexes. Cross-linking of activating FcγRs by immune complexes results in the triggering of intracellular Immunoreceptor Tyrosine-based Activating Motif (ITAM) signaling pathways that are present either in the cytoplasmic domain of the receptor (FcγRIIA and FcγRIIC), or in the associated FcR γ-chain (FcγRI and FcγRIIIA), resulting in cell activation (Nimmerjahn and Ravetch, 2008). FcγRIIB is a low affinity and the only inhibitory Fcγ-receptor that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. The cytoplasmic tail is also

associated with different tyrosine kinase (TK) proteins (Smith and Clatworthy, 2010) that modulate cell signaling cascades. Cross-linking of FcγRIIB and B cell receptors by immune-complexes results in phosphorylation of ITIM-associated protein kinases that negatively regulates other activating signaling in B cells and aborts B cell activation. FcγRs differ in their cellular distribution; myeloid cells express FcγRI, FcγRIIA and FcγRIIIA, whereas granulocytes express FcγRI, FcγRIIA and FcγRIIIB. In these cells, immune complex-mediated activation of these receptors is negatively regulated by FcγRIIB (Nimmerjahn and Ravetch, 2008). FcγRs bind different IgG subtypes with differing affinity. For example, FcγRIIB binds with highest affinity to IgG3, followed by IgG1 and IgG4, whereas activating FcγRs have higher affinity to IgG1 (Smith and Clatworthy, 2010).

Evidence has been presented suggesting that the immune-modulatory effects of IVIg in many autoimmune diseases are mediated by the Fc –FcR interaction rather than via the $F(ab')^2$ fragment (Nimmerjahn and Ravetch, 2008). The Fc fragment of IgG can saturate the neonatal receptor FcRn, a natural Fc receptor that binds to immunoglobulin and increases their half-life by protecting them from catabolism. It is proposed that IVIg saturation of FcRn might result in the prevention of pathogenic autoantibodies binding to receptors, therefore accelerating their natural degradation in serum (Fig. 4). In agreement with this, the effect of IVIg has been reversed in FcRn deficient mice. IVIg treatment of mice with ITP leads to clearance of anti-platelet antibodies, whereas in mice deficient in FcRn, IVIg failed to decrease pathogenic Abs (Fig. 4).

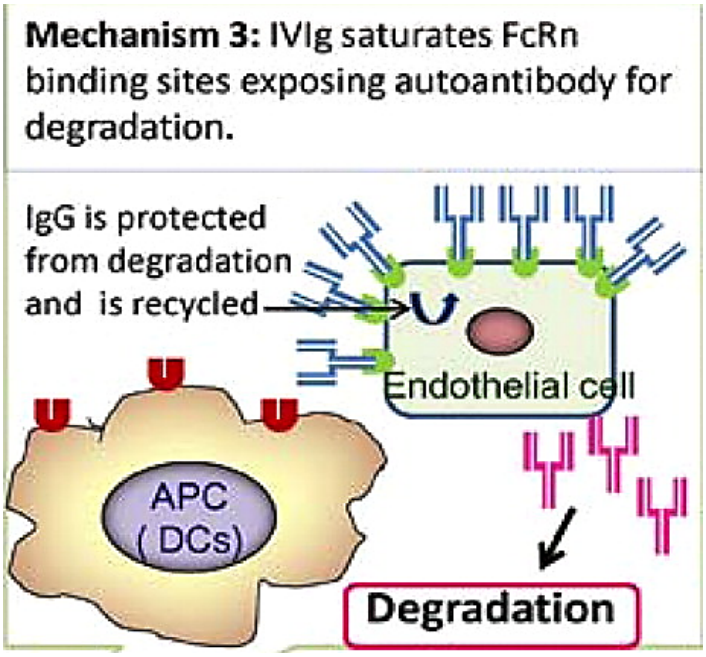

Figure 4. IVIg increases the clearance of pathogenic Abs by saturation of FcRn. Adopted from (Chong and Chong, 2010).

The neonatal Fc receptor (FcRn) is an Fc receptor which is similar in structure to MHC class I. FcRn has been well characterized in the transfer of passive humoral immunity from a mother to her fetus. In addition, throughout life, FcRn protects IgG from degradation, thereby explaining the long half-life of this class of antibody in the serum. IVIg can saturate binding sites on the neonatal FcR. This can lead to increased degradation of auto-Abs and reduction of auto-Ab titers.

Li et al. (Li et al., 2005) demonstrated that IVIg-treated wild-type, but not neonatal FcRn-KO mice, were protected from developing bullous skin disease when the animals were infused with antibodies from patients with pemphigus vulgaris. However, Crow et al. (Crow et al., 2011) have recently shown contrary data in a murine model of ITP, in which IVIg-treated FcRn deficient mice displayed no difference in increasing platelet numbers as compared to IVIg-treated C57Bl/6 wild type mice. They did not, however, assess the frequency of anti-platelet antibodies.

Another theory involves binding of IgG to activating FcγR or induction of FcγRIIB on APC. FcγRs are important in antibody-mediated effector functions of APCs, thus blocking of individual or all activating FcRs results in abrogation of antibody activity in triggering of inflammatory responses, as shown in a variety of autoimmune models such as ITP, nephrotoxic nephritis (NTN) and arthritis (Anthony et al., 2008b; Clynes and Ravetch, 1995) (Fig. 5).

FcγR-dependent actions of IVIg appear to involve APC (i.e. DC, macrophages and B cells). Activating and inhibitory FcγRs set a threshold for immune effector cell activation (Desai et al., 2007). While the activating FcγRs contribute to immune complex-mediated autoimmunity (Diaz de Stahl et al., 2002), FcγRIIB is the only inhibitory FcγR that contributes to the immune tolerance and regulates APCs activation (Fig. 6). A disturbed balance of activating and inhibitory FcγR has been implicated in the pathogenesis of many autoimmune diseases. In agreement with this concept, induction of FcγRIIB overexpression on B cells raises the threshold for B cell activation, which results in suppression of autoimmune disease, such as Lupus, despite the heterogeneity of factors influencing disease susceptibility (Svenson et al., 1998). IVIg has been shown to induce enhanced expression of FcγRIIB on macrophages and B cells, thereby increasing the threshold of immune complex-mediated cell activation.

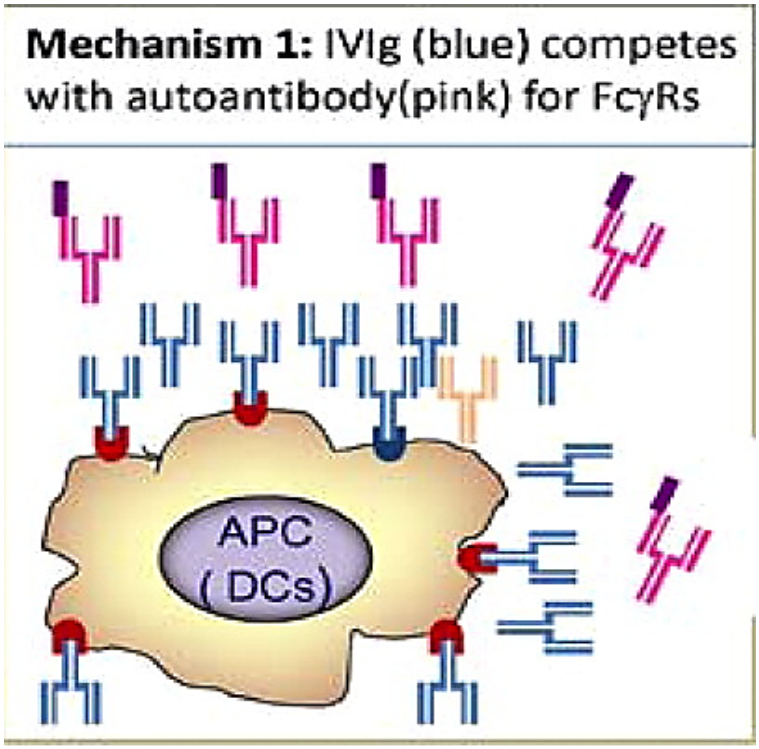

Figure 5. Natural immunoglobulins non-specifically block the FcRs by their Fc fragments. Adopted from (Chong and Chong, 2010).

Fc fragments of immunoglobulin bind to activating FcγRs on APC, and reduce the clearance of auto-antigens coated with autoantibodies, abrogating the associated inflammatory responses initiated upon the ligation of FcγRs.

Siragam et al. (Siragam et al., 2005) and Bazin et al. (Bazin et al., 2006) proposed in separate studies that artificial immune complexes can confer protection to mice with ITP, comparable to IVIg, dependent on activating FcγRIII. They also showed that IgG molecules do not interact with inhibitory FcγRIIB (Siragam et al., 2006). IVIg-primed leukocytes from wild type mice could confer protection to mice with ITP, whereas IVIg-primed leukocytes from FcRγ chain–deficient mice could not. In another study, the effectiveness of immune complexes in ameliorating ITP was assessed, by generating IgG specific to a soluble antigen (OVA). Immune complexes of OVA/anti-OVA Ab recapitulated the protective effects of IVIg in ITP as well as in the K/BxN serum-induced arthritis model, through a mechanism dependent on FcγRIIB (Siragam et al., 2005). Moreover, in separate studies it was demonstrated that inhibition of FcγRIIB receptors, either by genetic deletion or with blocking antibodies, inhibits the protective effect of IVIg in ITP (Crow et al., 2003; Jee et al., 2011).

IVIg contains monomeric and dimeric IgG populations which are in a dynamic equilibrium depending on concentration, pH, temperature, donor pool size and stabilizers added to IVIg (Wymann et al., 2008). The frequency of dimeric IgG molecules within IVIg is usually at a very low level. Moreover, IVIg preparations are acid treated and stabilized at low pH routinely in order to dissociate the dimeric IgG (section 2). In addition high-performance liquid chromatography-purified IVIg monomers do not have significant protective effects in the murine model of ITP (Siragam et al., 2005). Monomeric antibodies in IVIg are not able to compete with pathogenic immune-complexes for binding to activating FcγRs because of their low affinity for these receptors (Nimmerjahn and Ravetch, 2007b). Thus it is unclear if the anti-inflammatory effect of IVIg is attributed to multimeric IgG molecules.

In addition, conflicting results have been found regarding the requirement of inhibitory FcγRIIB for the immune-regulatory action of IVIg. FcγRIIB was not reported to be the physical target of IgG in IVIg (Samuelsson et al., 2001; Siragam et al., 2006; Zhuang et al., 2002). IVIg can abrogate B-cell responses without direct interaction with FcγRIIB (Zhuang et al., 2002). Inhibition of human tonsillar B cell proliferation and immunoglobulin production, in response to IL-4 and CD40 stimulation, was not inhibited by IVIg when cells were co-incubated with both IVIg and antibodies against FcγRIIB, suggesting that FcγRIIB is not targeted by IVIg. In addition, adoptive transfer of IVIg-primed FcγRIIB deficient DC to mice with ITP led to the improvement of disease severity in recipients (Siragam et al., 2006). Expression of FcγRIIB was required by the recipient mice to achieve anti-inflammatory activity. This suggests that although FcγRIIB is not itself the physical target of IVIg, it plays a role in the downstream phase of IVIg's function.

Aubin et al. (Aubin et al., 2010) have demonstrated that IVIg can inhibit antigen presentation to the same degree in bone marrow-derived DC from both wild-type and FcγRIIB-deficient mice. The overexpression of FcγRIIB on macrophages is not sufficient to achieve anti-inflammatory activity comparable to IVIg in murine models of arthritis and systemic lupus erythematosus (Baerenwaldt and Nimmerjahn, 2008; Diaz de Stahl et al., 2002). A recent study also indicated that there were strain-specific responses to IVIg. Leontyev et al. (Leontyev et al., 2012) demonstrated that both FcγRIIB-deficient and wild-type BALB/c recover from ITP with similar kinetics after IVIg treatment; while, this was not the case for C57BL/6 FcγRIIB-deficient mice. Thus, genetic factors might be important for the responsiveness to IVIg therapy.

Anthony et al. (Anthony et al., 2011a) recently proposed, using the K/BxN model of serum induced arthritis, that IVIg interacts with a C-type lectin immunorecetor (CLR). SIGN-R1 is expressed on the surface of marginal zone macrophages, and interaction with IVIg leads to the production of IL-33 by those cells and subsequently inducing production of IL-4 by basophils. IL-4 promotes increased expression of inhibitory Fc receptors (FcγRIIB) on effector APC (Fig. 6). This will be further elaborated in section 4-2-3-2.

Taken together, the inhibitory actions of IVIg on APC may involve interaction of IgG molecules with activating FcγRs, or unconventional FcγRs, resulting in induction of inhibitory signaling (Fig.6). Up-regulation of FcγRIIB on effector APCs may also contribute to the action of IVIg.

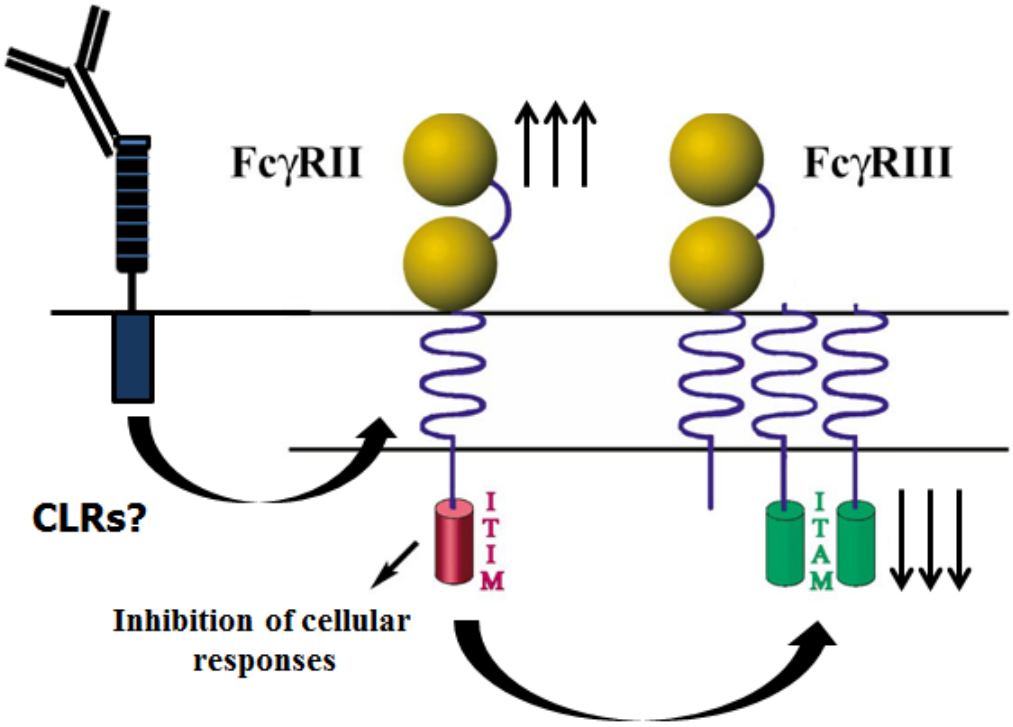

Figure 6. IVIg induces the up-regulation of FcγIIB expression and the associated ITIM signaling pathway.

IVIg may induce surface expression of FcγRIIB on splenic macrophages and other APCs. Modulation of inhibitory (FcγRIIB) signaling is a potent mechanism for attenuating autoantibody-triggered inflammatory diseases. Upregulation of FcγRIIB is believed to be the result of interaction of IgG with activating FcγRs or certain c-type lectin receptors.

4-2-3-2- The immune-regulatory activity of sialylated-IgG

Recently work examining the glycosylation structure of IgG has led to fractionation of IVIg based on glycosylation sites. Glycosylation of IgG can determine the affinity and binding characteristics of IgG for FcγRs (Anthony et al., 2012). For example, IVIg can be separated into extensively sialylated and nonsialylated IgG fractions. The sialylated fraction (SA-IVIg), comprising only 5% or less of the IgG pool, appears to contribute importantly to the anti-inflammatory activity. Oligosaccharide residues present on the Fc of normal polyclonal IgG can attach at Asn 297, and sialic acid can be linked to the oligosaccharide chain either via a α2,3 or α2,6 linkage. Recent studies showed that α2,3 sialylation provides a better protection to the protein than α2,6 sialylation (Fig. 7).

In murine models of inflammatory arthritis, administration of high-dose IVIg protects mice from developing joint inflammation. Pretreated of IVIg with neuraminidase, removing the terminal sialic acid residues, abrogates the expected anti-inflammatory activity. Conversely, sialic acid–enriched IVIg preparation showed equivalent protection at one-tenth the dose of unfractionated IVIg (Anthony et al., 2008a).

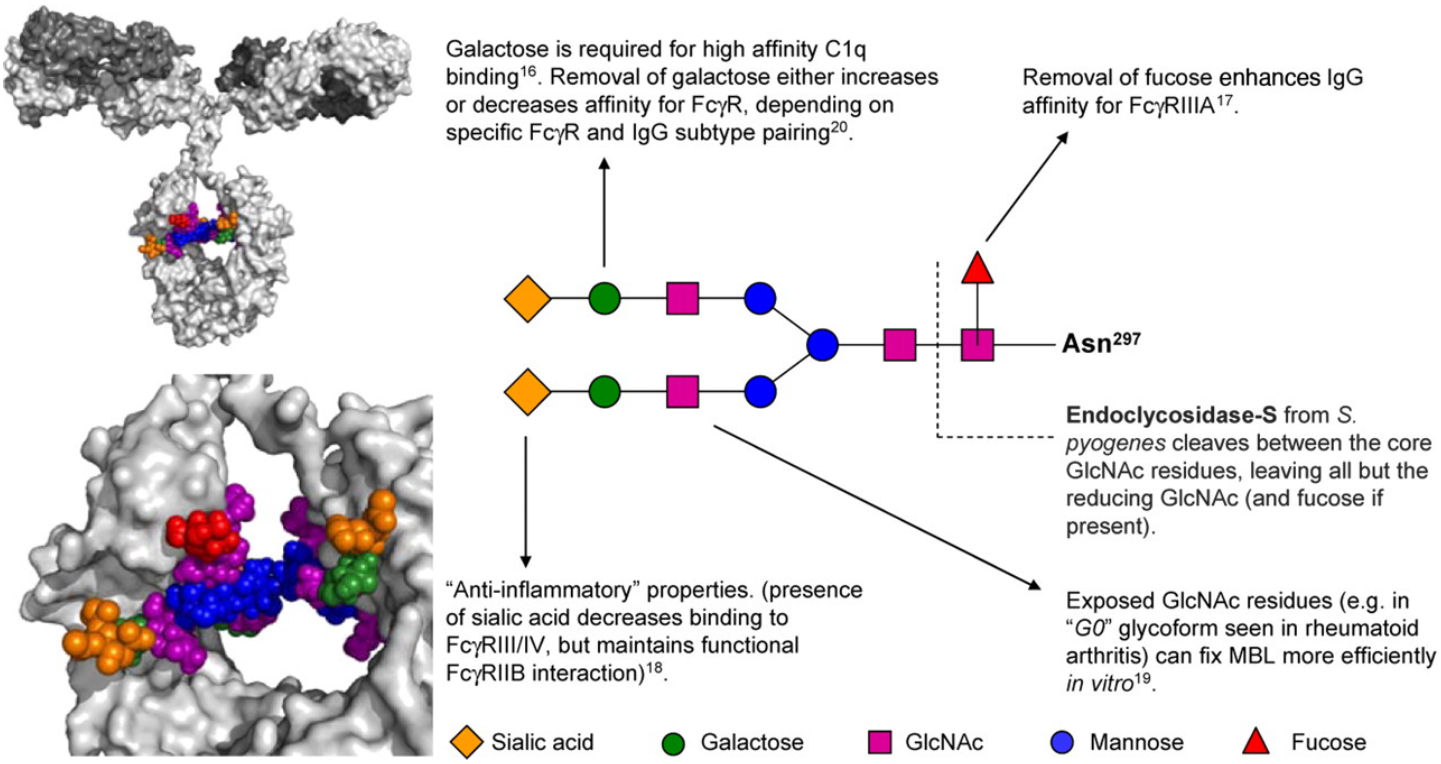

Figure 7. Schematic structure and glycosylation of IgG. Adopted from (Scanlan et al., 2008)

The two conserved N-linked carbohydrate chains of IgG are attached to Asn-297 of the Cγ2 domains of each IgG heavy chain. A sialylated biantennary glycan is shown, with nonreducing sialic acid residues modeled according to likely linkage constraints. A range of other glycoforms also exists on different subsets of IgG.

Anthony et al. (Anthony et al., 2011a; Anthony and Ravetch, 2010; Anthony et al., 2008b) demonstrated that SIGN-R1, a C-type lectin receptor on murine splenic marginal zone macrophages, recognizes and can mediate anti-inflammatory effects of SA-IVIg in a murine model of serum-induced arthritis. They also demonstrated that sialylated-Fc fragments can bind to DC-SIGN, the human orthologue of SIGN-R1, with the similar affinity, that results in triggering of an inhibitory response in the ligated cells. It is important to note that DC-SIGN and SIGNR1 differ significantly in their cellular and tissue distribution and DC-SIGN is expressed particularly on human dendritic cells rather splenic or intestine macrophages.

Kaneko et al. (Kaneko et al., 2006) demonstrated that between 1 to 5% of IgG molecules within IVIg contain sialic acid moieties at the Asn297. They proposed that Fc-sialylated IgGs bind to a unique receptor on macrophages or other APCs and this interaction leads to the up-regulation of inhibitory FcγRIIB (Fig. 6). In addition to SIGNR1, SA-IVIg interacts with other C-type lectin receptors, such as CD22 (Siglec-2), which modulates B cell receptor signaling and promotes apoptosis in mature human B lymphocytes (Seite et al., 2010).

In a recent study sialic acid-enriched IgG was biochemically and functionally characterized. It was shown that the sialylation of IgG is not restricted to the Fc portion; high levels of sialic acid residues were mainly found on the Fab fragments. *ex vivo* LPS or PHA-stimulated human monocytes treated with enriched sialylated-Fab exhibited reduced CD54 expression and reduced secretion of MCP-1 (CCL2) (Kasermann et al., 2012). These data suggest that the anti-inflammatory activity of IVIg may be dependent on sialylation sites on both Fab and Fc.

The potential importance of sialylation IgG was underscored by evidence that the balance of sialylated vs. de-sialylated IgG influences autoimmune conditions. For example, in patients with

rheumatoid arthritis an increased ratio of desialylated:sialylated IgG correlated with higher disease severity (Parekh et al., 1985; Rook et al., 1991). Recently, induction of tolerance in an OVA-driven murine model of allergic inflammatory diseases was associated with increased expression of 2,6-sialyltransferase in plasma cells (Ab-producing cells), and enhances sialylation of IgG (Oefner et al., 2012). These authors also demonstrated in 3 patients that birch pollen immunotherapy resulted in increased levels of sialylated serum-specific IgG to birch pollen.

5- The action of IVIg on the development and the activation of regulatory T cells

Our laboratory has proposed that the mechanism by which IVIg regulates immune responses is through induction of regulatory T cells (Treg). A small number of models of autoimmune diseases as well as clinical trials demonstrated that IVIg therapy is associated with increased frequency and/or functional activity Treg. The underlying mechanism is poorly understood.

Treg are specialized subpopulation of T cells that act to suppress activation of undesirable immune responses, and thereby maintain immune system homeostasis and tolerance to self- and non-self-antigens (Tang and Bluestone, 2008). Treg are generally characterized as expressing CD3, CD4, very high levels of CD25, and the forkhead box P3 (FOXP3), a transcription factor which acts as a master regulator of Tregs (Piccirillo and Shevach, 2004). Ectopic expression of Foxp3 in conventional $CD4^+$ T cells recapitulates the phenotype and function of Treg cells (Fontenot et al., 2003). Based on their developmental or functional differences, Treg are categorized into two main populations of naturally occurring (nTreg) and peripherally induced (iTreg) cells that are generated in the thymus and in peripheral lymphoid tissues, respectively. Thus far, no distinctive marker(s) has been identified to definitively distinguish these two subsets from one another, although Helios is more prevalent on nTreg.

Deficiencies in Treg are associated with severe autoimmune and lymphoproliferative inflammatory disorders (Sakaguchi et al., 2010). Pre-clinical studies have shown that infusions of freshly isolated or *ex vivo* induced Treg can confer protection in inflammatory settings (Pilat et al., 2010; Xu et al., 2012). However, human Treg infusion therapy has been difficult to implement in the clinic, and relatively few clinical trials have been initiated (Riley et al., 2009). Therefore, developing new therapeutic approaches with the capability to modulate the immune system through activation and/or expansion of Treg has been the goal of many recent studies. Several therapeutic immunosuppressive compounds, including corticosteroids and rapamycin, have been identified as promoting the expansion or suppressive activity of Treg. IVIg has advantage over immune suppressive drugs, in that it is not an immunosuppressor, but rather an immunomodulator, and is associated with few and relatively mild side effects.

Initial reports on the immune-regulatory effects of IVIg indicated that T lymphocytes purified from IVIg-treated individuals exhibit a suppressive capability when co-cultured *ex vivo* with pokeweed mitogen-stimulated T and B cells (Durandy et al., 1981). The relevance of those reports to the activation of Treg was supported in a more recent study by Kessel et al. (Kessel et al., 2007), who demonstrated that IVIg treatment of Treg purified from human peripheral blood increased intracellular TGF-β, IL-10, and *FOXP3* expression as well as enhanced suppressive function.

IVIg appears to restores reduced levels of circulating $Foxp3^+$ Treg and enhance their function in certain conditions. IVIg therapy of Kawasaki Disease patients increased the percentage of $Foxp3^+$ Treg, and enhanced the reduced expression of genes related to the Treg, such as *FOXP3*, *CTLA4*, *GITR*, as well as cell-surface-bound TGF-β (Anthony and Ravetch, 2010; Ephrem et al.,

2008). Likewise, in Guillain-Barre Syndrome (GBS), (Chi et al., 2007), IVIg was demonstrated to increase expression of *FOXP3* and production of inhibitory cytokines.

In Th-2 mediated inflammatory conditions, IVIg therapy might contribute in normalization of Treg. In eosinophilic granulomatosis with polyangiitis (EGPA) (a disease characterized by peripheral and tissue eosinophilia and followed by allergic granulomatosis and necrotizing vasculitis) therapeutic administration of IVIg led to increases of $Foxp3^+$ Treg, as well as increased percentage of IL-10 producing $CD4^+$ T cells (Tsurikisawa et al., 2012).

The effect of IVIg in increasing of $Foxp3^+$ Treg was reported in an experimental model of autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis, in which IVIg reduced the severity of the disease through a mechanism that involved expansion and enhancement of the suppressive capacity of $Foxp3^+$ Treg (Ephrem et al., 2008). Furthermore, administration of IVIg failed to confer protection in EAE mice that were depleted of Treg prior to treatment. More recently Aslam et al. (Aslam et al., 2012) demonstrated that in a murine model of ITP, administration of IVIg was associated with elevated of peripheral Treg. Transfer of IVIg-primed $CD4^+$ T cells blocked graft rejection in a murine model of transplantation, while depletion of Treg prior to IVIg-priming reversed this effect (Tha-In et al., 2010).

The majority of studies have indicated that Treg induction by IVIg is obtained using so-called high dose IVIg e.g. 1-2 gm/kg in humans or the equivalent in mice. However, Ramakrishna et al. (Ramakrishna et al., 2011) recently reported that the administration of low-dose IVIg had anti-inflammatory effects in mice with HSV-mediated fatal encephalitis. This is the only report indicating the efficacy of low-dose IVIg in the treatment of inflammatory conditions.

Taken together, these findings support the concept that the immune-regulatory action of IVIg, in associated with the potentiation and functional activation of Treg.

5-1- The mechanism by which IVIg affects the Treg compartment

The mechanisms by which IVIg may affect Treg compartment may involve direct interaction of IgG with effector T cells/Treg, or via interaction and modification of APCs. In this section, supporting evidence regarding the each of potential mechanism is discussed.

The action of IgG on DCs in the development of Treg was first proposed in a study at 1981 where the co-culture of DC/$CD4^+$ T-cells, in the presence of IVIg, led to enhancing of the suppressive capacity of T cells through a mechanism dependent on the secretion of prostaglandin E2, a specific myeloid DC mediator (Durandy et al., 1981).

De Groot et al. (De Groot et al., 2008) uncovered a number of promiscuous IgG-derived T-cell epitopes (Tregitopes) within the heavy chain (Fc) of different subsets of IgG with the capability of activating $Foxp3^+$ Treg. Tregitopes are T cell epitopes naturally located in immunoglobulins that bind to multiple MHC Class II alleles and induce regulatory T cell (Treg) responses. APCs present Tregitopes to Treg, engage feedback mechanisms promoting a tolerogenic APC phenotype, induce Treg expansion, and modulate antigen-specific effector T cell responses. (Cousens et al., 2012b; Elyaman et al., 2011) (Fig.8). De Groot and colleagues also postulated that the synthetic recombinant “Tregitopes” were able to induce the production of IL-10 and expand Treg when being presented on the context of MHC class II to T cells.

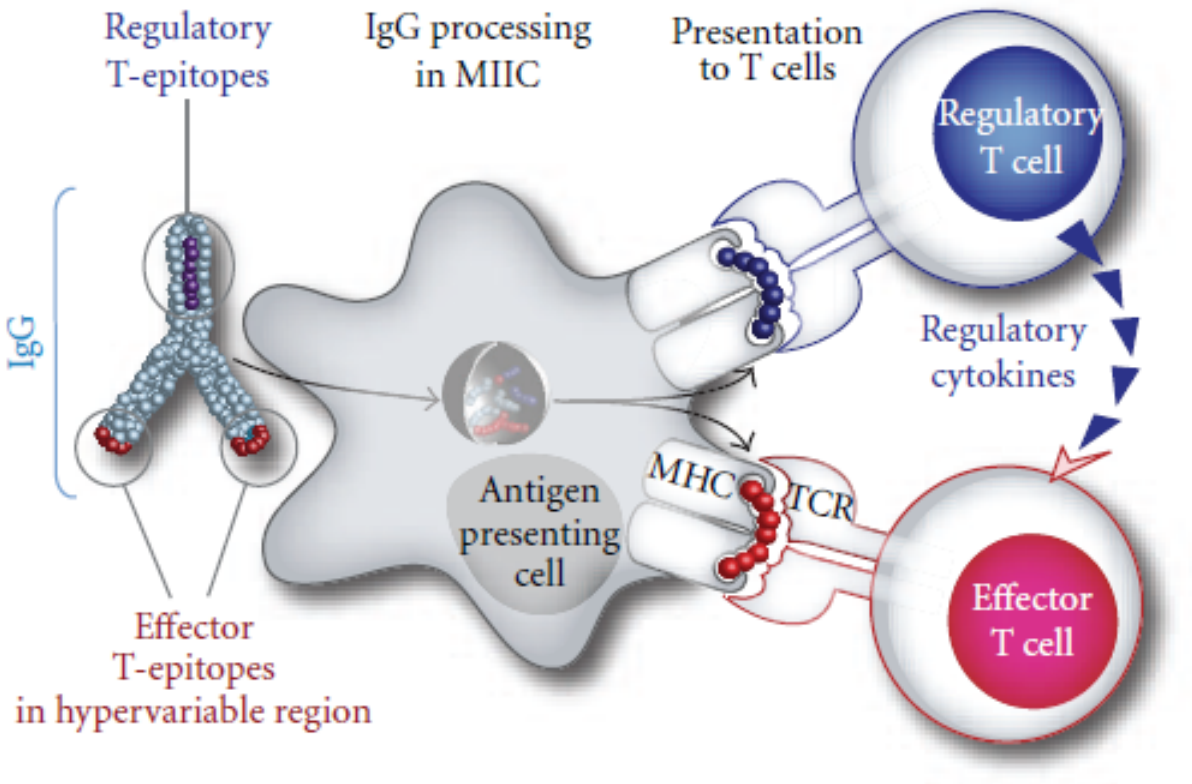

Figure 8. Proposed role of Tregitopes (Antibody-derived Treg epitope) in the immune-regulatory action of IgG. Adopted from (Kwekkeboom, 2012).

Tregitopes (dark blue) are presented in the context of MHC-II molecules to natural Treg and activates these cells to produce IL-10, which consequently leads to suppression of effector T cells (red).

In addition to the role of DCs in the development of Treg by IVIg, direct interaction of IgG with Treg might stimulate their expansion and activation. Tha-In et al. (Tha-In et al., 2010) showed that *in vitro* IVIg treatment of human Treg resulted in activation, as detected by increased expression of cell surface activation markers and enhanced capacity to suppress allogeneic effector T cell proliferation in a co-culture assay. They also reported that stimulated Treg exhibit phosphorylation of ZAP-70, an intracellular signaling molecule involved in activation of T cells. The implication of ZAP-70 in the development of Treg has not been fully characterized. A previous study has shown that stimulation of ZAP-70 phosphorylation by CD4 monoclonal Ab in Treg results in enhancing of their suppressive capacity (Becker et al., 2007).

Taken together, IVIg appears to affect Treg compartment through two main mechanisms:

1- Rendering tolerogenic DC that subsequently results in the peripheral induction, activation and expansion of Treg.

2- Direct interaction and functional activation of Treg.

The precise mechanism by which IVIg functions as an anti-inflammatory agent remains unclear. Studies in animal models have provided important insights, but for many of the proposed activities, the mechanisms remained to be validated in humans, as the animal models will not necessarily translate to human trials *per se*. The most validated inhibitory mechanisms of polyclonal IgG are summarized in the following table.

Table I. Potential Anti-inflammatory and Immunomodulatory Activities of IgG

Fab-mediated activities

- Suppression or neutralization of autoantibodies
- Suppression or neutralization of cytokines
- Neutralization of activated complement components
- Restoration of idiotypic–anti-idiotypic networks
- Blockade of leukocyte-adhesion-molecule binding
- Targeting of specific immune cell–surface receptors

Fc-dependent activities

- Blockade of the FcRn
- Blockade of activating FcγR and competitive inhibition of immune-complex binding to activating Fc-receptors
- Up-regulation of inhibitory FcγRIIB

Fc- or Fab-dependent activities

- Modulation of maturation and function of dendritic cells
- Functional activation of Treg
- Modulation of apoptosis and the cell cycle
- Sialylated-IgG immune modulation

6- Use of IVIg in Th-2 mediated atopic diseases

Allergic diseases are immunological disorders, caused by the immune response towards an innocuous antigen that usually leads to type I hypersensitivity reaction, a so-called Th2 mediated immunologic response that stimulates B cell production of IgE antibodies. The condition that underlies allergic diseases is atopy, which is characterized by raised serum IgE levels (Grammatikos, 2008).

In general, non-atopic adults mount an insignificant immunological response after exposure to allergens (such as those derived from pollen, house dust mite and cat). They produce allergen-specific IgG1 and IgG4 antibodies, whereas atopic individuals mount an exaggerated allergen-specific Th2 response associated with elevated IgE serum levels. Atopic patients also exhibit positive skin tests to extracts of common aeroallergens (a test done on the skin, in which a small amount of the suspected allergen is placed on the skin, followed by gently scratching with a needle, to identify the allergen that is the trigger for an allergic reaction) (Kay, 2000).

In the early stages of allergy, effector T cells produce elevated levels of Th2-type cytokines i.e. interleukin-4 (IL-4), IL-5 and IL-13 (Ebner et al., 1995) rather than cytokines of the Thl type (IFN-γ and IL-12) or regulatory T-cell cytokines IL-10 and TGFβ. Signals provided by Th2 cytokines cause B cells to undergo class switching and produce allergen-specific IgE antibodies.

With subsequent exposure to the same allergen, IgE molecules bound on the surface of the mast cells or basophils can be cross-linked. Cross-linking of Fcε receptors by IgE occurs when more than one IgE-receptor complex interacts with the same allergen molecule, which activates the "sensitized" cell. Activated mast cells and basophils undergo degranulation, and release

histamine and other pro-inflammatory mediators (histamine, kinins, leukotrienes, and prostaglandins) from their granules into the surrounding tissue, causing local and systemic effects, such as vasodilation, mucous secretion, nerve stimulation, and smooth muscle contraction. This results in rhinorrhea, itchiness, dyspnea, and potentially anaphylaxis. Depending on the individual, the allergen, and the mode of introduction, symptoms can be system-wide (classical anaphylaxis), or localized to particular organ. For instance, asthma is localized to the respiratory system (Janeway, 2001).

The most importantly step in the management of allergic diseases, is avoiding the allergen in question. Medications that inhibit the action of allergic mediators, or prevent activation of cells and degranulation, are used for treating allergic patients. These include antihistamines, glucocorticoids, epinephrine (adrenaline), theophylline, cromolyn sodium and anti-leukotrienes (Barnes, 1999). Corticosteroids are also used as important primary treatment for allergic diseases, although used long term are associated with serious side effects. Alternative therapies are often used by practitioners attempting to manage patients who suffer from severe, chronic asthma, defined as asthma that is difficult to manage by conventional therapies. IVIg has been therapeutically used off-labeled in the treatment of different Th2-mediated inflammatory diseases, including allergic asthma, chronic sinusitis, atopic dermatitis and urticaria.

Asthma is one of the most prevalent chronic inflammatory disorders of airways worldwide and is associated with airway hyperreactivity leading to recurrent episodes of wheezing, breathlessness, chest tightness and coughing. The main types of anti-inflammatory drugs administered for better asthma control, are inhaled and oral corticosteroids, mast cell stabilizers (Stockl et al., 2008), leukotriene modifier drugs (Berges-Gimeno et al., 2002), and immunomodulators (e.g. Anti-IgE

such as Omalizumab) (Sarinho and Cruz, 2006). Approximately 5% to 10% of patients with asthma suffer from severe type of the disease which is refractory or unresponsive to even high doses of inhaled corticosteroids. In these cases the immune-modulatory therapy may be required as a steroid sparing alternative.

IVIg is not currently approved by the FDA for the treatment of allergic asthma, although its beneficial effect in severe chronic steroid-dependent asthma has been reported in a number of small studies. The first clinical trial evaluating the effects of IVIg therapy in severe steroid-dependent asthmatic patients was carried out over 2 decades ago by Mazer and colleagues (Mazer and Gelfand, 1991; Mazer et al., 1989), who demonstrated that monthly administration of high dose IVIg (2g/ kg), for six consecutive months, significantly improved asthma symptoms. They demonstrated that IVIg therapy in these patients lead to the improvement of symptom scores and significantly reduced their doses of corticosteroids. Following that, in an open-label study of 11 adults with steroid-dependent asthma, Landwehr et al. (Landwehr et al., 1998), confirmed the protective effect of IVIg therapy with an improvement in FEV1 (forced expiratory volume, a parameter used in the diagnosis of obstructive and restrictive lung disease). In addition they identified the greatest benefit of IVIg in conjunction with steroid therapy. These results were in agreement with the findings by Salmun et al. (Salmun et al., 1999) who found the beneficial, steroid-sparing effect of IVIg, in patients with severe asthma, albeit with no improvement in FEV1. IVIg can enhance steroid receptor sensitivity, thus reducing the requirement for inhaled corticosteroid therapy (Marie et al., 2010; Spahn et al., 1999; Zandman-Goddard et al., 2007). Thus, it appears that the therapeutic efficacy of IVIg in steroid-dependent asthma enhances inhaled corticosteroid therapies.

The efficacy of IVIg therapy in severe asthmatic patients remains controversial. Jakobsson T, et al. (Jakobsson et al., 1994) tested the long-term effects of IVIg therapy in patients aged 6-20 years old with moderately severe bronchial asthma in 4-5 month vs. 14 month protocols. The patients on the 4-5 month IVIg protocol demonstrated significantly improved asthmatic symptoms. However, no differences in asthmatic symptoms were found between patients on the 14 month IVIg protocol vs. untreated controls, suggesting a lack of long-term effects of IVIg. In a following study by Kishiyama et al. (Kishiyama et al., 1999) no significant difference was found between the placebo group and the two IVIg treatment groups, e.g., 1 and 2 g/kg. However that study was terminated for severe adverse reactions, including 3 patients with aseptic meningitis syndrome. The incidence of adverse reactions was extraordinarily high compared to other studies using high-dose IVIg in patients with autoimmune disease and may be related to the particular preparation of IVIg used in this study. Another factor may also have compromised this study to lead to negative results. Patient population was principally adults with a mean age of 40, much higher than the study by others in which the study was composed of children and adolescents. Thus, the age group, children vs adults, may be an important factor in the response of the asthmatic inflammatory process to treatment with IVIg, and still more optimizations for the IVIg therapy of patient with severe asthma are required.

In addition to allergic asthma, the beneficial efficacy of IVIg therapy in the treatment of other Th2-mediated inflammatory disorders has been identified. Improvement in patients with severe dermatitis after IVIg therapy has been observed in a number of studies (Kimata, 1994; Noh and Lee, 1999) (Jee et al., 2011). The protective effect of IVIg in severe solar urticaria, an IgE-mediated hypersensitivity induced by exogenous photosensitization has been shown as well, when treatment with high-dose antihistamine did not demonstrate improvement (Adamski et al.,

2011; Correia et al., 2008; Hughes et al., 2009; Maksimovic et al., 2009). More recently, the protective effect of IVIg in drug-induced hypersensitivity syndrome (DIHS) mediated by Th-2 cells has been reported (Kito et al., 2012).

Taken together these data demonstrates that IVIg has immune-regulatory functions in Th-2 mediated inflammatory disorders and may be considered as an alternative therapeutic approach in these chronic conditions.

6-1- Proposed anti-inflammatory mechanisms of IVIg in allergic asthma

While there is compelling evidence that IVIg has an efficient therapeutic effect in Th2 type atopic inflammatory diseases, the modes of action of IVIg in these contexts have not been fully elucidated. IVIg can exert its ameliorative effects in Th-2 mediated immune disorders by modulation of cytokine networks, as mentioned earlier, however this effect appears not to be solely dependent on neutralizing antibodies (Mazer et al., 1989), and IVIg can directly modulate the cellular responses by T and B-lymphocytes or APCs (Durandy et al., 2009).

A potential alternative mechanism by which IVIg may enhance steroid responsiveness in T cells is by abrogation of IL-2, IL-4, and IL-13 production (Kito et al., 2012; Modiano et al., 1997), the three cytokines that skew T-cell differentiation to Th-2 type and may induce steroid-resistance in patients, which can be reversed by IVIg (Spahn et al., 1999). IVIg may restore steroid sensitivity and enhance the effectiveness of glucocorticoid therapy by decreasing the inflammatory cytokine production. Indeed, steroid-resistant patients who had decreased glucocorticoid receptor (GCR) binding affinity exhibited normal GCR binding affinity after IVIg therapy (Spahn et al., 1999).

The close association between IgE levels, allergy, and asthma has long been recognized. The approval of specific therapy against IgE provided new insights into the role of IgE in asthma (Davydov, 2005). *Ex vivo* assessment of the action of IVIg on B-lymphocytes showed that IVIg decreased stimulated B-cell proliferation and IgE production (Sigman et al., 1998; Zhuang and Mazer, 2001). Clinical observation in asthmatic patients treated with IVIg also showed that the serum IgE levels markedly decreases (Mazer and Gelfand, 1991)

Yamamoto et al. (Yamamoto et al., 2010) recently, in a mouse model of allergic airway disease, demonstrated that the administration of purified rabbit polyclonal IgG or the fragmented Fc portion of rabbit IgG attenuated airway eosinophilia, airway hyperresponsiveness, goblet cell hyperplasia, and serum levels of Th2 cytokines. They also demonstrated that this modulatory effect is due to the action of IgG on $CD11c^+$ DC, as IVIg-treated $CD11c^+$ DC attenuated the production of Th2 cytokines by *ex vivo* OVA-stimulated, OVA-specific $CD4^+$ T.

Other immune cell types which are involved in atopic disorders, such as iNKT cells, can be also affected by IVIg. iNKT cells are implicated in development of Th-2-mediated diseases such as allergic asthma and contact dermatitis (Akbari et al., 2003; Benlagha et al., 2002; Lisbonne et al., 2003). In a recent study, Araujo et al. (Araujo et al., 2011) demonstrated that IVIg therapy abrogated the activation and function of iNKT cells in a murine model of OVA-driven allergic airway disease. However, adoptive transfer of IVIg-primed iNKT cells, as opposed to IVIg-primed $CD11^+$ DC, did not confer protection to the recipients. IVIg also can target receptors on eosinophils and neutrophils (two key accessory cells in atopic conditions). In two recent studies (Schaub et al., 2011; von Gunten et al., 2007) the presence of anti-sialic acid-binding Ig-like lectin 8 and 9 antibodies (Anti-Siglec8 and Anti-Siglec9 Abs) was found in IVIg. These

antibodies were able to induce caspase-dependent and caspase-independent programmed cell death, by targeting their cognate receptors on eosinophils and neutrophils, respectively.

As mentioned in section 4-2-3-1, IVIg is able to confer protection in a murine model of arthritis, through a mechanism that involves the upregulation of Th2 type cytokines. This suggests that IVIg has the capacity to regulate immune responses through various mechanisms and that the initiating antigens and the nature of the immune response will direct the overall effect of IVIg.

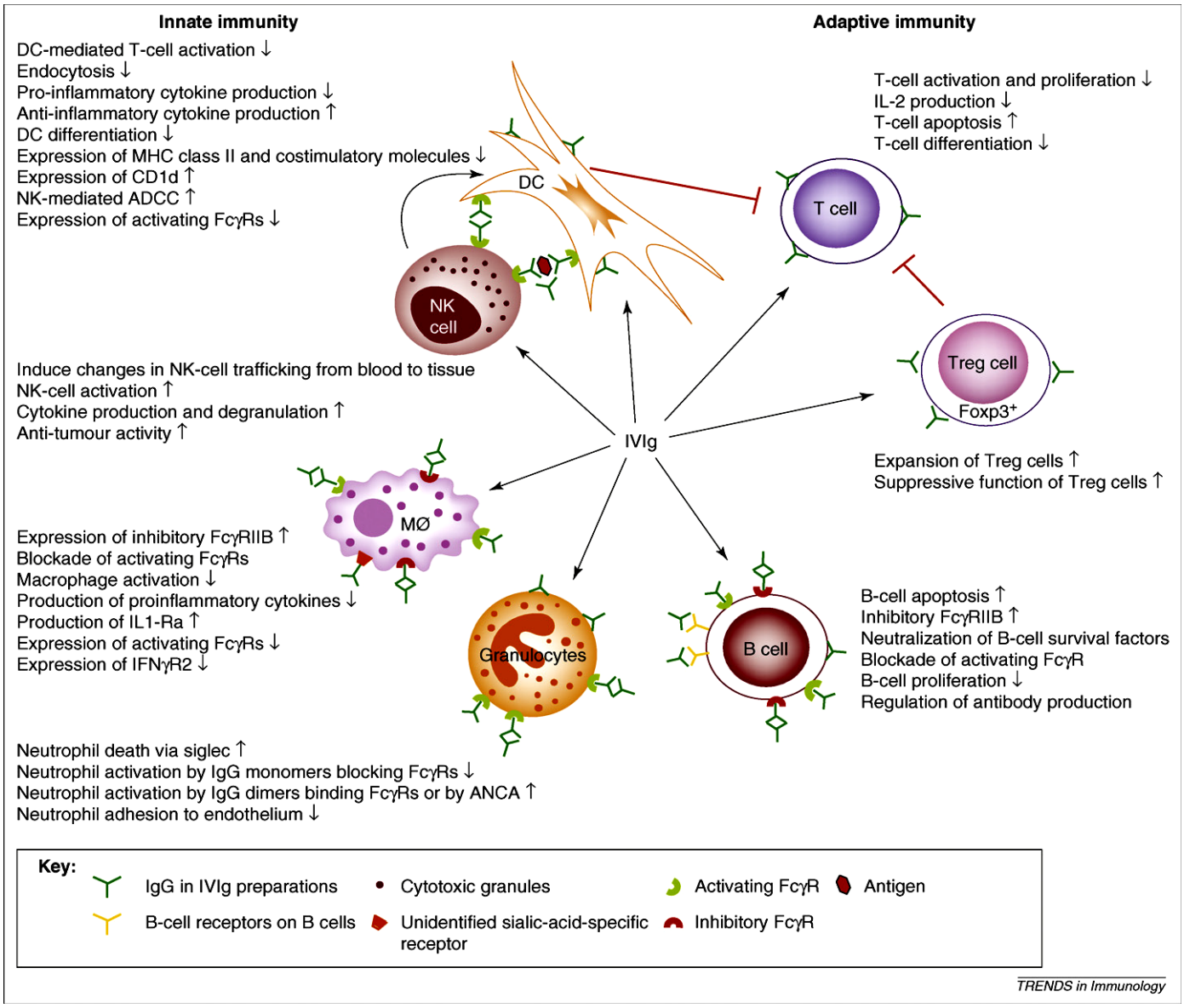

Figure 9. A schematic depiction of the cellular components of immune system that could be affected by IVIg. Adapted from (Tha-In et al., 2008).

Numerous potential targets for the effects of IVIg have been identified within the immune system ranging from the innate to the adaptive immune cells. IVIg has been shown to inactivate, silence or drive auto-reactive T-cells into apoptosis, acting to restore the balance of anti-inflammatory and pro-inflammatory cytokines, interfering with the production of antibodies via inhibition of B-cell activation. IVIg may also induce down-regulation of APCs activation, through mechanism discussed before.

7- Concluding remarks and rationales

IVIg is an extremely complex preparation that achieves immunomodulation through a number of synergistic mechanisms to provide therapeutic effects. The immune-regulatory effects of IVIg appear to be pleiotropic and involve multiple mechanisms, due to the complex interplay of IgG molecules with different cells and mediators. IVIg targets different cells, including innate and adaptive immune cells (Fig. 9).

IgG dimers, sialylated IgG, neutralizing antibodies and Tregitopes have been considered in various studies as the active moieties in IVIg with potential anti-inflammatory effects, however, their efficacy needs to be established in robust animal models.

IVIg is being increasingly used off-label in a number of pathological conditions. The advantage of IVIg over conventional "immune-suppressor" drugs is that IVIg functions as an immune-enhancer, and has few side effects and does not diminish responses to infectious agents. However, owing to cost of IgG purification processes and shortage in blood plasma resources, judicious use of IVIg is highly desirable. Identification of the precise mechanisms of action of IVIg in different conditions, and the pro-inflammatory vs. anti-inflammatory fractions of this compound, may help us to develop strategies to selectively target and optimize use of IgG-based therapies.

8- Hypothesis and objectives

The mechanism by which IVIg modulates the production of pro-inflammatory cytokines and suppresses allergic responses in Th-2 mediated allergic diseases remained ill understood.

Given the broad mechanism of action and targets of IVIg in different settings, we investigated the anti-inflammatory fraction of IgG and the potential molecular basis for the suppressive action of IVIg. We hypothesized that IVIg would inhibit airway inflammation and the production of pro-inflammatory Th-2 type cytokines, through a mechanism that involves the promotion of regulatory T cells.

To that end the effects of IVIg in inhibition of airway hyperreactivity (AHR), airway inflammation and regulation of cytokine production have been assessed in antigen-driven murine models of allergic airway disease. The effects of IVIg on induction, development and activation of Treg cells have been assessed. The mechanism by which IVIg regulates Treg cells or compartment of the immune system, and how IVIg interact with and render tolerogenic to the immune cells has been investigated.

Chapter II

Studies on the immune-modulatory effect of IVIg in a murine model of allergic airway disease

Article I.

Intravenous immune globulin attenuates airway hyper-responsiveness in a murine model of allergic asthma

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Abstract

Background: Intravenous Immunoglobulin (IVIG) has potent anti-inflammatory and immune modulating properties. IVIG has been utilized as a steroid sparing agent in severe asthma, but results of clinical trials have been conflicting. Objective: To determine if IVIG is able to attenuate bronchial reactivity, pulmonary inflammation and T-cell function using a murine model of allergic airways disease. Methods: BALB/C or C57BL/6 mice were sensitized to OVA or PBS control using local nasal sensitization, and then received 5 intranasal challenges on days 28-32 prior to sacrifice. Mice were treated intraperitoneally (IP) with either IVIG (1-2 gm/kg) or equivalent human serum albumin (HSA) 24 hours prior to the first OVA challenge. Bronchial reactivity to methacholine was examined using the Flexivent small animal ventilator. We evaluated pulmonary histology, mRNA from lung digests for Th2-related genes, and bronchoalveolar lavage (BAL) for cell counts and cytokines. Splenocytes were utilized to study OVA induced cell proliferation, cytokine production and dendritic cell maturation. Results: IVIG markedly attenuated the perivascular and peribronchial pulmonary inflammation, and decreased bronchial hyperresponsiveness to methacholine. IVIG treatment of splenocytes from sensitized animals diminished cellular proliferation to OVA, whereas IVIG treatment *in vivo* markedly attenuated OVA-driven splenocyte proliferation. This is accompanied by diminished IL-13 and TNF-α levels in splenocyte culture, decreased expression of Jagged 1, increased Delta 4 and decreased GATA3 mRNA levels, signs that IVIG has suppressed the expected Th2 response that accompanies repeated allergen exposure. Increased regulatory T-cells were found in draining pulmonary lymph nodes in IVIG treated mice but not in controls. Conclusion: IVIG was effective in ameliorating allergic airway disease in our model. Clinical relevance: IVIG may be a promising adjunct therapy requiring further study for patients with severe asthma.

Key words

Asthma, dendritic cells, IVIG, immune modulation, cell proliferation, bronchial responsiveness, murine model.

Abbreviations

IVIG: Intravenous Immunoglobulin; HSA: Human Serum Albumin; [3](Adamski et al.)thy: tritiated Thymidine; BHR: Bronchial hyper-responsiveness; Treg: Regulatory T-lymphocytes; IN: Intranasal.

Introduction

Intravenous immunoglobulin (IVIG) is the most commonly prescribed plasma derivative in North America. Each lot of IVIG contains a broad spectrum of antibodies pooled from the plasma of over 10,000 individuals. Although the primary indication for IVIG is supplementation for immune deficiency, the escalating use of IVIG is due largely to its potent action as an immune regulator. IVIG includes neutralizing antibodies against inflammatory proteins or surface molecules,(Kaveri et al., 1997) anti-idiotype antibodies that modulate cellular function, and immune complexes that may activate or inhibit the action of macrophages and dendritic cells.(Bayry et al., 2003a; Bayry et al., 2003c) Although progress has been made in understanding the mode of action of IVIG in conditions such as Immune Thrombocytopenic Purpura (ITP) (Crow and Lazarus, 2008), there are numerous questions to be clarified(Crow et al., 2009).

Murine models of disease have been effectively employed to examine the mechanism of action of IVIG. The majority of this work has addressed the action of IVIG in autoimmune diseases, such as ITP or immune mediated arthritis, as well as infectious diseases. While data exists regarding the action of IVIG on Th1 mediated conditions, there are no models that examine the action of IVIG on inflammation engendered by allergens or by Th2 immunity. However, allergen driven models of allergic airway disease provide an excellent vehicle to determine if IVIG can attenuate Th2 driven inflammation. Clinically, IVIG has been employed as a therapeutic modality for severe steroid dependent asthma (Corrigan, 1997; Gelfand et al., 1996; Mazer and Gelfand, 1991; Schwartz et al., 2006). In spite of both positive and negative results in small clinical trials, there is insufficient data regarding efficacy and mechanism of action; IVIG was

recommended for use in asthma in the context of clinical trials in a recently published guideline (Orange et al., 2006).

In this study, we address the action of IVIG on murine allergic airway disease using a model of upper airway sensitization and challenge (McCusker et al., 2002; McCusker, 2004). We demonstrate that IVIG, administered prior to allergen challenge, can attenuate bronchial hyperresponsiveness in OVA-sensitized mice, improve the degree of pulmonary inflammation, and diminish cellular proliferative responses to OVA and Th2 cytokines. These changes are associated with alteration in the genes for Th2 related co-stimulatory molecules and the transcription factor GATA3, as well as with recruitment of regulatory T-lymphocytes to the draining lymph nodes of IVIG treated animals. These studies demonstrate that IVIG is capable of diminishing the effects of allergen-driven Th2 inflammation, and may provide an explanation for the positive effects of IVIG observed in studies on severe asthmatics.

Material and Methods

Animals, Sensitization and Challenge.

Male Balb/c or C57 Black-6 mice, 5-6 weeks old, were purchased from Harlan Sprague-Dawley (Indianapolis, Indiana). All animals were kept in conventional animal facilities under standard conditions, and all experimental protocols were approved by the McGill University Institutional Animal Care Committee. Mice were sensitized to OVA according to the method of McCusker *et al.* (McCusker et al., 2002). Mice received intranasal (IN) 1% ovalbumin (Sigma, Oakville, Ontario) in PBS, or PBS alone daily over a 10-day period, consisting of two 10-μL intranasal instillations in each nostril. A booster dose of IN allergen was administered on day 22, and allergen challenges were carried out by similar daily IN administration on days 29 through 33. Mice were divided into three groups: negative control (PBS-HSA-PBS, sensitized and challenged with phosphate-buffered saline, treated with human serum albumin), positive control (OVA-HSA-OVA; sensitized and challenged with ovalbumin, treated with HSA), and treatment (OVA-IVIG-OVA; sensitized and challenged with ovalbumin, treated with intravenous immune globulin). Some groups also received IVIG after PBS sensitization/challenge alone. For treatment, on day 28, mice were injected intraperitoneally with 400 μL (approximately 1.75 g/kg) of either 10% human intravenous immune globulin (Gammunex, Bayer Biological Products, Research Triangle Park, North Carolina) or 5% human serum albumin in normal saline (Bayer Biological Products).

Histological Assessment.

Formalin-fixed, paraffin-embedded histological lung sections were prepared. Animals were sacrificed at day 33, lungs were inflated with neutral buffered formalin, dissected, processed and embedded in paraffin, and sectioned at 5 μm. Lung sections were stained with Hematoxylin and Eosin according to standard protocols and were examined by light microscopy for signs of airway inflammation by 2 blinded examiners and graded according to a 0-4 point scale validated in our laboratory (Salehi et al., 2009). Sections were also stained for immunohistochemistry using anti-CD3 and anti-B220 antibodies (Biolegend, Cedarlane, Toronto, ON, Canada) following antigen retrieval. Antigen retrieval was performed by microwaving samples in sodium citrate buffer, pH 6 for 10 min, and the endogenous peroxidase activity was blocked by hydrogen peroxide, 0.5% for 30 min.

Evaluation of Airway Responsiveness.

On day 34, airway hyper-responsiveness was assessed by intravenous methacholine challenge, as described previously (Koohsari et al., 2007; Wang and McCusker, 2005). Mice were anaesthetized by i.p injection of xylazine hydrochloride (12 mg/kg) and sodium pentobarbital (39 mg/kg). A tracheostomy was performed and a cannula inserted into the Y-adaptor of a FlexiVent small animal ventilator (SCIREQ, Montreal, Quebec). Mice were ventilated at a tidal volume of 10 mL/kg, a respiratory rate of 150 breaths/min, and an I:E ratio of 3:2. Post-expiratory end pressure (PEEP) was kept between 1.50 and 2.00 cm H_2O. Doubling doses of methacholine chloride (MCh) was injected i.v. (16-512 μg/ml), and resistance was measured.

Mononuclear cell proliferation.

Spleens were dissected in an aseptic manner, and mononuclear cells were isolated by collagenase digestion. Antigen-induced proliferative response was evaluated by ^{3}H-thymidine (^{3}(Adamski et al.)thy) incorporation assays in 96-well plates. Splenocytes were seeded at a density of 2 x 10^{5} cells in 200 μL of complete RPMI 1640 medium (4 mM L-Glutamine, 1% Penicillin-Streptomycin, 1 mM sodium pyruvate, 15 mM HEPES, 10 % FBS, 55 μM β−mercaptoethanol) and incubated in the presence or absence of 1 mg/mL OVA at 37ºC, 5% CO_2 for 5 days. Cells were labeled with 1 μCi/well of ^{3}H-thymidine (MP Biomedicals, Montreal, Quebec) 24 hours prior to harvesting onto glass fiber filters (Wallac, Chicago, Illinois). Thymidine incorporation was then quantified by liquid scintillation counting (Wallac). Proliferation is reported as a stimulation index (SI), the ratio between sample and control counts per minute of ^{3}(Adamski et al.)thy.

Bronchoalveolar lavage.

Bronchoalveolar lavage was performed using 1 ml of ice cold saline for 2 instillations. Cytospin slides were prepared, and differential cell counts were obtained under light microscopy by 2 blinded examiners. The fluid was analyzed by ELISA for cytokines IL-4, IL-5, IL-13, and IFN-γ, as directed by the manufacturers (BioLegend for IL-4, IL-5, TNF-α and Biosource for IFN-γ and IL-13 from Cedarlane, Toronto, ON).

Molecular analysis and quantitative real-time PCR.

RNA was extracted from lungs post sacrifice using TRIzol (Invitrogen). cDNA was prepared by reverse transcription and mRNA was quantified by SYBR Green RT-qPCR (LightCycler, Roche)

according to previously published protocols (Adamski et al.). The following primers were used: Gata3: 5'-CTGCGGACTCTACCATAAA-3' (sense) and 5'-CTGGATGCCTTCTTTCTTC-3' (antisense); Jagged-1: 5'-AGAAGTCAGAGTTCAGAGGCGTCC-3' (sense) and 5'-AGTAGAAGGCTGTCACCAAGCAAC-3' (antisense), Delta-4: 5'-AGGTGCCACT-TCGGTTACACAG-3'(sense) and 5'-AATCACACACTCGTTCCTCTCTTC-3' (antisense). GAPDH was used as a reference gene for normalization, with the primers 5'-AGCAATGCCTCCTGCACCACC-3' (sense) and 5'-GAGGCTGGTAAGGAACTGG-3' (antisense). All samples were normalized to the corresponding individual GAPDH level, and the results were calculated according to specific internal standards.

Dendritic cell phenotyping.

For dendritic cell phenotyping, splenocytes were pooled and dendritic cells purified using MACS Pan DC microbeads (anti-CD11c, clone N418, and Anti-mPDCA-1, clone JF05-1C2.4.1) (Miltenyi Biotech, Auburn, California). The isolated dendritic cells were blocked with Fc-Block (BD Biosciences Mississauga, Ontario) and stained for the presence of the H-2 histocompatibility complex, I-A^d haplotype (clone AMS-32.1, BD) and CD80 (clone 16-10A1, BD) or CD86 (clone GL1, BD). After surface staining, cells were permeabilized with BD Cytofix/Cytoperm, and stained for intracellular IL-12/IL-23 p40 (clone C15.6, BioLegend) or IFN-γ (clone XMG1.2, BioLegend). Data was acquired and analyzed by flow cytometry using a BD FACSCalibur.

Statistical Methods.

Comparative statistics were performed with Prism 5.0 using ANOVA with Dunnet's or Bonferroni multiple comparisons post-tests conducted in between experimental groups. A *p* value less than 0.05 was considered statistically significant evidence of differences between groups. Directionality was determined by the sign of the mean difference.

Results

IVIG inhibits cellular infiltration in murine allergic airway disease.

Sensitized mice were treated with either IVIG or HSA one day prior to OVA challenge. They were sacrificed on Day 33, and lung tissues were examined for histological features of allergic airway disease. Airway inflammation was present in OVA-HSA-OVA mice, with a perivascular infiltrate of mononuclear cells and granulocytes including eosinophils (Figure 1C). There was pronounced disruption of the brush border and evidence of goblet cell hyperplasia (Figure 1C). Conversely, decreased inflammation was noted in the lungs of IVIG-treated animals (Figure 1B). The peribronchial and perivascular mononuclear cell infiltrate included significant numbers of $CD3^+$ T-lymphocytes in the OVA-HSA-OVA mice but not in the IVIG group (Figure 1F). B-lymphocytes, identified by B220 staining, were frequently present in peribronchial organized follicular structures, as well as single cells in the OVA-HSA-OVA group (Figure 1G), but not in IVIG treated mice or PBS controls (data not shown). There was a significant difference in the inflammation score between IVIG and HSA treated animals (Figure 1H). Higher baseline inflammation was noted in the IVIG treated group (Figure 1H) than PBS, as IVIG was administered after sensitization and a booster dose of antigen; however, it was mild, patchy in nature and there was more variability between animals. In keeping with treatment post sensitization, there was no significant decrease in systemic IgE between the groups (data not shown). The results were similar in Balb/C and C57BL/6 mice (data not shown).

Allergen induced Airway Hyper-responsiveness is attenuated by IVIG treatment.

In order to determine if IVIG treatment was able to diminish airway hyperresponsiveness, airway mechanics were measured by FlexiVent in OVA-sensitized and challenged mice treated with IVIG or HSA. Typically, OVA sensitized and challenged animals exhibited a dose dependent increase in pulmonary resistance to methacholine (MCh). Figure 2 illustrates the responses of each group to doubling doses of inhaled MCh. IVIG treatment was able to attenuate airway hyper-responsiveness, bringing it down to near-baseline levels.

IVIG inhibits OVA-induced Cell Proliferation.

To assess if IVIG was effective in inhibiting bronchial responsiveness and pulmonary inflammation by interfering with cell-mediated responses to OVA, we evaluated the action of IVIG on OVA-driven T-cell proliferation. We initially examined the *in vitro* effect of IVIG or HSA added to cultured splenocytes from animals that had been sensitized, but not challenged to OVA. Spleens were removed on day 22, and cultured with and without OVA. A response to OVA, as indicated by an increase in cell proliferation measured by ^{3}(Adamski et al.)thy uptake, was present in OVA sensitized mice and was not inhibited by the addition of HSA. However, addition of IVIG diminished the proliferation of cells in a dose dependent manner (Figure 3A), with IVIG doses below 1mg/ml being ineffective. The effect of IVIG was specific for antigen-driven proliferation; with no significant effect on CON A stimulated proliferation (data not shown).

Moreover, we evaluated splenocyte proliferation in response to OVA following *in vivo* IVIG treatment. Mice treated with IVIG had significant attenuation of cell proliferation in response to

OVA (Figure 3B) with stimulation indices comparable to non-sensitized animals. Proliferation to CON A was again not decreased by *in vivo* IVIG treatment (data not shown).

IVIG attenuates inflammatory cytokine release.

Because IVIG inhibited cell proliferation to antigens, we hypothesized that IVIG would inhibit the elaboration of inflammatory and regulatory cytokines. Supernatants from splenocytes cultured with and without OVA from sensitized, treated and challenged mice were harvested at 4 and 7 days. OVA-sensitized mice produced significant IL-4, IL-13 and TNF-α at both 4 (Figure 4) and 7 days of culture (data not shown) when stimulated with OVA *in vitro*. Cells treated with IVIG had lower baseline levels of all cytokines than cells from OVA sensitized, HSA treated mice at both time points. IVIG treatment of OVA sensitized mice significantly reduced the production of the Th2 cytokines IL-4 and IL-13 and the inflammatory cytokine TNF-α. IVIG did not significantly alter *in vitro* splenocyte IL-10 production (Figure 4).

Pulmonary Th2 responses are altered by IVIG treatment.

We examined if dampening of pulmonary inflammation by IVIG was due to alteration in the interaction between T-cells and antigen presenting cells such as dendritic cells. We examined mRNA from homogenized pulmonary tissues to study key co-stimulatory molecules in the Notch pathway, Delta-4 and Jagged-1, which mediate Th1 and Th2 development respectively (Amsen et al., 2009a). Following OVA sensitization and challenge, there was a large increase in expression of Jagged-1 (Figure 5A), which is characteristic of Th2 responses. This was accompanied by an increase in GATA-3, the transcription factor that is necessary for induction of Th2 cytokines such as IL-13 (Figure 5C). Mice treated with IVIG prior to challenge exhibited

mRNA levels of Jagged-1 (Figure 5A) and GATA-3 (Figure 5C) that were similar to PBS-HSA-PBS mice. In addition, IVIG treated animals expressed over 2-fold more mRNA for Delta-4, the ligand expressed on DC that augments Th1 responses (Figure 5B). This suggests that IVIG treatment may alter the co-stimulatory interaction between APC and T-cells.

In keeping with these findings, we evaluated dendritic cell maturation and cytokine production from spleens of the mice from each group. IVIG treatment diminished the number of Ia^+CD80 and Ia^+CD86^+ DC compared to the OVA-HSA-OVA groups (Figure 6). Furthermore, we detected increased intracellular Th-1 cytokines IFN-γ and IL-12 in the $CD11c^+$ DC of IVIG treated mice (Table 1), in keeping with the phenotype of DC with increased Delta4 mRNA expression.

IVIG treatment induces T regulatory cells in local draining lymph nodes.

To further address the anti-inflammatory effects of IVIG, we examined mice post-sacrifice for the presence of regulatory T-cells. Spleens and draining (cervical and mediastinal) lymph nodes were dissociated and CD4 populations isolated by flow cytometry. We then targeted $CD4^+$ cells that expressed CD25, the high affinity IL-2 receptor, as well as the regulatory transcription factor FOXP3. Figure 7A indicates that IVIG treated mice had a significant increase in the $CD4^+CD25^+FOXP3^+$ population. The increase was primarily evident in draining cervical lymph nodes (Figure 7C). In contrast, little change was exhibited systemically, with minimal differences in the numbers of Treg in the spleen of IVIG treated mice vs. the other groups (Figure 7B).

Discussion

Severe asthma is a difficult condition, with both high morbidity and resource utilization (Taylor et al., 2008). Individuals with steroid resistant or difficult to control asthma have been treated with remittive agents to enhance the efficacy of corticosteroids or to minimize side effects. The difficulty in finding effective adjunct treatments is exemplified in recent trials employing TNF-α antagonists that were relatively ineffective (Morjaria et al., 2008),(Matera et al., 2009). Intravenous immunoglobulin is cited frequently as a potential steroid sparing agent for severe asthma, although results of small clinical trials have been mixed, and a mechanism of action is unclear (Orange et al., 2006). Due to its favorable risk benefit ratio and efficacy in other inflammatory conditions, IVIG remains a potential alternative therapy in severe asthma. Moreover, determining the mechanism of immune modulation in an antigen-driven disease such as allergic asthma is important for understanding of the role of IVIG in other inflammatory conditions.

We demonstrate that administration of high-dose IVIG to allergen-sensitized- mice attenuates the inflammatory infiltrate, decreases bronchial hyperresponsiveness to methacholine, and diminishes cellular proliferation to OVA. This is accompanied by favorable alterations in co-receptor expression for the Notch ligands Delta-4 and Jagged-1, a decrease in GATA3 mRNA, and diminished Th2 cytokine production by cultured lymphocytes ; signs that IVIG can suppress Th2 responses accompanying repeated allergen exposure. IVIG did not decrease systemic serum IgE, which was induced by OVA sensitization. However, IVIG therapy increased the numbers of regulatory T-cells were found in the draining LN of IVIG treated mice.

These results contribute to an increasing number of studies demonstrating the complexity of immune regulation by IVIG. For example, IVIG is highly effective in treating Immune Thrombocytopenic Purpura (ITP). In murine models of ITP, inhibition of established disease is dependent on the presence of FcγRIIb, the inhibitory Fc receptor. IVIG treatment upregulates inhibitory Fc receptors, particularly on monocytes, macrophages and dendritic cells (Anthony et al., 2008b; Crow et al., 2003; Kaneko et al., 2006; Siragam et al., 2006). Sialylated n-glycan residues linked to asparagine at position 297 on the terminal Fc portion of IgG molecules have been hypothesized to contribute prominently to the anti-inflammatory action of IVIG in ITP as well as in murine models of joint disease (Anthony et al., 2008b). Other possible mechanisms include interference with Fc receptor function attenuation of cellular proliferation, decreases in cytokine production and direct effects on T-lymphocytes, B-lymphocytes, and dendritic cells.

In our study, IVIG significantly attenuated bronchial hyper-responsiveness (BHR) and antigen specific cell proliferation, as well as OVA-induced histological changes including cellular infiltration, goblet cell hyperplasia and epithelial disruption. BHR is dependent on several factors, including the cellular infiltrate and cytokine milieu. Th2 cytokines, specifically IL-4, IL-5 and IL-13, contribute to the establishment and maintenance of BHR (Wang and McCusker, 2005). IL-13 is well recognized to be an important contributor to the inflammation in asthma. Gene linkage studies have pointed to IL-13 and IL-13 receptor polymorphisms among the most reproducible genes that increase susceptibility to asthma. IL-4 and IL-13 mediate the production of IgE. IL-13 activates epithelial cells, smooth muscle, and with IL-9 increases mucous production and goblet cell hyperplasia. (Brightling et al., 2009; Wills-Karp, 1999; Wills-Karp et al., 1998), (Fawaz et al., 2007). The source of IL-13 is predominantly T-lymphocytes, with

contributions from mast cells, eosinophils and potentially B-lymphocytes (Hajoui et al., 2004), and is dependent on the transcription factor GATA3.

Initiation of the Th2 response is commonly considered IL-4 dependent. However, as DC rarely produce IL-4, alternative pathways have been evaluated to explain the induction of Th2 responses(Amsen et al., 2009a). The Notch pathway, which includes Notch and Notch ligands (Delta and Jagged) plays a crucial role in many developmental processes. The interaction between the different Notch molecules and ligands regulate vascular, morphologic and hematopoietic development. Indeed, thymic development is highly dependent on the presence of Notch on T-cell precursors and Delta on thymic epithelial cells, and both marginal zone B-cells and dendritic cells require expression of Notch for normal development. In directing naïve T-cells towards the Th2 lineage, the initiating signal from APC, especially dendritic cells, appears to be the Notch ligand Jagged-1(Amsen et al., 2009a; Amsen et al., 2009b). Downstream Notch-1 signals following this interaction lead to the induction of GATA-3, and subsequent production of IL-4, IL-5 and IL-13 and signaling via IL-4R (Amsen et al., 2007; Fang et al., 2007; Okamoto et al., 2009). Conversely, the presence of Delta isoforms, especially Delta-4, can induce Th1 cells in an IL-12 independent manner. The expression of Delta-4 also can inhibit the production of Th2 cells (Amsen et al., 2009b; Fukushima et al., 2008). Delta-1 may be able to upregulate ROR□ and thus induce the IL-17 family of cytokines and Th17 cells (Higashi et al., 2010). Our data on lung homogenates indicates that the Notch receptor ligands respond to OVA sensitization and challenge by following an expected pattern, with upregulation of Jagged-1 and no change in Delta-4, indicating a preferential Th2-type response. This leads to the induction of GATA-3, and the production of Th2-type cytokines. The administration of IVIG appears to alter the interaction of Notch on T-cells with the cognate ligand; there is a decrease in Jagged-1, an increase in Delta-

4, suppression of GATA-3, and increased intracellular IFN-γ in DC. Clearly, this data must be explored further, with determination of the Jagged and Delta expressing cells and determination if other, downstream signaling pathways are implicated in the action of IVIG.

There was no significant effect on the production of IgE, showing that in spite of the presence of the pre-existing immune sensitization, IVIG could still be effective. Although IVIG may directly influence IgE production by B-cells (Sigman et al., 1998; Zhuang and Mazer, 2001) the anti-inflammatory effect from its acute use in already sensitized allergic airways disease is not due to inhibition of IgE production.

Another prominent effect of IVIG was its ability to decrease antigen specific proliferation. Splenocytes from sensitized mice, cultured *ex vivo*, did not respond to exogenous OVA when IVIG was either present in culture or when IVIG had been administered prior to OVA-challenge. This was accompanied by attenuated production of Th2 cytokines *in vitro*. Possible targets for IVIG thus include direct action on T cells or impairment of antigen presentation. A direct action on T-cell proliferation *in vitro* has been previously documented, as has (Amran et al., 1994; Tha-In et al., 2006) inhibition of antigen presentation by DC (Tha-In et al., 2007), through impairment of DC maturation and increases in immunoregulatory cytokines (Bayry et al., 2003b; Crow et al., 2009). Decreases in antigen presentation have been correlated with decreased expression of Ia and other MHC-Class II molecules (Bayry et al., 2003a). The best explored mechanism for the inhibition of DC maturation appears to be via FcγRIIb ligation, leading to negative signaling and decreased DC activation (Siragam et al., 2006). DC express a specific receptor, DC-SIGN, that appears to bind the sialylated IgG molecules and provide anti-

inflammatory signals (Anthony et al., 2008a) which may be important in the decreased DC maturation that we and others have documented.

An important issue to examine more extensively is whether the effects of IVIG are antigen specific. In our cell proliferation experiments, IVIG only inhibited splenocyte response to OVA, but not to the mitogens such as CON A or to GM-CSF^{+}IL-4 (data not shown). In addition, we did not detect any effect of IVIG on the overall bronchial responsiveness, when animals were only exposed to PBS. If the action of IVIG is antigen-specific, this would increase the rational for targeted use of this therapy in antigen-driven inflammation.

Finally, we have found that the inhibition of BHR and pathologic changes of airway responsiveness are associated with local, but not necessarily systemic increases in CD25+FoxP3+ Treg cells. This implies that administration of IVIG systemically can alter the local immune responses in this model of allergic inflammation. Ephrem et al (Ephrem et al., 2008), using a murine model of autoimmune experimental encephalitis, observed increases in $CD4^{+}CD25^{+}$ cells in IVIG treated animals, which acted as classical regulatory/suppressor cells in *in vitro* assays. Individuals treated with IVIG for various autoimmune diseases were found to respond with increased Treg in their circulation, and IVIG also induced Treg in culture (Kessel et al., 2007). Experiments are currently underway to determine if Treg found in these studies represent inducible Treg or recruitment of naturally occurring Treg, and to determine the characteristic cytokine responses of these cells. The discovery of Tregs in the local immune response as a consequence of IVIG treatment does not preclude other regulatory mechanisms, including alteration of DC phenotype and cytokine profile, induction of SOCS proteins or recruitment of IL-10 producing B-lymphocytes, among others. Notch-Delta interaction has been

suggested to be able to induce Treg (Huang et al., 2009; Rutz et al., 2008; Rutz et al., 2005). Interestingly, Notch signaling is essential in the development of MZ B cells expressing CD1d (Scheikl et al., 2009); these cells have recently been implicated in anti-inflammatory processes by producing IL-10 and facilitating Treg recruitment (Amu et al., 2010).

Taken together, these data provide evidence for an anti-inflammatory action of IVIG in allergic airways disease, and is the first demonstration of an anti-inflammatory effect of IVIG in a model of disease associated withTh2-type inflammation. The reproducibility and flexibility of this model allows for further study of the mechanism of IVIG and extending these findings to other antigen induced inflammatory conditions. In addition, the effects that IVIG has on BHR, antigen-induced cell proliferation and cytokine production may lead to a re-evaluation of antibody based immune modulation therapies that may attenuate the ongoing allergic inflammation in severe asthma.

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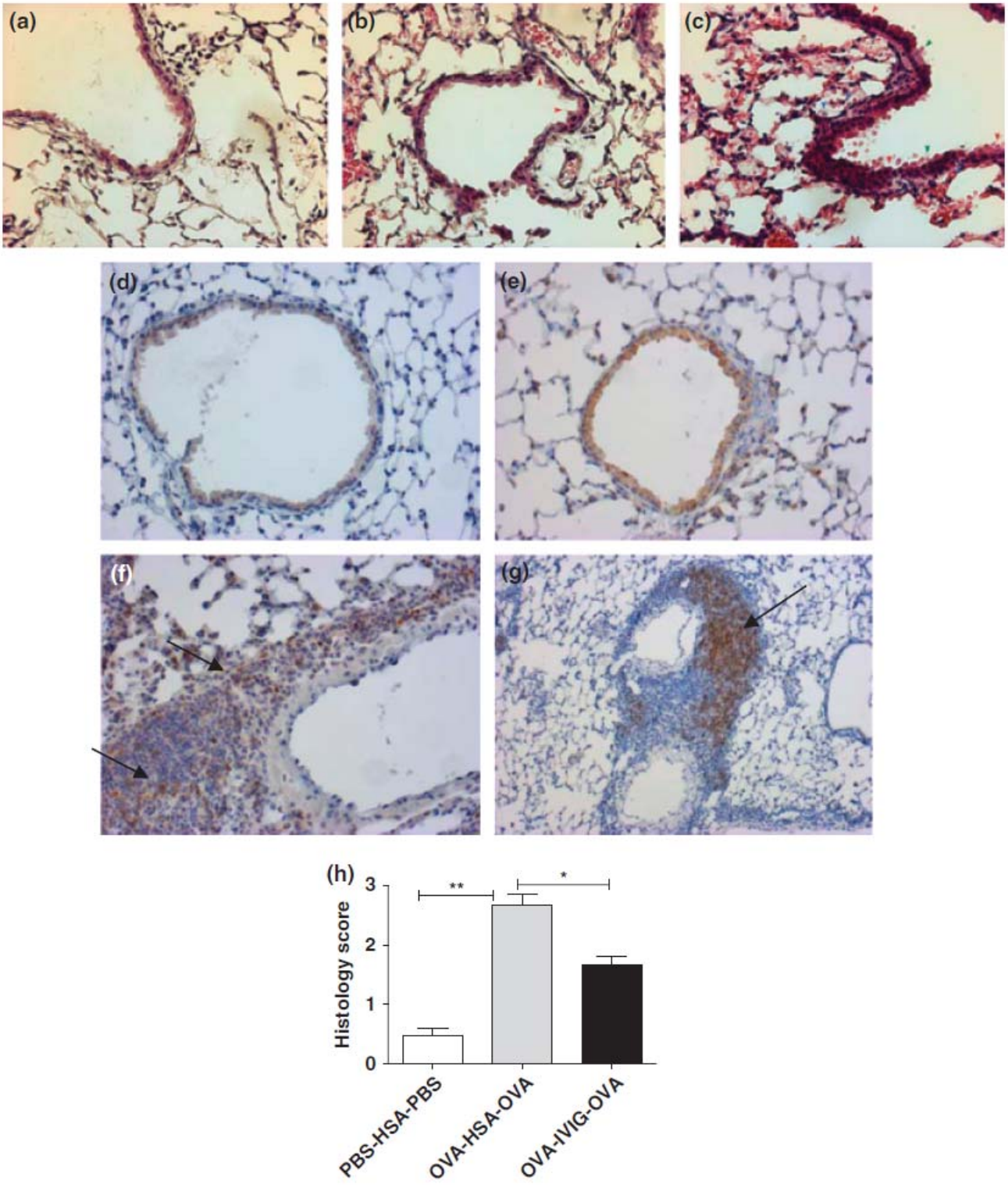
Figure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OVA-IVIG-OVA

Figure 1. IVIG diminishes lung inflammation in allergic airways disease.

H&E staining of mouse lung sections (original magnification 100x.) (A) PBS-HSA-PBS; (B) OVA-IVIG-OVA; (C) OVA-HSA-OVA. Red arrowheads, brush border disruption; green arrowheads, goblet cells; blue arrowheads, granulocytes. D-F: Immunohistochemical detection of $CD3^+$ T cells; D: OVA-IVIG-OVA; E: OVA-IVIG-OVA; F: OVA-HSA-OVA. Arrows indicate $CD3^+$ cells staining brown. G: Immunohistochemical detection of B220+ B-lymphocytes in the OVA-HSA-OVA group. Arrows indicate $B220^+$ follicles, staining brown. Representative of 12 mice in each experimental group. Very few $B220^+$ cells were found in sections from control or IVIG treated animals. H: Cumulative histological scoring of 25 sections from 5 mice in each group, based on a 4 point scale. * $p<0.05$ comparing OVA-IVIG-OVA and OVA-HSA-OVA; ** $p<0.01$ compared to PBS-HSA-PBS, by ANOVA with Dunnett's t-test.

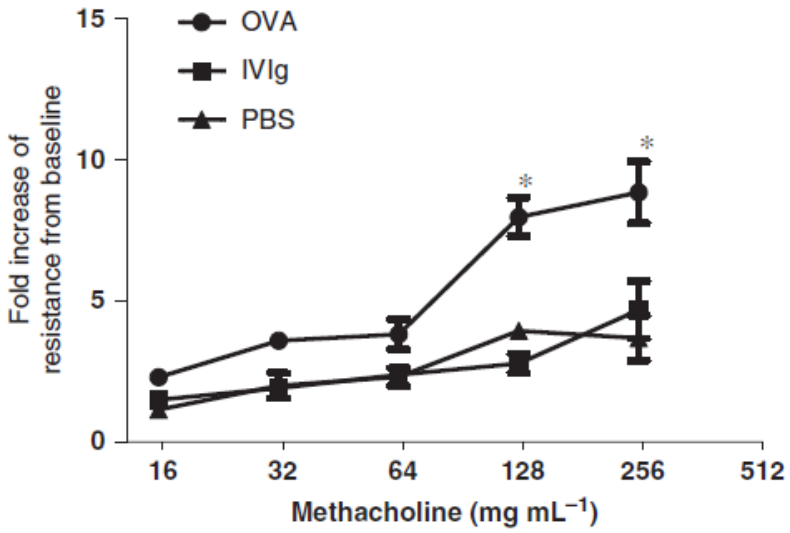

Figure 2. IVIG inhibits Bronchial responsiveness to Methacholine.

Following day 5 of challenges, bronchial responsiveness to methacholine was measured by FlexiVent. IVIG treatment attenuated the increase in pulmonary resistance to near baseline levels. n=9 per group, *= $p<0.05$ by ANOVA with Bonferonni's correction.

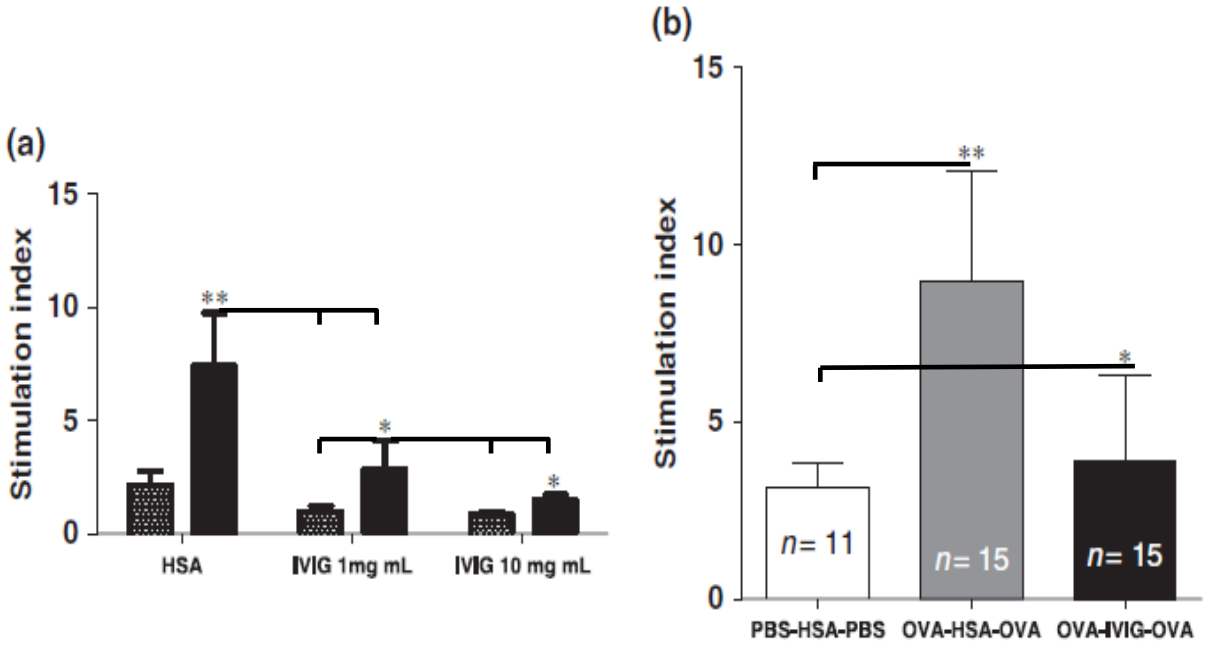

Figure 3. IVIG attenuated OVA-driven splenocyte proliferation.

Panel A: Splenocytes were purified from mice sensitized to OVA following the booster dose on day 22, and cultured with PBS vehicle (hatched bars) or OVA (solid bars) *in vitro* with or without IVIG or HSA. IVIG treatment impaired the response to OVA in a dose dependent manner. **p<0.01, *p<0.05 n=6 by one-way ANOVA. B. Splenocytes from mice sensitized, treated with IVIG and then challenged were purified, and exposed to OVA *in vitro* without added IVIG. *In vivo* IVIG treatment also attenuated the response to OVA; * p<0.05, compared to both PBS-HSA-PBS and OVA-IVIG-OVA. as indicated on graph.

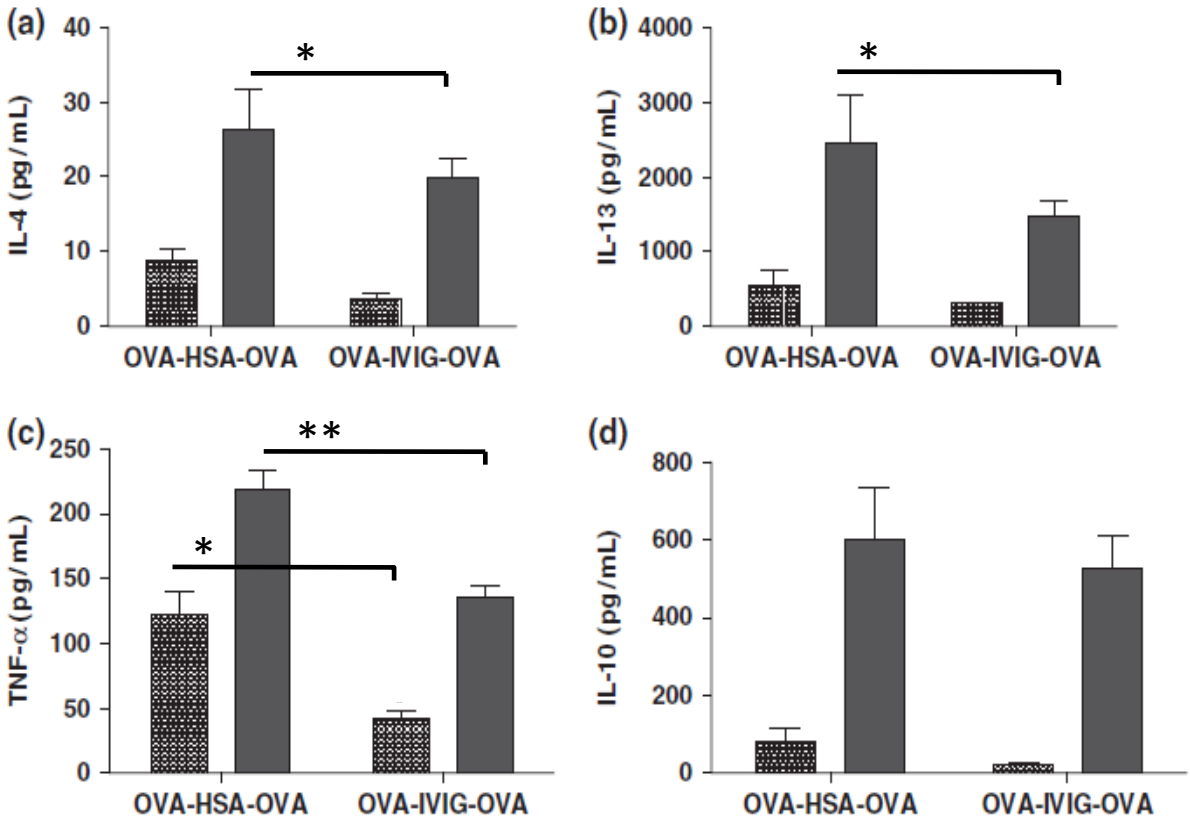

Figure 4. IVIG attenuates *in vitro* cytokine production.

Splenocytes from animals treated with IVIG or HSA control were cultured for 7 days with medium alone (hatched bars) or OVA (solid bars). Splenocytes from IVIG treated mice produced significantly less IL-4, IL-13 and TNF-α in response to OVA than splenocytes from HSA treated animals. *$p<0.05$ **<0.005, n=5 mice per group.

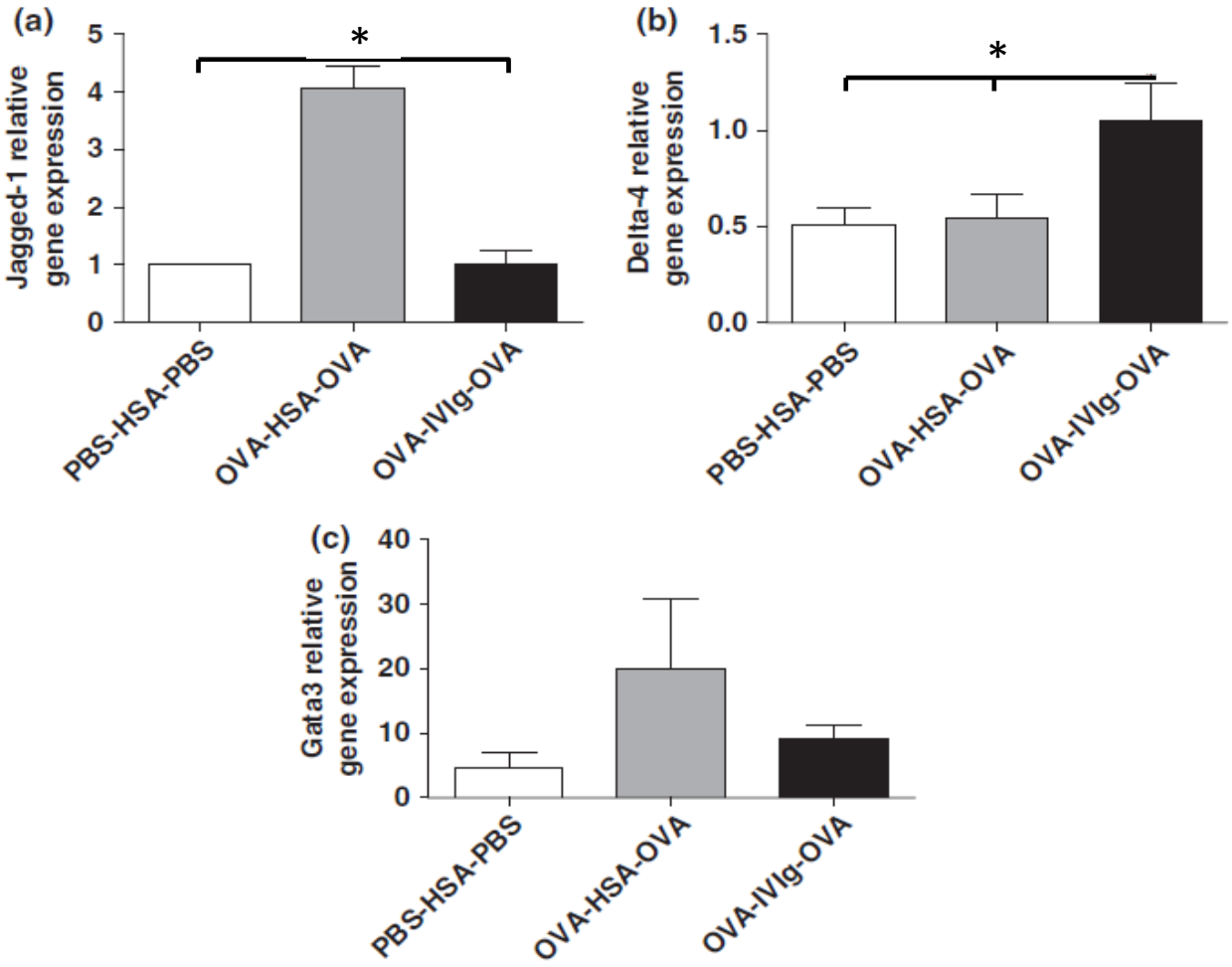

Figure 5. Induction of mRNA for Notch ligands and Th2 transcription factor GATA3.

A and B) Detection of A) Jagged-1 and B) Delta-4 by quantitative Real-Time PCR from lung homogenates. n=6, ** $p<0.01$; * $p< 0.05$ by one-way ANOVA. C) induction of mRNA for the Th2 transcription factor GATA3 detected by fluorescent real-time PCR from lung homogenates. p value = 0.057 by one-way ANOVA with Dunnett's T-test, comparing GATA3 in OVA-HSA-OVA with the other 2 groups.

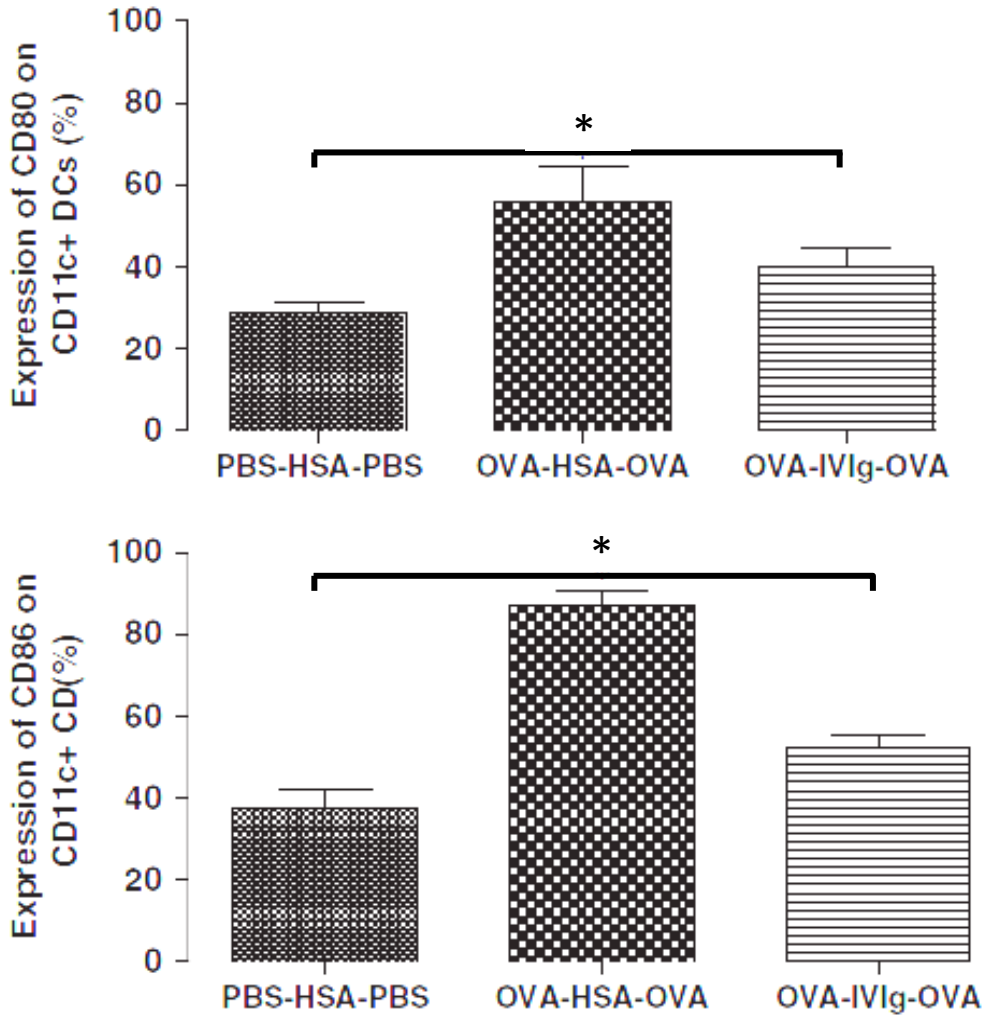

Figure 6. IVIG treatment diminished dendritic cell maturation.

$CD11c^+$ cells were purified from pulmonary digests by magnetic bead separation, and stained for Ia and co-stimulatory molecules CD80 and CD86. Upper panel, CD80+ DC; lower panel, CD86+ DC. *p=0.05 by one-way ANOVA with Dunnett's t-test.

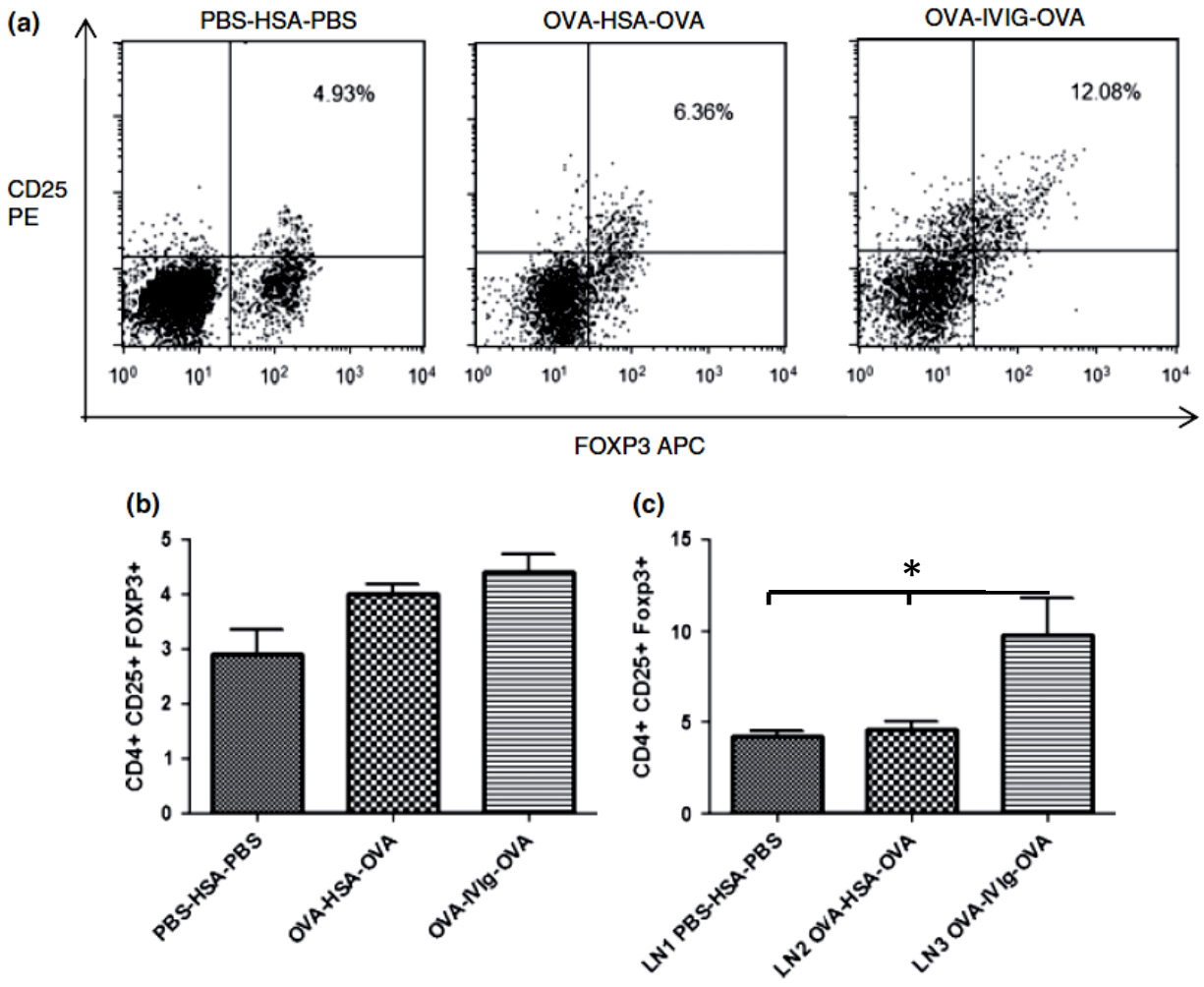

Figure 7. Assessment of T-regulatory cells in local and systemic tissues:

Cell suspensions were prepared from spleens and pulmonary lymphoid tissues and evaluated for the presence of $CD4^{+}CD25^{+}FOXP3^{+}$ cells. A) Representative dot plot of detection of Treg in draining lymph nodes in each of the three treatment groups, PBS-HSA-PBS, OVA-HSA-OVA and OVA-IVIG-OVA. . B) Cumulative histograms of Treg detected in spleens from the three treatment groups; C) Cumulative histograms of Treg detected in the draining lymph nodes from the three treatment groups. n=4 sets of 3 mice. *p=0.05 by one-way ANOVA with Dunnett's t-test.

Dendritic cell marker	PBS–HSA–PBS	OVA–HSA–OVA	OVA–IVIG–OVA
$Ia1^+CD11c^+CD86^+IFN\text{-}g^+$	15.75.4	22.73.4	30.75.5*
$Ia1^+CD11c^+CD80^+IFN\text{-}g^+$	20.46.8	28.52.8	41.76.3*
$Ia1^+CD11c^+$ $CD86^+IL\text{-}12^+$	15.54.5	21.83.8	25.86.0
$Ia1^+CD11c^+$ $CD80^+IL\text{-}12^+$	18.86.2	27.34.8	30.15.4*

Table 1. Cytokine measurements within purified splenic $CD11c^+$ dendritic cells.

Mouse spleens were removed following 5 days of OVA challenge, the cells were dissociated and $CD11c^+$ dendritic cells were purified using magnetic beads. Cytokine measurements were performed on n = 5 sets of mice, three pooled spleens for each. The data represent per cent of all $Ia^+CD11c^+CD86^+$ or $CD80^+$ cells detected by flow cytometry, following surface staining for co-stimulatory molecules and intracellular staining for IFN-γ or IL-12. *$P<0.05$ vs. other control groups, by one-way ANOVA.

Article II

Intravenous immunoglobulin attenuates airway inflammation via the induction of Foxp3^{+} regulatory T cells in a murine model of allergic asthma

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Abstract

Intravenous immunoglobulin (IVIg) is an important disease modifying therapy for a large spectrum of autoimmune and inflammatory diseases, yet the mechanisms of action are incompletely understood. Using a robust murine model of antigen-driven allergic airways disease, we demonstrated that IVIg markedly improved OVA-induced airway hyper-responsiveness (AHR), accompanied by a 4-to 6-fold increase in regulatory T-cells (Treg). In this study we address the cellular interactions that lead to induction of Treg by IVIg. IVIg-therapy of OVA-sensitized and challenged mice led to induction of antigen-specific $Foxp3^+$ Treg from non-Treg precursors. The induced Treg home specifically to lungs and draining LN, and have highly potentiated suppressive activity compared to Treg purified from sham-treated controls. Induction of Treg is mediated by tolerogenic dendritic cells (DC) generated following IVIg treatment. Compared to sham-treated OVA-exposed mice, IVIg-primed DC express altered surface Notch-ligands including increased Delta-4 and reduced Jagged-1, reflecting decreased Th2 polarization. Furthermore, IVIg primed DC can stimulate Treg differentiation from uncommitted T cell precursors *ex vivo*, and adoptive transfer of IVIg-primed DC abrogates AHR and induces Treg. Thus, the well-documented anti-inflammatory effects of IVIg therapy may be mediated in part by the immunomodulation of DC and subsequent induction of antigen-specific, highly suppressive regulatory T cells.

Introduction

Intravenous Immunoglobulin (IVIg) is the most commonly used plasma derivative. It is the mainstay of therapy for primary and secondary immune deficiencies, and is most extensively used in the treatment of autoimmune and inflammatory diseases. Although IVIG therapy has been employed for close to 3 decades, the mechanism of action is incompletely understood (Ballow, 2011). Direct effects of IVIg on B-cells, T-cell, DC, neutrophils and Natural Killer cells have been described, and the action of IVIg may be mediated by both Fc and Fab portions of the IgG molecule. Most recently, crucial moieties that mediate the anti-inflammatory action of IgG have been elucidated, including Fc receptors such as FcγRIIb, and glycosylation and sialylation sites that are an integral part of the Fc receptor of IgG molecules (Nimmerjahn and Ravetch, 2007b). The impressive progress made in determining the structural basis of the action of IVIg must be complemented by further study into the cellular basis of IVIg-induced tolerance.

We have recently demonstrated that murine allergic airways disease is significantly attenuated by injection of therapeutic, human IVIg (Kaufman et al., 2011). We observed that this treatment leads to a significant inhibition of pulmonary inflammation and airway hyper-reactivity (AHR) (Kaufman et al., 2011). This was accompanied by a decrease in maturation of dendritic cells (DC) and significant alterations of Notch-ligands, from a phenotype that predisposes to Th2 inflammation to one that inhibits Th2 responses. A most striking finding of this study was the observation of a highly significant increase in Treg in the lungs and draining lymph nodes of IVIg treated mice. IVIg has been employed in severe steroid dependent asthmatics, but the reasons for its efficacy are not well defined and further trials are required to better understand its potential use (Ballow, 1999; Orange et al., 2006).

Regulatory T cells (Treg) are essential mediators of normal immune responses. Imbalances in Treg number or function are implicated in autoimmune and allergic diseases. This is manifest in its extreme by the IPEX syndrome, whereby mutations in the critical transcription factor Foxp3 causes a syndrome characterized by severe skin, endocrine and allergic manifestations and enteritis with X-linked inheritance (d'Hennezel et al., 2009). Induction of Treg is a characteristic of successful injected allergen immunotherapy treatment for airway disease (Akdis and Akdis, 2007) and oral immunotherapy for food allergy (Vickery et al., 2011). In murine models of allergic airways disease, the infusion of Treg to sensitized mice is associated with abrogation of pulmonary inflammation and airways reactivity (Huang et al., 2009; Schmidt-Weber and Blaser, 2005), and other maneuvers that induce Treg minimize allergic inflammation (Preston et al., 2010). A goal of therapy in severe, chronic asthma is to design treatment approaches that can improve the balance between effector T (Teff) and Treg subsets.

Therapy with IVIg has been associated with increased levels of Treg in both human diseases and in murine models (Ephrem et al., 2008; Kessel et al., 2007; Olivito et al., 2010). In this paper we have focused on this novel regulatory property of IVIg therapy. We have demonstrated for the first time that IVIg treatment promotes the induction of Treg from non-regulatory precursors. These Treg are highly suppressive and antigen specific. The IVIg-mediated induction of Treg is a direct consequence of its action on a subset of $CD11c^{+}$ pulmonary DC. Thus, polyclonal IgG acts to bridge the innate and adaptive immune systems, modifying DC phenotype, and generating $Foxp3^{+}$ Treg cells, which then modulate subsequent inflammatory responses.

Material and Methods

Wild type (WT) and reporter mouse stains.

C57BL/6 mice were obtained from Charles River Laboratories (Senneville, QC). C57BL/6 GFP-Foxp3 knock-in reporter mice (B6.Foxp3^{GFP}) were kindly provided by A. Y. Rudensky (Memorial Sloan-Kettering Cancer Centre, NY). OT-II transgenic mice expressing the MHC class II-restricted, OVA-specific Vα2/Vβ5.1 TCR were obtained from Taconic Laboratories, and were mated to B6.Foxp3^{GFP} reporter mice to produce transgenic mice expressing OVA-specific Foxp3^{+} Treg cells. All mice were bred and maintained in a specific pathogen-free animal facility at McGill University, and generally used at 6-8 weeks of age according to procedures accepted by the Animal Ethics Committee at McGill University.

Murine model of allergic airway disease.

Mice were sensitized intranasally (i.n.) to OVA as in (McCusker et al., 2002) with modifications as in (Kaufman et al., 2011). One day prior to antigen challenge, (day 28) IVIg, 2g/kg (Gammunex, Talecris Biotherapeutics, Missisaugua ON) or an equal volume of 3% HSA (Talecris Biotherapeutics) as a control, was administered intra-peritoneally (i.p.). Control mice received i.n. PBS only and were also treated with either IVIg or HSA. Ragweed (RW) sensitization was performed by administering two i.p injections of RW (80 μg/injection) (Greer Laboratories, Lenoir, NC) in 25 μl of Imject Alum (Pierce, Rockford, IL) and 75 μl of PBS 4 days apart. Seven days after the second i.p. injection, mice were challenged by i.n. administration of 8 μg of RW in PBS. 24 h after the final challenge, mice were sacrificed by ketamine/xylazine overdose with subsequent exsanguination.

Histological analysis.

Lungs were removed, fixed with 10% buffered formalin, processed and embedded in paraffin. 0.5-µm sections from all lobes were stained with H&E and examined by standard light microscopy. For immunohistochemistry (IHC), antigen retrieval was performed in sodium citrate buffer, pH 6, by heating in a microwave for 10 minutes. Tissues were permeabilized using 0.2% Triton 100X in TBS. Sections were immersed in serum-free protein block (Dako, Burlington, ON, Canada), followed by incubation with primary anti-GFP antibodies (Sigma Aldrich), diluted 1/200 in Dako antibody diluent at 4□C overnight. The second antibody was a biotinylated anti-rabbit IgG antibody (Cell Signaling Technology, Boston, MA) (1/100 dilution). The signal was amplified by applying streptavidin–HRP-streptavidin (Vector Lab. Burlingame, CA) for 45 min. Peroxidase was developed using diaminobenzidine (DAB1) (Dako,). Counter-staining was performed with hematoxylin and sections evaluated by light microscopy.

Airway responsiveness to methacholine.

Mice were exposed to saline, followed by doubling concentrations of aerosolized methacholine, (MCh, 16-256 mg/ml, Sigma-Aldrich, Mississauga ON, Canada) using a flexiVent small animal ventilator (Scireq, Montreal, QC, Canada) and Aeroneb lab nebulizer adjusted to a 4 second nebulization time at a cycling rate of 50%, synchronized with inspiration. A 1.2 second, 2.5 Hz single-frequency forced oscillation maneuver (SFOT; SnapShot-150 perturbation) was performed at 10 sec intervals and respiratory system resistance (R) and elastance (E) were calculated with commercial software by fitting the equation of motion of the linear single compartment model of lung mechanics to the SFOT data using multiple linear regression. The

relative peak response at each MCh dose compared to the baseline was calculated (Shalaby et al., 2010).

Preparation of cell suspensions from lymphoid and non-lympoid tissues.

Lungs were removed, perfused with PBS and inflated with 1ml Collagenase (0.2 Wunsch units/ml from *Clostridium histolyticum* (Type XI-S), Sigma-Aldrich) in PBS and 0.5 mM Ca^{2+}. Deep cervical draining lymph nodes (dLN) were removed before lung digestion. The dissociated lungs were minced and incubated with stirring for 45 min at 37°C in 3ml collagenase, DNAse (1000D Unit/ml) (Sigma-Aldrich) in PBS and 0.5 mM Ca^{2+}. The slurry was passed through a 40µm nylon cell strainer. The cells from spleens and other lymphoid organs were mechanically dissociated through a 90µm mesh. Single cell suspensions were resuspended in complete medium consisting of RPMI-1640 (Invitrogen, Toronto, ON, Canada) supplemented with 10% FBS (Hyclone, Logan UT), l-glutamine, Penicillin/Streptomycin, Hepes, and b-mercaptoethanol.

Cell separation and adoptive transfer.

$CD4^+$ T cells were isolated from spleen and lymph nodes of B6.$Foxp3^{GFP}$ or OT-II-$Foxp3^{GFP}$ reporter mice by positive separation of magnetically labeled $CD4^+$ cells (Miltenyi Biotec, Vancouver, BC) followed by FACS sorting of GFP^+ cells. $3x10^6$ $CD4^+Foxp3^{-GFP-}$ cells were adoptively transferred through the tail vein to WT mice. Plasmacytoid DC (pDC) were separated from digested lung cells by positive separation, using a cocktail of antibodies (CD304 and CD141) (Miltenyi Biotech). Magnetically labeled $CD11c^+$ DC were removed from the pDC-depleted cells (Miltenyi Biotec). The overall purity of isolated $CD11c^+$ cells was typically ≥ 95%.

Flow cytometric analysis.

Cell samples were washed in PBS containing 2% FBS and FC-receptor binding was blocked with IVIg for 20 minutes (100µl/50ml). The antibodies utilized included anti-CD62L PE, anti-CCR7 PE, anti-CCR4 PE-Cy7, anti-CD11c APC, anti-CD11c FITC, anti-CD8α FITC, anti-Jagged-1 PE, and anti-Delta4-APC, purchased from Becton-Dickinson. 10^6 cells were incubated with appropriate antibodies for 30 min at 4°C. After staining, the cells were washed with PBS-2% FBS. Some cell samples were fixed and permeabilized with Cytofix/Cytoperm (eBiosciense) and incubated with anti-Ki67 PE (BD-Biosciences), or anti-Foxp3 APC (eBioscience). Intracellular cytokine staining was performed on phorbol-12-13-dibutyrate (5 ng/ml) (PDB, Sigma-Aldrich) and ionomycin (50 ng/ml) (Sigma Aldrich) stimulated cells, in the presence of BD GolgiPlug™ (BD-Bioscience) After 6 hours of culture, the cells were permeabilized and stained with anti-IL-10 APC or anti-IFN-γ APC (BD-Biosciences,) for 30 min at 4°C Relative fluorescence intensities were determined on BD FACS Calibur flow cytometer.

Assessment of Treg suppressor activity.

$CD4^+$ T cells were purified from spleens and dLNs of naïve OT-II mice and incubated with CFSE (1µM) for 5 min at 37°C. Cells were washed and resuspended in culture medium for 15 min. GFP^+-Treg cells were recovered from lungs and dLN by cell sorting. CFSE-labeled OT-II $CD4^+$T cells were mixed with Treg in ratios of 1:1 to 1:16 and co-cultured in six well plates. Cells were stimulated with 1 mg/ml OVA, with $2x10^5$ purified $CD11c^+$DC added as APCs. After 3 days the cells were harvested and CFSE dilution analyzed by flow cytometry.

Statistics. Data analyses were performed using the Prism 5 (GraphPad Software, San Diego, CA). One or two-way analysis of variance (ANOVA) with the Tukey post-test was used to determine statistical differences compared to control groups. A P value < 0.05 was considered statistically significant. Columns and error bars represent mean ± SEM.

Results

The *in vivo* protective effect of IVIg is associated with induction of Foxp3+ Treg cells from non-regulatory $CD4^+$ T cell precursors.

We demonstrated that IVIg attenuated AHR in a murine model of allergic airway disease, associated with a significant increase in $Foxp3^+$ Treg cells detected in the lungs of IVIg treated mice. We thus investigated if IVIg could induce Treg from $CD4^+Foxp3^-$ precursors, or if treatment with IVIg was associated with expansion of pre-existing $Foxp3^+$ cells. $3x10^6$ purified $CD4^{+GFP-}$ T cells were adoptively transferred to WT syngeneic mice prior to OVA or sham (PBS) sensitization. 24 hours prior to antigen challenge, mice received either IVIg, or HSA as a control. Following 5 days of OVA or sham challenge, the mice were sacrificed and expression of $Foxp3^{GFP}$ was assessed by flow cytometry. IVIg treatment led to a 4 to 6-fold increase in the expression of $CD4^+Foxp3^{+GFP+}$ cells within spleen, lungs and dLN (Fig. 1a, b). Of note, there was also an increase in the percentage of endogenous ($CD4^+Foxp3^{+GFP-}$) Treg in IVIg treated mice, compared to the other groups (Fig. 1a, b). These results indicate that IVIg treament can induce Treg from conventional $CD4^+Foxp3^-$ T cells as part of its immunoprotective effects *in vivo*.

We have previously shown that OVA-sensitized and challenged mice exhibit a significant peribronchial and perivascular infiltrate of $CD3^+$ cells, which was significantly reduced by IVIg (Kaufman et al., 2011). The distribution of iTreg in lung tissue of recipient mice was determined histopathologically by detecting $Foxp3^{GFP+}$ cells in lung sections. As illustrated in Figure 2, there is a substantial reduction in the overall peribronchial cell infiltrate in OVA-IVIg-OVA mice compared to the OVA-HSA-OVA group. This was accompanied by a 10-fold increase in

Foxp3^{GFP+} iTreg within the perivascular and peribronchial infiltrate in the lungs of OVA-IVIg-OVA mice (Fig. 2d). The striking increase in iTreg in the IVIg treated mice was mediated by expression of homing receptors that would predispose to migration from dLN to the pulmonary parenchyma. Specifically, we assessed the expression of CD62L (L-selectin) on total cells and Foxp3^{+} Treg obtained from spleen, dLNs and lung tissue. A majority of Treg found in dLNs expressed CD62L in OVA-IVIg-OVA mice, while expression of this receptor was down-regulated on Foxp3^{+} Treg in lung (Fig. 3a), as would be expected in cells that were homing to tissues. Furthermore, iTreg detected in the peribronchial/perivascular infiltrate of IVIg treated mice expressed 3 to 4-fold higher levels of CCR4, critical for the localization of cells into lung tissue (Fig. 3b,). In parallel, the expression of CCR4 on leukocytes from dLNs (Fig. 3) and distal, non-draining LN (data not shown) remained at basal levels. IVIg did not alter CCR7 expression in lung or dLN (data not shown). iTreg from IVIg treated mice showed increased proliferation with significantly higher Ki67 staining compared to HSA treated mice (Table I). These data suggest that IVIg induces iTreg, and that CCR4 expression may be a key chemokine receptor for mediating homing of iTreg to arrest OVA-induced pulmonary inflammation.

Induction of Foxp3^{+} Treg cells by IVIg is antigen-specific.

We questioned whether the iTreg induced by IVIg were antigen specific or were due to polyclonal expansion of Treg. To investigate the action of IVIg in the induction phase of antigen specific Treg, OT-II mice were bred onto the B6.Foxp3^{GFP} reporter background to provide a source of OVA-specific T-cells that would express GFP if converted to Treg. CD4^{+}Foxp3-$^{GFP-}$ cells were purified from OT-II.Foxp3^{GFP} mice and adoptively transferred to WT C57BL/6 syngenic mice. The mice were sensitized and challenged with either OVA or RW, and IVIg or

HSA were again administered 1 day prior to challenge. RW sensitization and challenge induced significant pulmonary inflammation as well as increased AHR (data not shown). A 6-fold increase in Foxp3+$^{GFP+}$ iTregs was observed following challenge in OVA-IVIg-OVA mice (Fig. 4a & c). However, while there was a significant increase in endogenous Foxp3^{+} Tregs in RW-IVIg-RW mice compared to other groups (Fig. 4b), only baseline levels of GFP^{+} cells were detected in ragweed challenged mice (Fig. 4b & d). This experiment indicates that IVIg treatment leads to the preferential induction of antigen-specific Treg *in vivo*.

IVIg induces Foxp3^{+} Treg cells with increased suppression activity.

Since IVIg therapy was able to strongly influence the induction of Treg cells, we asked if the potency of suppression of antigen-specific iTreg was augmented by IVIg treatment. To this end, the suppressive capacity of IVIg iTreg was compared to Treg harvested from the control groups using an *ex vivo* suppression assay. As depicted in Figure 5, Treg obtained from OVA-IVIg-OVA mice were consistently more effective in suppressing proliferation of OVA stimulated OT-II T-cells than Treg from PBS-HSA-PBS or OVA-HSA-OVA mice. Treg from IVIg-treated mice suppressed OVA-induced CD4 proliferation at ratios of 1:1-1:16 (Fig. 5). Furthermore, IVIg iTreg demonstrated higher frequencies of IL-10^{+}and IFN-γ^{+} cells (Table II) compared to Foxp3^{+} Tregs from control groups. Taken together, these data suggest that treatment with IVIg to sensitized and challenged mice not only favors the development of Treg, but also robustly increases the potency of the effector phase of Treg activity.

IVIg primes CD11c^{+} DC to generate Treg.

Recent studies implicate DC in the elaboration of peripheral T cell tolerance, through induction and expansion of antigen-specific Treg cells (Akbari et al., 2001; Huang et al., 2010). To test the ability of IVIg to induce tolerogenic DC, splenic CD11c^{+}DCwere purified from naïve mice and primed with IVIg (10 mg/mL) for 3 hours, then incubated with OVA (2mg/ml) for 3 hours. DC primed with OVA alone or IVIg alone were used as controls. IVIg+OVA primed DC, co-cultured with purified CD4^{+} T cells from OTII.Foxp3^{GFP} mice, generated a 2-fold increase in expression of CD4^{+}Foxp3+$^{GFP+}$ compared to DC that were pulsed with OVA or IVIg alone or CD4^{+} cells cultured with IVIg alone (Fig. 6a). Thus iTreg induction is mediated, at least in part, by DC, and both antigen and IVIg are required for optimal Treg induction.

We then assessed whether DC from OVA-IVIg-OVA mice induced Treg *ex-vivo*. CD11c^{+}DC, purified from digested lungs of the four experimental groups, were co-cultured with 10^{6} Foxp3-CD4+ T-cells from OT-II.Foxp3^{GFP} mice, stimulated with OVA for 5 days. Only DC harvested from IVIg treated mice increased the expression of Foxp3^{GFP+} in cultured CD4^{+} cells (Fig. 6). We also fractionated CD11c^{+} myeloid DC and CD11c^{-} plasmacytoid DC to determine which DC population was primarily responsible for the tolerogenic effects of IVIg (Dunne et al., 2009; Huang et al., 2010; Ito et al., 2007). CD11c^{+}DC from OVA-IVIg-OVA treated mice induced Treg from conventional T-cells, while pDC from OVA-IVIg-OVA did not (Fig. 6). Furthermore, neither pDC nor CD11c^{+}DC from OVA-HSA-OVA treated mice induced Foxp3 expression in CD4+ T cells (Fig. 6b). Collectively, our results show that myeloid CD11c+ DC are selectively modulated by IVIg, and are responsible for the generation of iTreg *in vivo*.

The tolerogenic DC from IVIg treated mice differed from the other DC populations. We previously demonstrated that CD80 and CD86 expression were reduced on DC following IVIg treatment (Kaufman et al., 2011). Compared to OVA-HSA-OVA mice, IVIg treated mice had increased numbers of pulmonary DC that expressed intracellular IL-10 as well as surface expression of CD8α. We also previously found that IVIg treatment was associated with changes in Notch ligands, including elevated Delta4 mRNA and diminished Jagged-1 mRNA in digested lung tissue (Kaufman et al., 2011). Pulmonary DC from all treatment groups were thus examined for Jagged and Delta isoforms by flow cytometry. The expression of Delta-like-4 increased from the baseline on OVA-IVIg-OVA pulmonary DCs, while the expression of Jagged-1 decreased in OVA-IVIg-OVA DCs as compared to OVA-HSA-OVA (Table III). These results suggest that the induction of Treg by IVIg may involve modulation of Delta-4 expression and/or activity on tolerogenic $CD11c^+$ DC.

Adoptive transfer of DCs from OVA-IVIg-OVA mice recapitulates the protective effects mediated by IVIg treatment *in vivo*.

To determine if the induction of Treg by IVIg was mediated by its actions on DCs, we hypothesized that adoptive transfer of DCs from OVA-IVIg-OVA mice would inhibit AHR and induce Treg in OVA sensitized and challenged mice. Pulmonary $CD11^+c$ DC from PBS-HSA-PBS, OVA-HSA-OVA, PBS-IVIg-PBS and OVA-IVIg-OVA mice were purified, and transferred intra-tracheally to WT syngeneic mice 24 hours prior to the OVA challenges. DC from OVA-IVIg-OVA mice promoted $Foxp3^+$ Treg in recipients, while adoptively transferred DC from OVA-HSA-OVA mice did not appear to increase Tregs in sensitized mice (10%-11.5% vs 6.5%-7.5%) (Fig.7a and b). The recipients of OVA-IVIg-OVA or PBS-IVIg-PBS DC also

showed significantly reduced hyperreactivity to methacholine challenge (Fig. 8), and substantially less inflammatory changes in the airways (Fig. 8), compared to recipients of DC from OVA-HSA-OVA or PBS-HSA-PBS mice. The results demonstrated that adoptive transfer of DC from OVA-IVIg-OVA mice induces Treg cells which subsequently inhibit OVA-driven airway reactivity.

Discussion

IgG is a key player in immune networks. Beyond its major property in the defense against pathogens, IgG regulates immune function via idiotype-anti-idiotype networks, feedback inhibition of Ig production, and modulation of cytokine responses (Gupta et al., 2001). IVIg therapy is employed for a variety of diseases, including Immune thrombocytopenic purpura, Kawasaki syndrome, dermatomyositis and polymyositis, and various neurological conditions (Ballow, 2011). Despite the consistent efficacy of polyclonal IgG in a large number of unrelated conditions, no unifying mechanism of action has been determined. Experimental evidence exists for IVIg-mediated Fc receptor blockade, inhibition of lymphocyte and DC proliferation (Nimmerjahn and Ravetch, 2008), decreases in cytokine production, and impaired neutrophil migration (Macmillan et al., 2010). In both animal models and through direct observation in humans, important anti-inflammatory effects of high dose IgG appear to be mediated by the Fc portion of the IgG molecule, (Nimmerjahn and Ravetch, 2007a; Nimmerjahn and Ravetch, 2008). Whereas many effects of IVIg are FcγRIIb dependent, direct ligation of FcγRIIb has been difficult to demonstrate (Zhuang et al., 2002), and it is more likely that IVIg contributes to a pathways that upregulates FcγRIIb (Anthony et al., 2008a; Anthony and Ravetch, 2010). Fc interaction via glycan linkages such as sialylation sites may explain the ant-inflammatory action of IVIg. (Anthony et al., 2008a; Anthony and Ravetch, 2010) Our study broadens this rapidly emerging field by dissecting the links between IVIg, innate and adaptive immunity to ascertain its anti-inflammatory effects.

In this study, we utilize a well-described model of allergic airways diseases to dissect the mechanism of regulatory action of IVIg (McCusker et al., 2002; McCusker, 2004). The allergic

airway model has many advantages for evaluating the immune regulatory properties of polyclonal IgG, in that it is antigen-driven, requires antigen presentation and both T-and B-cell responses, and is highly amenable to examination of relevant tissues and physiological measurements. Furthermore, small clinical trials make a case for the study of IVIg in the treatment of individuals with severe asthma (Ballow, 1999; Orange et al., 2006). We have described that IVIg abrogates airway responsiveness, antigen specific T-cell proliferation, and cytokine production in murine allergic airways disease. This was accompanied by an increase in $Foxp3^+$ Treg cells in the lungs and draining lymph nodes of IgG treated mice (Kaufman et al., 2011). In this study, we address the dynamics and cellular basis of Treg cell induction by IVIg *in vivo*. More specifically, we ask whether IVIg can generate Treg from conventional $CD4^+$ T cells, the antigen specificity of IVIg-iTreg, and relative contributions of DC to this process.

Our data clearly demonstrate that high dose IgG induces antigen-specific Treg. Using adoptive transfer of CD4 cells from B6.$Foxp3^{GFP}$ reporter mice, we demonstrate that IVIg can induce Treg *de novo* from Foxp3-$^{GFP-}$ T-cells, suggesting that there is not simply an expansion of the endogenous Treg. Infusion of IVIg requires the presence of antigen to induce Treg, as IVIg alone does not increase Treg populations, though changes in DC phenotype can be documented. Furthermore, the adoptive transfer of $CD4^+$ T cells from OT-II-$Foxp3^{GFP}$ mice followed by exposure to a relevant antigen (OVA) or an irrelevant antigen (RW) revealed that IVIg only induced GFP^+ cells in the presence of the relevant antigen, OVA. Not surprisingly, the total number of Treg was increased in the tissues of mice sensitized and challenged to RW in the context of IVIg treatment. However, there was a conspicuous absence of OVA specific Foxp3GFP^+ cells in RW-sensitized mice. This implies that antigen specificity is a key feature of the anti-inflammatory effect of IVIg in this model.

The induction of antigen-specific Treg cells has important implications. It would suggest that Treg interact with the same antigen as Teff cells at the DC interface, and that the suppressive action is antigen-specific. This fact is emphasized by our observation that OVA-induced proliferation was inhibited in T-cells from IVIg treated mice, but mitogen induced T-cell proliferation was not affected (Kaufman et al., 2011). This antigen specificity favors the induction and migration of Treg to antigen-exposed sites, in this case the airways and draining LN. Moreover, the pattern of chemokine receptor expression induced by IVIg, with elevated CCR4 and decreased L-selectin in the lung, predisposed to homing to inflamed lungs. Lymph node-associated Treg expressed higher levels of L-selectin as well as CCR7. It remains to be determined if the trafficking of Treg cells is from the inflamed tissues to the dLN as proposed by Zhang et al (Zhang et al., 2009); and what other factors, including chemokines and cytokines, facilitates this pattern of circulation.

The highly suppressive effects of IVIg-induced Treg interacting with Teff (Fig. 5) provide an explanation for the marked decrease in pulmonary inflammation described in our initial report (Kaufman et al., 2011). IVIg-induced Treg suppressed OVA-mediated proliferation of OTII cells at a 4 to 8-fold lower dilution compared to identically prepared Treg from sham-sensitized and challenged or OVA-sensitized and challenged mice. Thus, exposure to high levels of polyclonal IgG is not simply associated with an increase in number of induced Treg, but an increase in suppressive potency. The increase in potency may be due to the increased percentage of antigen-specific Treg in the $CD4^+$ population, or to the higher percentage of Treg expressing IL-10 in IVIg treated mice. This implies that upon exposure to antigen, more Treg are likely to be activated by OVA in IVIg treated cell populations. This will be tested as we further investigate the action of IVIg-induced Treg.

The second novel finding that we present is the crucial link between IVIg primed DC and induction of Treg. Previous work (Anthony et al., 2008a; Bayry et al., 2003a; Bayry et al., 2003b; Crow et al., 2009; Samuelsson et al., 2001) documented that IVIg can modify maturation and surface receptor expression on DC. Modulation of FcγRIIb expression on DC may be due to direct interaction of IVIg with receptors on DC (Bayry et al., 2003a; Bayry et al., 2005; Crow et al., 2009; Crow and Lazarus, 2003), or be indirect via splenic macrophages (Anthony et al., 2008a). Our data indicates that IVIg primed DC, both *ex vivo* or adoptively transferred to OVA-sensitized mice, can replace IVIg, diminishing AHR and increasing Treg populations in the lung. IVIg treated DC have several important properties that may predispose to the induction of Treg. DC harvested from IVIg treated mice expressed lower levels of co-stimulatory molecules CD80 and CD86, as well as reduced Class II HLA molecules (Kaufman et al., 2011) (Bayry et al., 2003a). Diminished levels of co-stimulatory molecules are associated with immature or tolerogenic DC, which can induce anergy and increase inducible Treg (Steinman et al., 2003; Steinman and Nussenzweig, 2002). It remains to be demonstrated if the DC phenotype following exposure to IVIg represents a true arrest of maturity, or strictly an alteration of characteristics that predispose to tolerogenic activity. Studies that directly evaluate antigen processing and presentation will be crucial to determine this. In addition, significant changes in expression of Notch ligands are also induced by IVIg, including decreased Jagged-1 mRNA (Kaufman et al., 2011) and surface expression and reciprocal increases in Delta-4-like mRNA and protein. Jagged-1 and -2 induce Th2 responses, (Amsen et al., 2004; Amsen et al., 2009b) whereas Delta-4 can induce regulatory responses. Thus, the overall phenotype consistently reflects the ability of IVIg to power tolerogenic responses.

The receptor that IVIg interacts with to induce tolerogenic DC populations is unknown. Previous work with IVIg suggests that FcγR are important for the anti-inflammatory action in ITP. Prime candidates for the mediating the action of IVIg include the inhibitory receptor FcγRIIb and the activating receptor FcγRIII. Using rabbit IgG, Yamamoto et al (Yamamoto et al., 2010) abrogated AHR in a murine model of allergen–induced reactive airways disease in a FcγRIIb dependent manner (Yamamoto et al., 2010). Although experimental models suggest a crucial role for FcγRIIb, the adoptive transfer of IVIg-treated splenocytes or DC from FcγRIIb$^{-/-}$ mice is reproducibly able to duplicate the action of IVIg (Anthony et al., 2008a; Bruhns et al., 2003; Crow and Lazarus, 2003; Siragam et al., 2006). Immune complexes can also duplicate the action of IVIg, suggesting interaction with an activating FcγR on DC can induce anti-inflammatory responses (Crow et al., 2009; Crow et al., 2001). Another possible portal through which IVIg may regulate immune responses is via direct ingestion of specific epitopes on Fc receptors that can directly activate naturally occurring Treg. "Tregitopes" synthesized based on expression libraries of Fc receptors, can inhibit antigen specific serological and T-cell responses in mice (De Groot et al., 2008). Recently, it has been proposed that glycosylated regions of the Fc receptor may be crucial in conferring the anti-inflammatory action of IVIg. Specifically, sialylation of ASP 297 may be a crucial feature of the action of IVIg, in *in vivo* models such as ITP and serum-induced arthritis (Anthony and Ravetch, 2010; Anthony et al., 2008b; Nimmerjahn et al., 2007; Nimmerjahn and Ravetch, 2007a; Nimmerjahn and Ravetch, 2008), and in direct inhibition of cellular response such as B-cell activation (Seite et al., 2010).

The action of sialylated IgG may be mediated via engagement of Sign-R1 or the human homologue DC-SIGN. In an arthritis model, IVIg treatment of mice increased IL-4 levels following ligation of DC-SIGN. The anti-inflammatory effects of IVIg in the arthritis model

appeared to be dependent on Th2 pathway cytokines, IL-4 and IL-33 (Anthony et al., 2011a). Importantly, our model is a Th2 driven model, and Th2 cytokines were diminished in cells from IVIg treated mice (Kaufman et al., 2011). Different model systems may lead to differing anti-inflammatory effects of IVIg; there have been no description of induction of Treg in the K/BxN serum induced model of arthritis.

The action of IVIg in this model of allergic airways disease demonstrates an important link between innate and adaptive immunity. Polyclonal IgG appears to act to bridge development of tolerogenic DC and the production of targeted, antigen-specific Treg. Therapeutically, this represents an important paradigm to comprehend how so-called tolerogenic DC develop, and what crucial signals are necessary for the induction and expansion of antigen-specific Treg. In addition, the crucial role of humoral immunity in mediating both inflammatory responses, but perhaps more importantly, in effecting the balance between inflammation and essential regulatory events are now being elucidated.

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Figures

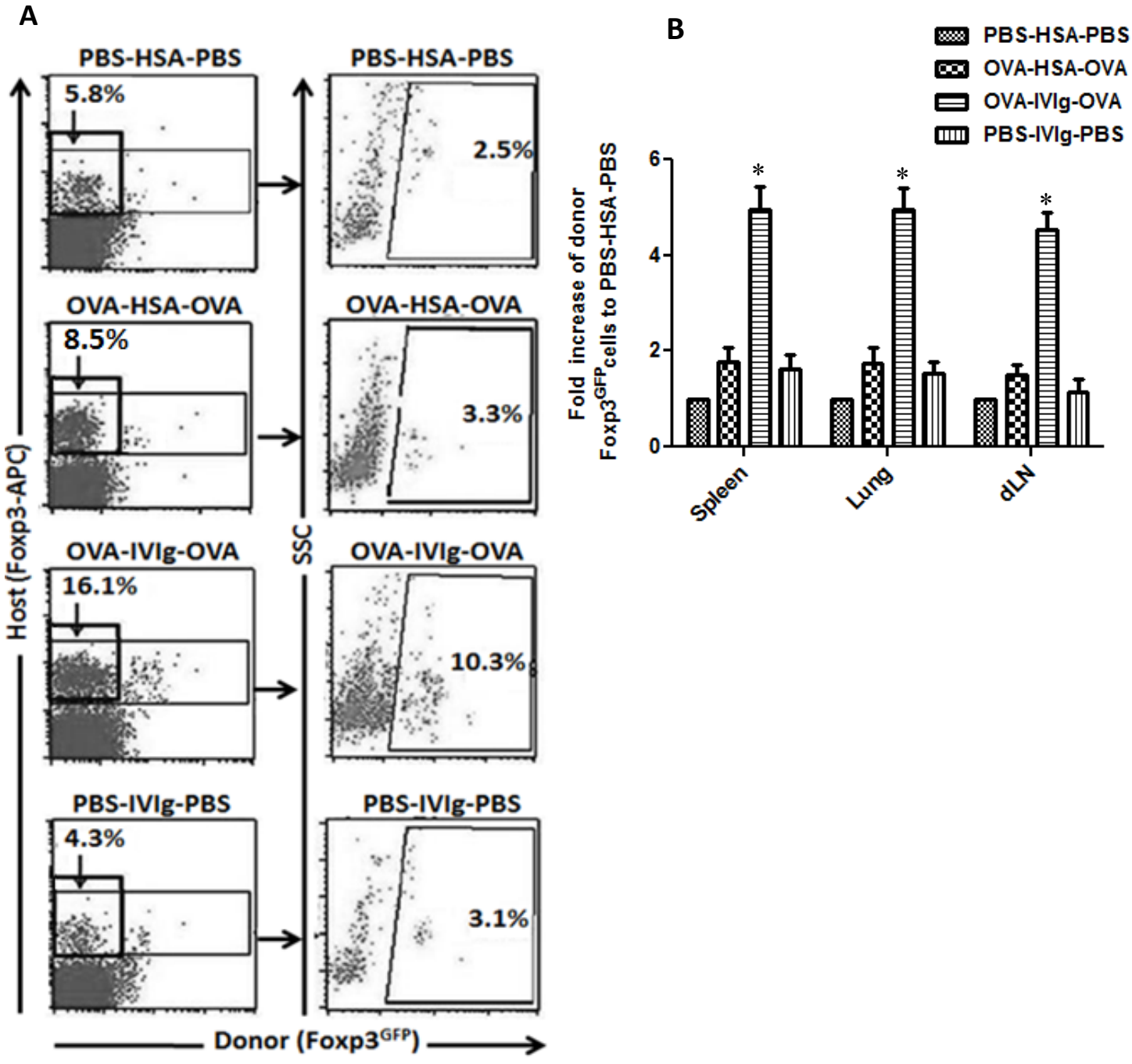

Figure 1. IVIg induces de novo Tregs from Foxp3 negative T-cells.

Representative flow cytometric analysis of endogenous, host Foxp3^{APC+} and induced, donor Foxp3$^{APC+GFP+}$ Treg cells within the lungs of mice with allergic airways disease. A: frequency of

(host) Foxp3^{APC+} Treg cells and (donor) Foxp3$^{APC+GFP+}$ cells. OVA-IVIg-OVA treatment induced Treg cells from Foxp3^{GFP-} precursors. Representative of three identical studies with n=9 for each condition. B: Bar graphs demonstrating the fold increase of induced Foxp3^{GFP+} cells among all Treg cells within spleens, draining LNs and lungs. *$p<0.05$. The results represent 3 identical experiments with 9 mice in each group.

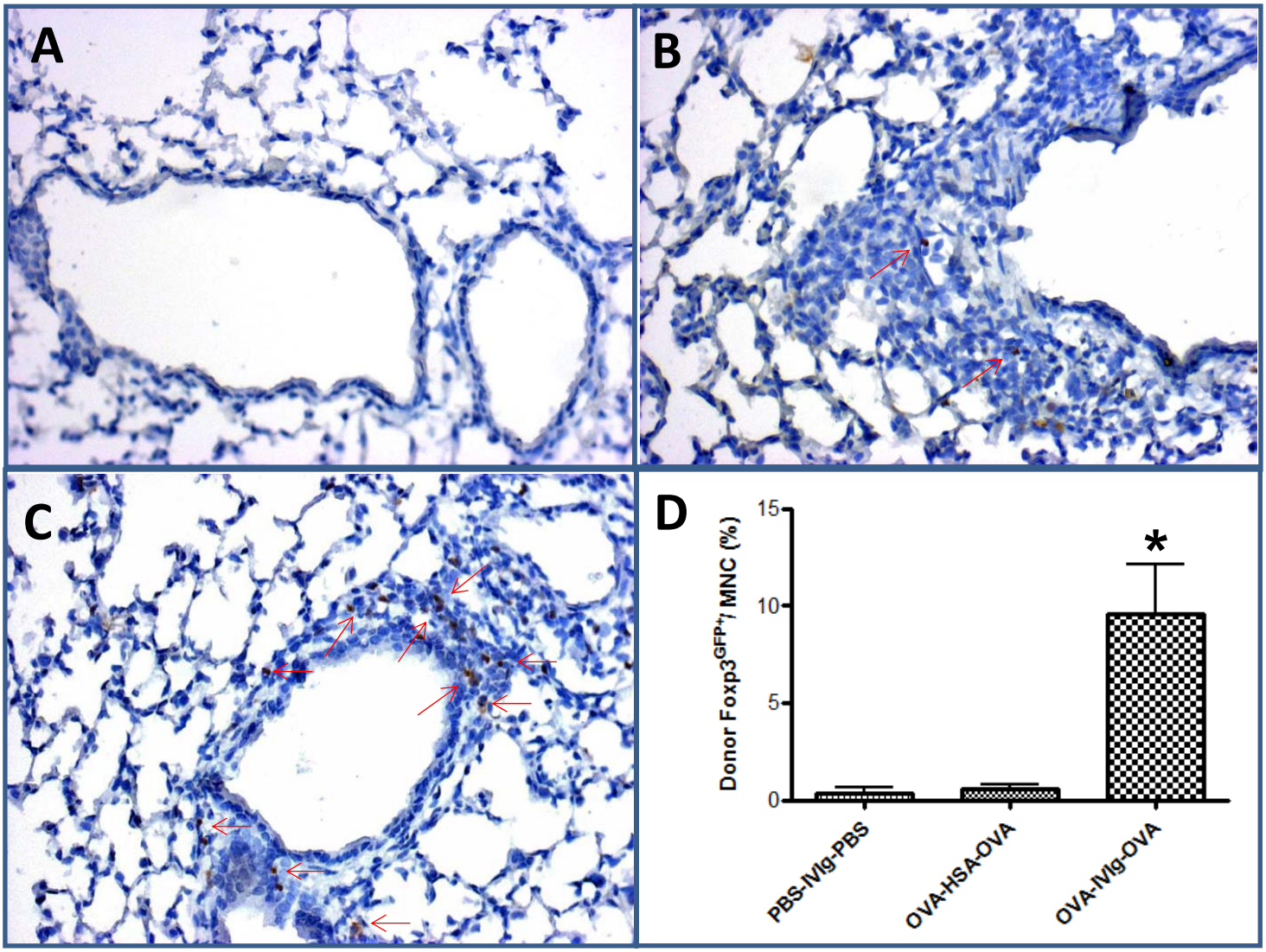

Figure 2. IVIg induced Treg are greatly enriched in inflamed pulmonary tissues.

Immunohistopathological analysis of donor Foxp3^{GFP+}cell in lung sections using anti-GFP Ab. OVA-IVIg-OVA mice (C) have decreased mononuclear cell infiltration compared to OVA-HSA-OVA mice (B). Within the mononuclear infiltrate, the frequency of induced GFP^{+} Treg in OVA-IVIg-OVA (C) mice is increased compared to other groups. D: Histogram indicating percentage of Treg within the mononuclear cell infiltrate from of each condition. Slides were prepared from 6 lungs for each experimental condition, representing three experiments. *$p<0.05$ vs. OVA-HSA-OVA and PBS-IVIg-PBS. The results represent 3 identical experiments with 6 mice in each group

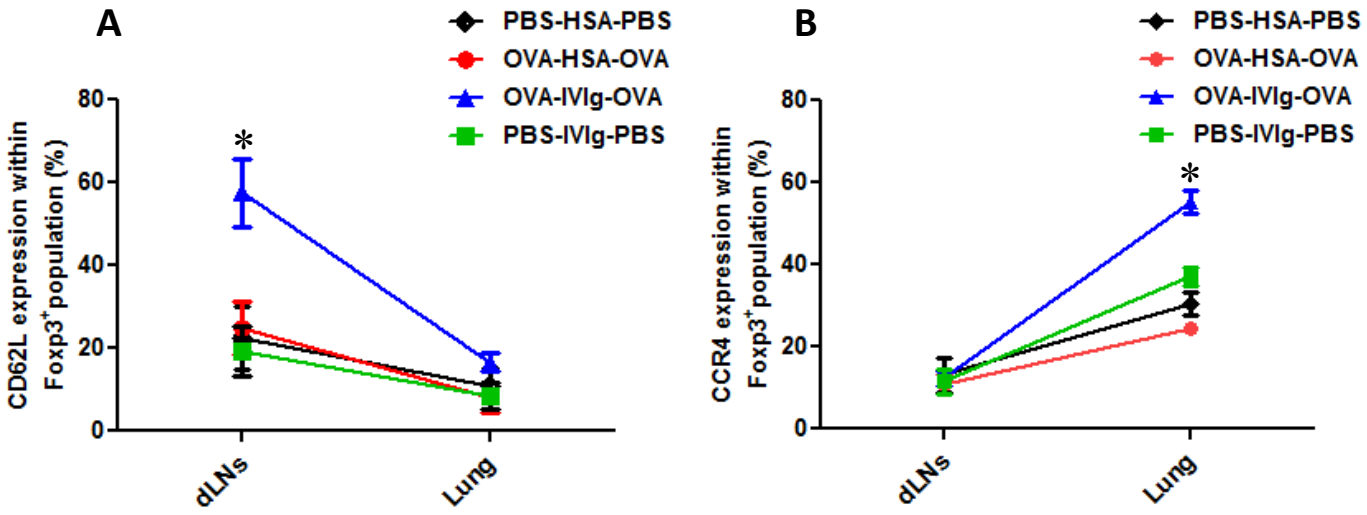

Figure 3. IVIg modulates homing receptors to encourage trafficking of Treg to pulmonary tissues.

A: CD62L expression was assessed on Treg from lung and dLN by flow cytometry. IVIg treated mice had diminished CD62L expression on Treg in lung compared to LN. B: The expression of CCR4 was determined as above. Treg populations within the lung of OVA-IVIg-OVA had significantly higher CCR4 expression than those in dLN, and compared Treg in all other experimental conditions. *$p<0.05$. Representative of 2 separate experiments with 6 mice in each group.

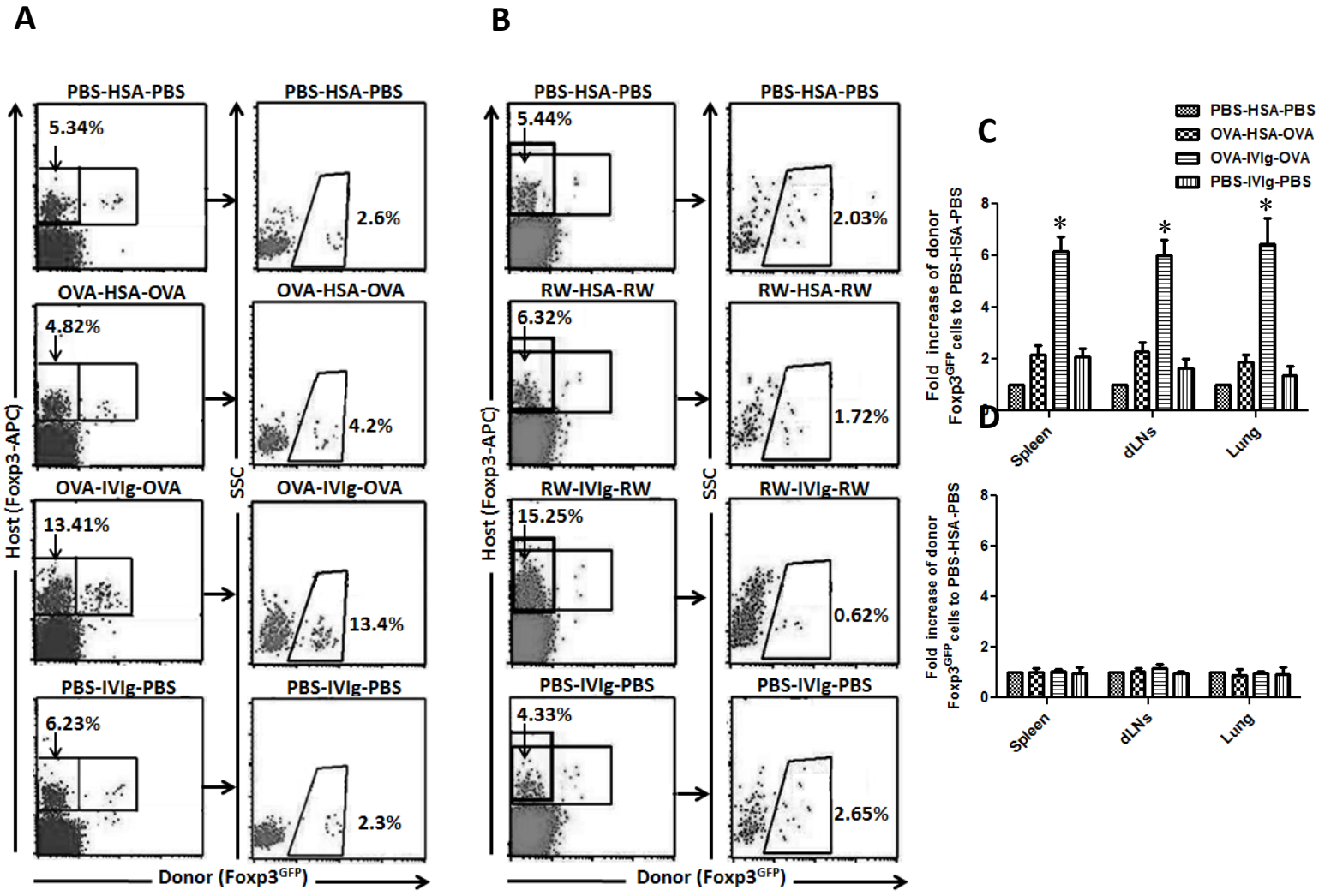

Figure 4. IVIg induces antigen specific Treg.

A, B: Representative flow cytometric plots of (donor) Foxp3$^{GFP+\ APC+}$ and (host) Foxp3 $^{APC+}$ Treg cells within the lungs of WT recipient mice. Prior to sensitization, all groups received 3 x10^6 CD4+$^{GFP-}$ T-cells from OT-II-Foxp3^{GFP} reporter mice. Only OVA-IVIg-OVA mice demonstrate an increase in OVA-specific CD4+Foxp3+$^{GFP+}$ cells (A) among the total Treg induced by IVIg. RW-IVIg-RW mice (B) demonstrate an increase in CD4+Foxp3+ cells compared to other groups, but no increase in Foxp3+$^{GFP+}$ cells. C , D) Fold increase of OVA-specific Foxp3^{GFP+} cells among all Treg cells within spleens, dLNs and lungs, compared to sham treated PBS-HSA-PBS control. *$p<0.05$. Data from two experiments with n= 6 mice per group.

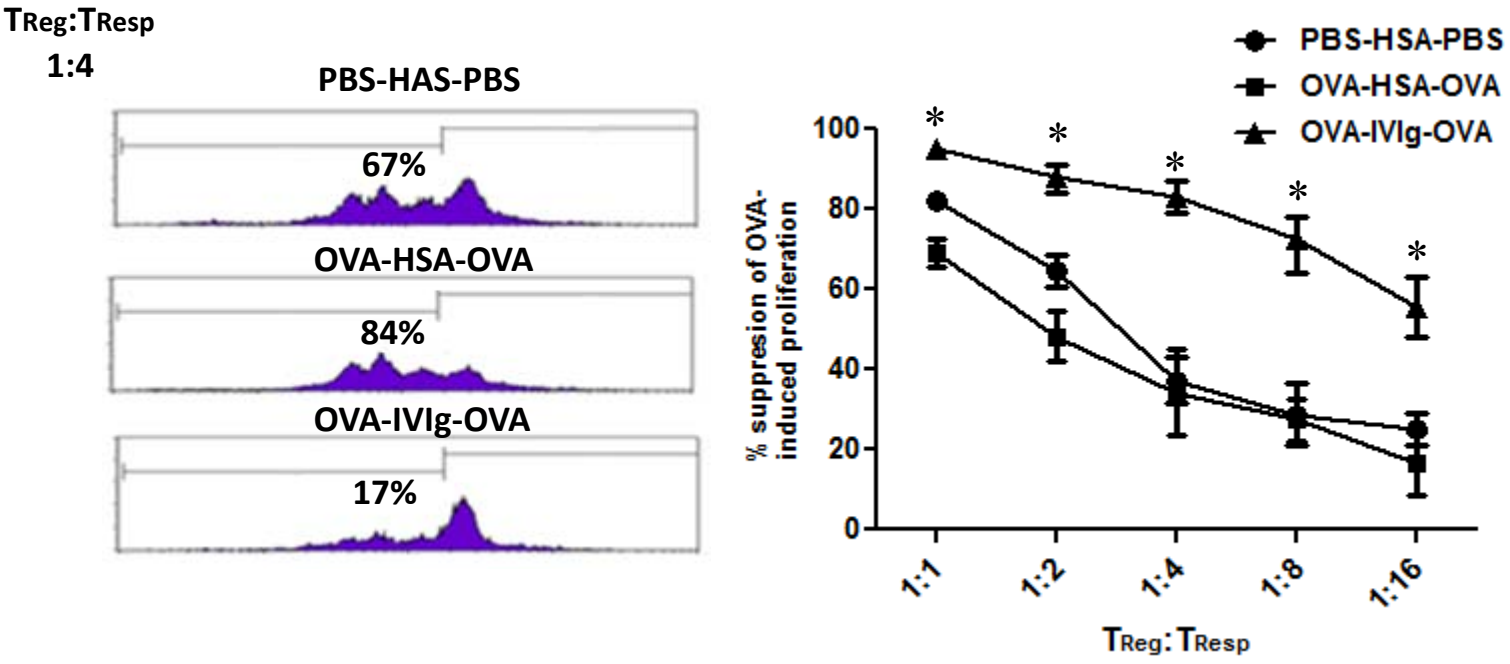

Figure 5. Treg from IVIg treated mice are highly suppressive.

The ability of Treg from the experimental animals to suppress OVA-induced proliferation was evaluated by an *ex vivo* suppression assay (see Materials and Methods), and monitored by CFSE dilution. Foxp3^{GFP+} cells from OVA-IVIg-OVA mice suppressed the proliferation of T-responder cells (T_{Resp}) to OVA stimulation to a greater degree at all ratios from 1:1- 1:16 compared to the other control groups. The left panel is a representative histogram plot for data obtained at a ratio of 1Treg: 4 Tresp. The right panel demonstrates the survival graph of suppression by donor Treg from each experimental group at 1:1 to 1:16 (T_{Reg}:T_{Resp}) ratios. *$p<0.05$. Representative of 2 experiments, n= 6 mice/group.

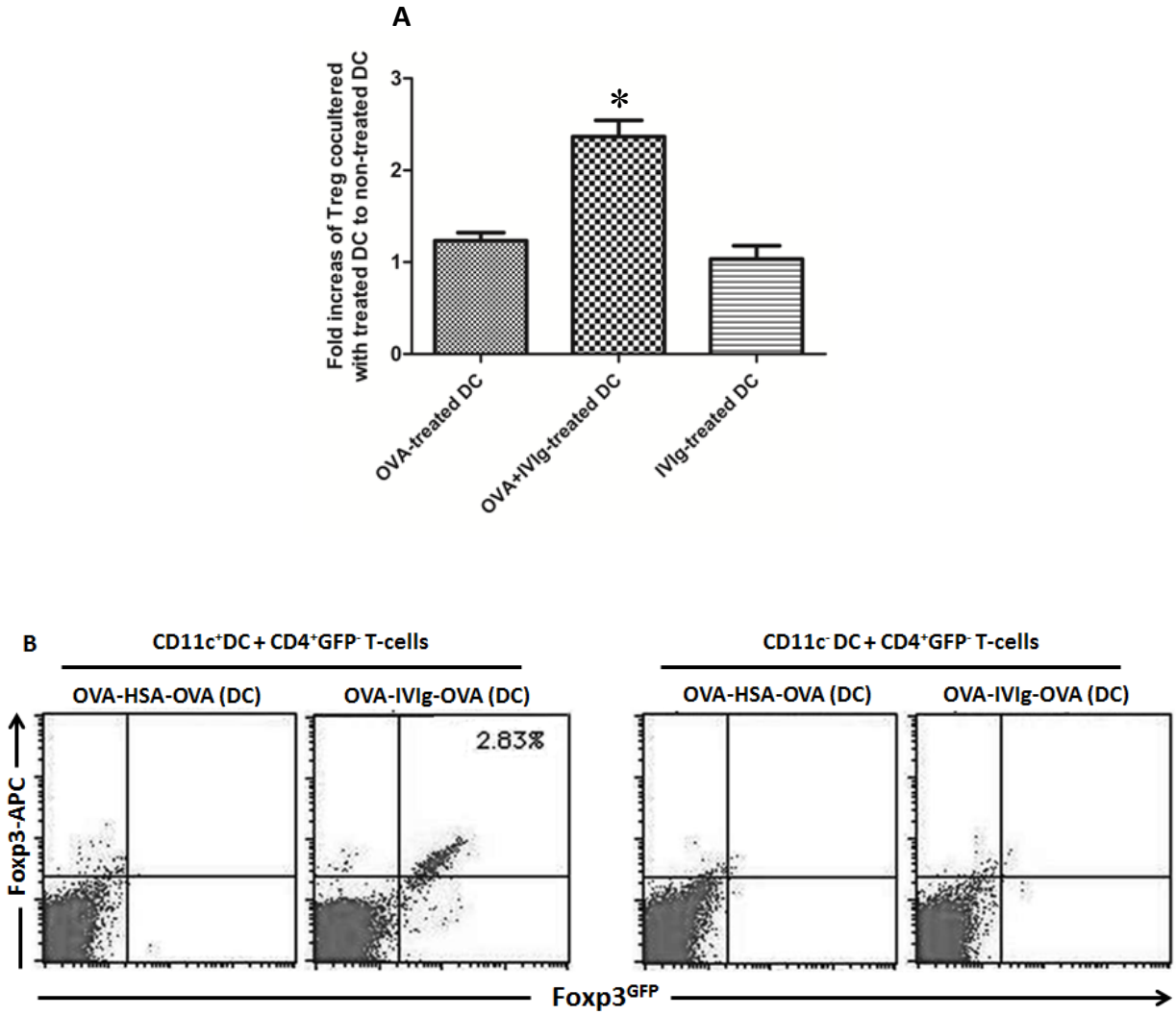

Figure 6. IVIg primes CD11c^{+} pulmonary dendritic cells to induce Treg.

A: DC were purified from naïve mice, and pulsed with IVIg with and without OVA, then co-cultured with CD4+ cells from OT-II-Foxp3^{GFP} reporter mice. The induction of *de novo* Treg cells was assessed by measuring the percentage of Foxp3$^{GFP+APC+}$ cells in the CD4^{+} population by flow cytometry. Only OVA-IVIg pretreated DCs induced the expansion of Treg. B: Purified CD11c^{+}DCs or plasmacytoid CD11c^{-}DCs from the lungs of OVA-IVIg-OVA and OVA-HSA-OVA mice were co-cultured with CD4+$^{GFP-}$ T-cells from OT-II.Foxp3 reporter mice. Only pulmonary CD11c^{+}DCs from OVA-IVIg-OVA mice induced expression of Foxp3. *$p<0.05$ n=6 mice for each experimental condition.

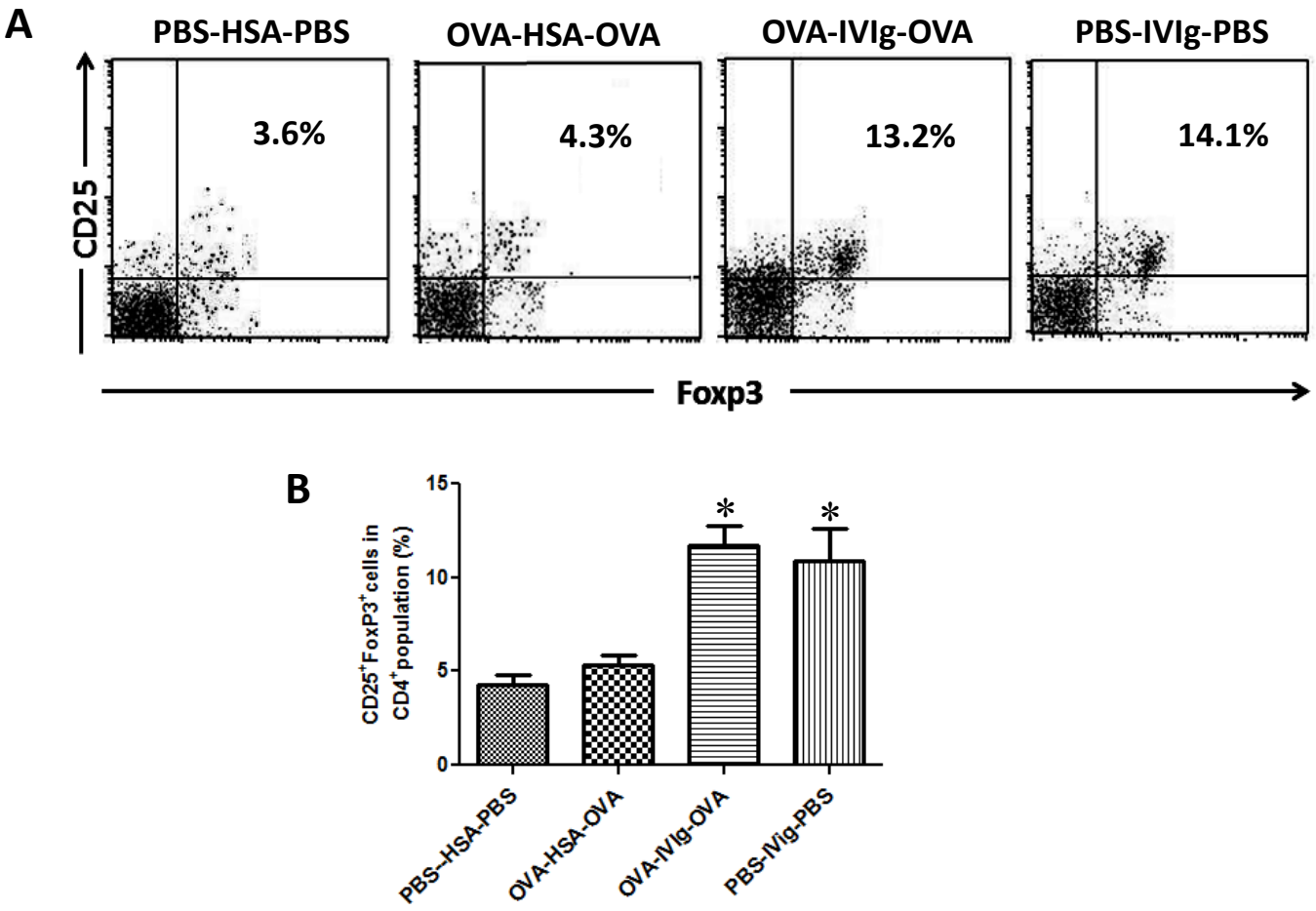

Figure 7. Adoptive transfer of pulmonary DCs from IVIg treated-mice recapitulates the protective effect of IVIg therapy by inducing Treg.

Pulmonary dendritic cells were purified from the lungs of mice from each experimental group following the completed challenge protocol. Recipient mice received adoptive transfer of purified dendritic cells one day prior to challenge (day 28) in place of IVIg or HSA treatment. A: Representative flow cytometric plots and B: quantification of $CD4^{+}CD25^{+}Foxp3^{+}$Treg cells within digested lung tissue of recipient mice following challenge. Adoptive transfer of pulmonary DCs from OVA-IVIg-OVA and PBS-IVIg-PBS mice to OVA-sensitized animals induced Treg cells comparably to IVIg treatment. $p<0.05$; representative of two experiments, n=6 mice for each experimental group.

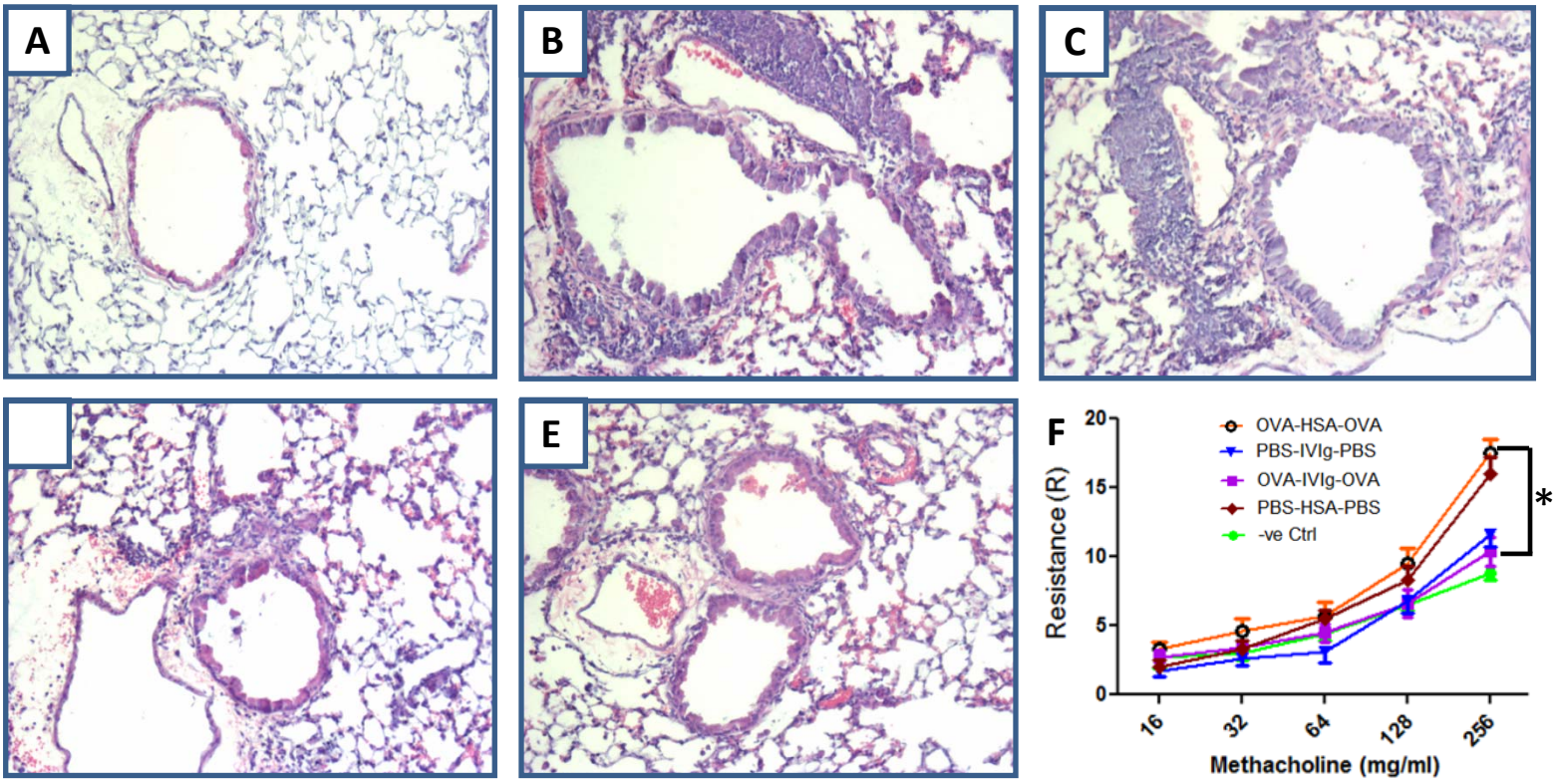

Figure 8. Adoptive transfer of pulmonary DCs from IVIg treated-mice protects against increased AHR and pulmonary inflammation.

Pulmonary dendritic cells were purified from the lungs of mice from each experimental group following the completed challenge protocol. Recipient mice received adoptive transfer of purified dendritic cells one day prior to challenge (day 28) in place of IVIg or HSA treatment. A-E: Histologic examination of H and E stained representative lung sections, demonstrating the protective effect of DC adoptively transferred from IVIg treated mice to OVA-HSA-OVA mice. A) Untreated control; B) DC from PBS-HSA-PBS; C) DC from OVA-HSA-OVA; D) DC from OVA-IVIg-OVA; E) DC from PBS-IVIg-PBS. F) AHR to Mch (pulmonary resistance) was measured by flexiVent as in Material & Methods. DC from IVIg treated mice substantially attenuated Mch induced AHR. $p<0.05$, representative of two experiments, n=6 mice for each experimental group.

Tables

Condition	Ki67 expression
PBS-HSA-PBS	16.7 ± 1.43
OVA-HSA-OVA	15.67 ±2.03
OVA-IVIg-OVA	30.67± 2.96*
PBS-IVIg-PBS	14.83± 0.73

Table I. Evaluation of Treg cell proliferation.

Ki67 expression was monitored by flow cytometry on $CD4^{+}CD25^{+}Foxp3^{+}$ Treg from the lungs under each experimental condition. IVIg treated mice had consistently higher Ki67 expression. *$p<0.05$, n=3 experiments with 3 mice in each group.

Cytokine	PBS-HSA-PBS	OVA-HSA-OVA	OVA-IVIg-OVA	PBS-IVIg-PBS
IL-10	0.41% ± 0.1%	0.55% ± 0.2%	3.2% ± 1% *	0.25% ± 0.15%
IFN-γ	0.21% ± 0.07%	0.1% ± 0.07%	1.8% ± 1.3% *	0.12% ± 0.05%

Table II. Measurment of cytokine production by induced-Tregs.

Cells from digested pulmonary tissue and draining LNs from recipient mice were assessed for the production of IL-10 and IFN-γ by flow cytometry. Frequency of IFN-γ^{+} and IL-10^{+} cells was significantly higher within donor $Foxp3^{GFP+}$cells from OVA-IVIg-OVA mice compared to other experimental groups. * $p<0.05$. n=6 mice in each group

	PBS-HSA-PBS	OVA-HSA-OVA	OVA-IVIg-OVA	PBS-IVIg-PBS
IL-10	2.1% ± 1%	1.9% ± 1.3%	7.9% ± 2.1% *	9.3% ± 4.2% *
IFN-γ	0.8% ± 0.5%	7.9% ± 3.2% **	1.9% ± 1.5%	1.8% ± 1.2%
Delta like-4	27.2% ± 4 %	28.5% ± 4.4%	42.5% ± 7.5% *	37% ± 5.4% *
Jagged-1	11.8% ± 3.5%	28.9% ± 5.5% **	16.4% ± 3.6%	8.5% ± 2.7%
CD8α	30% ± 5.5%	12% ± 4.2%	59% ± 10% *	14% ± 3.5%

Table III. Phenotypic characterization of $CD11c^+$ DC under each experimental condition.

Phenotyping of $CD11c^+$ DC is indicating increased expression of IL-10, Delta-like -4, and CD8α following IVIg treatment. IVIg also decreased Jagged-1 expression as well as IFNγ compared to OVA-HSA-OVA mice. *$p< 0.05$ comparing IVIg to HSA-treated groups. **$p<0.05$ for OVA-HAS-OVA compared to all other groups. n=6 mice in each group.

Article III

C-Lectin Receptor Dendritic Cell (DCIR) mediates the tolerogenic effects of Intravenous Immunoglobulin in Pulmonary Inflammation

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Abstract

IVIg is a polyclonal IgG preparation with potent immune-modulating properties. We have demonstrated that administration of IVIg attenuates airway hyperreactivity (AHR) and inflammation in allergic airway disease, accompanied by significant increases in peripherally-induced $Foxp3^+$ regulatory T-cells (T_{reg}). Because induction of T_{reg} was dependent on generation of tolerogenic dendritic cells (DC) by IVIg, we investigated potential receptors on DC mediating these events. Administration of sialic-acid enriched IgG (SA-IVIg), but not nonSA-IVIg, to OVA-sensitized and challenged mice induced T_{reg} and attenuated AHR and airway inflammation comparable to IVIg. Bone marrow-derived DC (BM-DC) cultured with SA-IVIg or IVIg, then adoptively transferred to mice prior to OVA-challenge, induced Treg and inhibited AHR equally. IVIg-treated BM-DC from FcγR-KO mice inhibited AHR, suggesting IVIg's action was not due to FcγR-mediated events. Fluorescent-labeled IVIg or SA-IVIg bound to DC, and co-localized to the C-Type-lectin dendritic cell immunoreceptor (DCIR). IVIg binding to DCIR induced phosphorylation of SHP-2 and SHIP-1 and internalization of IVIg into DC. Inhibition of IVIg binding to DCIR by siRNA completely abrogated induction of T_{reg}. Furthermore, abrogation of internalization by clatherin inhibitors rendered IVIg ineffective. DCIR is a novel receptor for IVIg, mediating the interaction of innate and adaptive immunity in tolerogenic responses.

Introduction

Intravenous immunoglobulin (IVIg) is a preparation of human polyclonal IgG obtained from pooled plasma samples, taken from thousands of healthy blood donors. It is widely used as a treatment for primary immune deficiency, but in terms of sheer volume, IVIg is employed extensively for the treatment of a wide range of autoimmune and inflammatory diseases.

IgG is a glycosylated molecule with various oligosaccharides, endowing it with distinctive properties, including the ability to bind to various innate immune receptors (Arnold et al., 2007). The structural properties of IgG and the interaction with multiple sites has led to the proposal of a number of mechanisms for the immune modulatory actions of IVIg, including blockade of phagocytic Fcγ receptors, up-regulation of inhibitory FcγR (FcγRIIB) on macrophages, neutralization of pro-inflammatory mediators, modulation of cytokine secretion, inhibition of cell proliferation and inhibition of DC activity (Vani et al., 2008). The glycosylation sites on IgG molecules may also participate in the immune regulatory activities of IVIg compound (Anthony et al., 2012).

Using a robust antigen-driven model of allergic airway disease in the mouse, our laboratory recently demonstrated that IVIg markedly improved OVA-induced AHR, associated with induction of T_{reg} from non-regulatory T cells in pulmonary tissues (Massoud et al., 2012). This mechanism appears to be dependent on $CD11c^{+}$ DC, as the action of IVIg could be completely replicated by adoptive transfer of DC from IVIg-treated mice to OVA-sensitized and challenged recipients. IVIg has been shown to modulate DC maturation and promotes IL-10 production by those cells (Bayry et al., 2003a; Kaufman et al., 2011; Massoud et al., 2012). It is unknown how IVIg interacts with DC in order to confer the observed tolerogenic phenotypes.

The doses of IVIg required in order to be effective as an immune modulator are 3-4 times of those used for immune supplementation. This suggests that a minor component of polyclonal IVIg is responsible for its immune-modulatory effects; such as the sialylated fraction which represents 2%-5% of the total IgG pool. As described by Anthony et al. (Anthony et al., 2008b) and Kaneko et al.(Kaneko et al., 2006) 2-6 sialic acid linkages have potent immune-regulatory effects in murine models of serum-induced arthritis and immune thrombocytopenic purpura (ITP). In these models, the anti-inflammatory effects of sialylated-IgG were suggested to involve SIGN-R1 (the murine ortholog of DC-SIGN), a lectin receptor expressed on splenic marginal zone macrophages. Séïté et al. (Seite et al., 2010) also demonstrated that SA-IVIg can bind to another lectin receptor, CD22 (Siglec-2), which promotes apoptosis in B-cells.

We have explored the mechanism by which IVIg interacts with DC and renders them tolerogenic. We demonstrated a requirement for IgG sialylation in the modulation of DC function and have identified a novel DC receptor for sialylated IgG molecules, dendritic cells immunoreceptor (DCIR), a C type lectin receptor that contains an immunoreceptor tyrosine-based inhibition motif (ITIM). DCIR appears to be required for induction of tolerogenic DC. We also demonstrated that DCIR-mediated internalization of IgG molecules is a crucial step for induction of T_{reg} and subsequent abrogation of inflammation by IVIg.

Material and Methods

Fractionation of IVIg.

Fractionation of IVIg was carried out using a sambucus nigra agglutinin (SNA)-lectin affinity column, according to the manufacturer's protocol. Briefly, whole IVIg (Talecris Biotherapeutics, Missisaugua ON.) was loaded onto a 2ml SNA column. The flow-through fraction (SA-IVIg depleted IVIg or nonSA-IVIg) was collected by washing the column with 10ml TBS. The sialic acid enriched fraction was eluted, using 4ml of 0.5M lactose. The nonSA-IVIg fraction was further treated with neuraminidase (Sigma-Aldrich Oakville, ON.) to completely deglycosylate the IgG_ENREF_8.

Induction of allergic airway inflammation.

C57BL/6 wild type and Fcγ-chain deficient (Fcγ-KO) mice were purchased from Jackson Laboratories (Bar Harbor, ME.). Mice were sensitized intraperitoneally (i.p.) with 100mg/mouse OVA (Sigma-Aldrich) in 2mg Aluminum hydroxide (Alum) on day 0 and 14 followed by intranasal (i.n.) OVA-challenges from day 29 to 33 (20µl of a 10mg/ml OVA solution). One day prior to antigen challenge, IVIg (2g/kg), SA-IVIg (0.1g/kg), nonSA-IVIg (2g/kg), or 2g/kg of 3% human serum albumin (HSA, Talecris Biotherapeutics), was administered to mice (i.p.).

In an alternative protocol (Koya et al., 2009), 1X 10^6 bone marrow-derive DC (BM-DC) were primed with OVA (1mg/ml), or OVA+ (10mg/ml) IVIg, or OVA+ (0.5mg/ml) SA-IVIg, or OVA+ (10mg/ml) nonSA-IVIg *in vitro*. Cells were stimulated for 3 hours with OVA, followed by further incubation for 18 hours with IgG preparations and 3 washes. The conditioned-DC were then instilled intratracheally into naïve mice, followed by 3 days (i.n.) OVA-challenges,

beginning 5 days post DC transfer. Experiments were performed 24 hours after the last challenge.

Airway responsiveness to methacholine.

AHR was measured as previously described (Kaufman et al., 2011). Mice were exposed to saline, followed by doubling concentrations of aerosolized methacholine (16-256 mg/ml, Sigma-Aldrich) using a FlexiVent small animal ventilator (Scireq, Montreal, QC, Canada). The relative peak response at each MCh dose compared to the baseline was calculated (Shalaby et al., 2010).

Preparation of cell suspensions from lung tissues.

Lungs were removed postmortem, minced and incubated for 45 min at 37$^{\circ C}$ in 3ml collagenase (Sigma-Aldrich) in 0.5 mM Ca^{2+}PBS and then passed through a 40μm cell strainer. Single cell suspensions were resuspended in complete medium RPMI-1640 (Invitrogen, Toronto, ON.).

Cytokine detection.

Intracellular cytokine staining was performed on lung digests stimulated for 6 hours with phorbol-12-13-dibutyrate (5 ng/mL)- and ionomycin (50 ng/mL, Sigma-Aldrich) in the presence of GolgiPlug (BD-Bioscience). Cells were permeabilized and stained _ENREF_4 with anti-IFN-γ-APC, anti-IL-4-PE or anti-IL-17-PerCP5.5 for 30 minutes at 4$^{\circ C}$. For detection of T_{reg}, cells were first stained with anti-CD4-FITC and anti-CD25-PE, followed by permeabilization and staining with anti-Foxp3-APC (all antibodies from BD-Bioscience). Relative fluorescence intensities were determined on a LSR-II FACSCalibur flow cytometer. IL-4 and IL-17 from supernatants of stimulated lung mononuclear cells were measured by ELISA (eBioscience, San Diego, CA).

Differentiation of dendritic cells from bone marrow (BM-DC).

DC were generated from bone marrow cells of naive (C57BL/6) mice as previously described (Lutz et al., 1999)_ENREF_12. Briefly, bone marrow cells from femurs ($2x10^6$/100mm petri dish) were cultured in complete RPMI-1640 medium, supplemented with 10 ng/ml recombinant murine GM-CSF (PeproTech Montreal QC.). On days 3, 5, 7 and 9, 10 ml medium was changed, and non-adherent DC were harvested at day 10. The purity of DC was tested by flow cytometry and was more than 90% by $CD11c^+F480^-$ staining.

Transfection of DCIR.

The DCIR expressing pCMV6-AC vector, containing a neomycin resistance gene (OriGene, Rockville, MD.) was transfected into CHO cells with lipofectamine (Invitrogen, Burlington, ON.) as described by the manufacturer. CHO cells expressing DCIR were screened by adding neomycin (Invitrogen, Burlington, ON Canada.) to the cultured cells, 48 hours after initiation of transfection.

Co-localization of DCIR and IgG by Fluorescent microscopy.

BM-DC from WT mice or Fcγ-KO mice were incubated with Alexa488-conjugated-SA-IVIg (10 mg/ml) or the same volume of Alexa488-conjugated-nonSA-IVIg, and Alexa555-conjugated anti-DCIR antibody (Biolegend, Burlington ON), at $4^{\circ C}$ for 60 min or 37 $^{\circ C}$ for 10 min, 30 min or 60 min in the presence of Fc-block (Biolegend, Burlington ON). Inhibition of clathrin-mediated internalization was performed by incubation of cells with Dynasore (100 μM in 0.02 DMSO per million cells, 30 min, Sigma-Aldrich).The co-localization and internalization of DCIR/IgG was visualized under the fluorescent microscope at 400X magnification, or by flow cytometry.

DCIR gene silencing by targeting siRNA.

The CLEC4a2-Mouse (DCIR) siGENOME siRNA reagent (Thermo Scientific, Ottawa, ON) was used (50 nM siRNA/ 1million cells) at 37°^{C}) for silencing the DCIR gene expression for 24 hours in BM-DC. A non-targeting GFP-conjugated siRNA was used to examine the delivery of RNA into the cells, using flow cytometry (Figure 2F). A scrambled siRNA was also utilized as a control. DharmaFECT reagent (0.5 nM) was used to increase the efficiency of transfection. DCIR gene expression in transfected-DC was analyzed by PCR and flow cytometry.

Western Blot Analysis.

Proteins were extracted from DC (2 x 10^6 cells) and immunoblotting was performed and probed with anti-phospho-SHP1, anti-phospho-SHP-2, and anti-phospho-SHIP-1 antibodies (New England Biolabs Ltd. Whitby, ON) and developed using the enhanced chemoluminescence (ECL) system (Amersham Biosciences Piscataway, NJ).

Molecular analysis and quantitative real-time polymerase chain reaction.

RNA was extracted from DC or dissected lungs, using TRIzol (Invitrogen). cDNA was prepared by reverse transcription and mRNA was quantified by SYBR Green RT-qPCR (Applied Biosystems 7300 Real-time PCR system). The following primers were used: DCIR: 5'-GATCTAAGAAAGCCTGGTTC-3'(sense) and 5'-GCAAGAGATATCGTTCCAGC-3'(antisense), SIGN-R 5'-CTGCAAGAAGTCTGCAACCC-3'(sense) and 5'-TGGCAGAATGGCATGAAGGT-3'(antisense). GAPDH was used as a reference gene for normalization, with the primers AGCAATGCCTCCTGCACCACC(sense) and GAGGCTGGTAAGGAACTGG(antisense).

Statistical Analysis. Statistical analysis was performed using Prism software (GraphPad). Statistics were calculated using one- or two-way analysis of variance (ANOVA) followed by the Tukey post-test. Asterisks (*P < 0.05, **P < 0.01, ***P < 0.0001) indicate significant difference from the control group. Columns and error bars represent mean ± SEM.

Results

Sialylation of IgG is required for the anti-inflammatory effect of IVIg.

We have reported that IVIg has protective effects in a murine model of allergic airway disease (Kaufman et al., 2011; Massoud et al., 2012). To address if the sialylated fraction of IgG was the active moiety in this process, IVIg, SA-IVIg or nonSA-IVIg were administered to OVA-sensitized mice one day prior to allergen-challenge.

Administration of SA-IVIg attenuated methacholine-induced AHR in OVA-challenged mice comparable to intact IVIg (Figure 1A), albeit at a 10 fold lower dose. The inhibition of AHR in both IVIg and SA-IVIg-treated mice was accompanied by a significant increase in the frequency of T_{reg} within the lung (Figure 1B-C). Administration of nonSA-IVIg did not prevent the elevation in AHR in OVA-sensitized and challenged mice, nor did it induce T_{reg}.

We then examined total $CD4^+$ T-cells in lung digests for production of the intracellular pro-inflammatory cytokines. Administration of IVIg or SA-IVIg to OVA-exposed mice, but not-nonSA-IVIg, resulted in a decreased frequency of both IL-4 and IL-17 producing $CD4^+$ T-cells (Figure 1D-E). The frequency of IFN-γ producing T-cells were not significantly changed in any of the experimental groups (Figure 1F). These results demonstrate that the actions of IVIg and SA-IVIg are comparable in our model.

DCIR acts as a specific receptor for SA-IVIg on DC.

$CD11c^+$ DC play an integral role in induction of T_{reg} and abrogation of AHR by IVIg in our model (Massoud et al., 2012). Sialylated-IgG is known to interact with lectin receptors such as

SIGN-R1on APCs, e.g. splenic macrophages (Anthony et al., 2008b; Anthony et al., 2012). However, we were unable to find significant SIGN-R1 mRNA or protein expression on $CD11c^+$ pulmonary DC (Figure 2D and data not shown). In an attempt to isolate a receptor on DC with the potential binding capacity for SA-IVIg, we focused our attention on the recently described dendritic cell immunoreceptor (DCIR), a member of C-type lectin receptor family (Kanazawa et al., 2002). There was low, constitutive expression of DCIR on pulmonary DC (15-20%) (Figure 2A). Treatment with either IVIg or SA-IVIg, but not nonSA-IVIg, enhanced both mRNA and protein expression of DCIR on pulmonary $CD11c^+$ DC (Figure 2A-B-C).

We determined if IVIg binding could be co-localized with DCIR by expressing the full length DCIR construct in CHO cells. Using flow cytometry and fluorescent microscopy we observed that Alexa488-labeled SA-IVIg, but not nonSA-IVIg, consistently co-localized with DCIR on transfected CHO cells (Figure 2E and data not shown).

DCIR mediates internalization of SA-IVIg into DC.

Most C-type lectin receptors are involved in rapid and efficient internalization of their cognate ligands into the cytoplasm (Meyer-Wentrup et al., 2008; Tacken et al., 2005). To examine the potential effect of DCIR in delivering sialylated-IgG into the cytoplasm, we utilized BM-DC, which are reported to express DCIR abundantly on their cell surface (Kanazawa et al., 2002).

SA-IVIg, but not nonSA-IVIg, colocalized with DCIR on FcγR-blocked wild type BM-DC (WT-DC) when incubated at either $4^{\circ C}$ or $37\ ^{\circ C}$ (Figure 3Ai-ii). Importantly, nonSA-IVIg did not colocalize with DCIR on WT-DC (Figure 3Aiii). At $37^{\circ C}$ both Alexa488-SA-IVIg and DCIR were simultaneously internalized into the cells over a 60 minute period (Figure 3Aii). To

ascertain the specificity of this binding DCIR expression was blocked using specific siRNA, which diminished mRNA expression and induction of DCIR after IVIg treatment (Figure 2C-F). SA-IVIg was not able to bind to DCIR-knockdown BM-DC (KD-DC), nor be internalized into KD-DC (Figure 3Aiii-iv). BM-DC transfected with non-specific (scrambled) siRNA behaved similar to WT-DC in terms of binding and internalization of SA-IVIg into the cytoplasm (Figure 3Av, vi). To better quantify these observations, BM-DC were incubated with biotinylated SA-IVIg as well as anti-DCIR antibodies for 10, 30, and 60 min at 37°C followed by flow cytometric detection of cell-bound SA-IVIg with streptavidin-Alexa488. As illustrated in Figure 3B-C, incubation of WT-DC at 37°C decreased the amount of cell surface bound SA-IVIg by more than 80% compared to cells incubated at 4°C over 60 minutes, whereas SA-IVIg neither bound nor internalized into the KD-DC. Taken together, these results indicate that DCIR serves as a portal for internalization of IgG molecules into the cytoplasm of DC.

Inhibition of AHR by IVIg is dependent on DCIR.

We investigated the requirement of SA-IVIg/DCIR interaction on the reversal of AHR. To this end, we utilized a protocol of rapid induction of airway inflammation, described by Koya et al. (Koya et al., 2009). We employed BM-DC conditioned with OVA or OVA+IVIg *in vitro* and intratracheally administered to syngeneic animals, followed by allergen-challenge 5 days later. Transferring of OVA-primed BM-DC markedly enhanced AHR after OVA challenge, comparably to systemic sensitization. In contrast, BM-DC conditioned with OVA+IVIg did not confer increased AHR and airway inflammation (Figure 4A and data not shown). Similarly to *in vivo* treatment with IVIg, transfer of OVA+IVIg primed BM-DC also increased T_{reg} in the lungs of OVA challenged mice (Figure 4C).

We subsequently incubated BM-DC with siRNA against DCIR prior to conditioning. KD-DC conditioned with OVA+IVIg or OVA+SA-IVIg had no protective effect against OVA induced AHR. In fact, transfer of KD-DC significantly enhanced AHR compared to the mice that have received OVA-primed WT-DC (Figure 4B). OVA+IVIg and OVA+SA-IVIg primed KD-DC also failed to enhance T_{reg} in recipients (Figure 4C). Lung digests from OVA+IVIg and OVA+SA-IVIg KD-DC recipients produced higher levels of pro-inflammatory IL-4 and IL-17 cytokines following *ex vivo* OVA re-stimulation compared to WT-DC (Figure 4D-E). Delivery of scrambled siRNA-treated and conditioned DC had the same protective effects as WT-DC (data not shown).

We investigated whether the action of IVIg and SA-IVIg were mediated by interaction with FcγR. Fcγ chain deficient BM-DC bound to and internalized SA-IVIg similarly to WT-DC (Figure 5D). In addition, transfer of Fcγ chain deficient BM-DC, conditioned with OVA+IVIg or OVA+SA-IVIg, fully attenuated AHR and increased T_{reg}, similarly to the corresponding WT-DC recipient groups (Figure 5A-C). Fcγ chain deficient BM-DC treated with DCIR siRNA prior to OVA+IVIg priming were unable to confer protection (Figure 5B). These data suggest that IVIg and SA-IVIg interact with DC in an FcγR independent manner.

Inhibition of IgG internalization into DC abolishes IVIg-mediated induction of T_{reg}.

Next we sought to determine if internalization IgG to DC was required for the immune-regulatory effects of IVIg. Internalization of DCIR-ligand complexes is mediated by a clathrin and dynamin-dependent pathway(Meyer-Wentrup et al., 2008) and "_ENREF_16dynasore", a cell-permeable and reversible noncompetitive dynamin 1 and dynamin 2 GTPase activity inhibitor, disrupting clatherin-mediated endocytosis of cell surface receptors (Macia et al., 2006). As

demonstrated by fluorescent microscopy as well as flow cytometry, pretreatment of BM-DC with dynasore attenuated DCIR-mediated internalization of SA-IVIg into DC (Figure 6A-B), with the majority of bound IgG remaining on the DC surface.

Furthermore, intratracheal transfer of dynasore pretreated BM-DC conditioned with OVA+IVIg or OVA+SA-IVIg, neither attenuated AHR following OVA challenge nor exhibited induction of T_{reg} in lungs of recipients (Figure 6C-E-F). Mice transferred dynasore treated DC had higher levels of activated effector T cells ($CD4^+CD25^+Foxp3^-$) (Figure 6D) and the frequency of IL-4 and IL-17 producing $CD4^+$ T cells were comparable to those from OVA-treated WT-DC recipients (Figure 6G-H).

DCIR ligation by SA-IVIg triggers phosphorylation of inositol phosphatase SHIP-1 and tyrosine phosphatase SHP-2.

The intracellular cytoplasmic tail of DCIR is associated with ITIM that may regulate cell activation (Meyer-Wentrup et al., 2008). Phosphorylation of tyrosine residues within ITIM form docking sites for other proteins involved in the intracellular signaling pathways, including SHP-1, SHP-2 and SHIP-1. We examined whether IgG treatment of DC can affect the phosphorylation status of these three proteins. (Phosphorylated-SHIP-1) p-SHIP-1, p-SHP-1 and p-SHP-2 protein levels were detected in the lysates of conditioned BM-DC by immune-blotting. OVA+IVIg and OVA+SA-IVIg conditioned BM-DC exhibited phosphorylation of SHIP-1 and SHP-2 (Figure 7A), but not SHP-1(data not shown), whereas no increase in phosphorylation was observed in OVA+ nonSA-IVIg conditioned BM-DC. Silencing of DCIR, using siRNA, in BM-DC abolished the action of IVIg or SA-IVIg on the phosphorylation of SHIP-1 and SHP-2 (Figure 7A). A similar pattern of SHIP-1 and SHP2 phosphorylation was found when we

examined lysates prepared from lungs of OVA-IVIg or OVA-SA-IVIg treated mice (Figure 7B), but SHP-1 was again not detected (data not shown).

To determine if these phosphorylation events were dependent on internalization of IVIg, we pretreated BM-DC with dynasore. This did not inhibit the effect of IVIg or SA-IVIg on the phosphorylation of SHIP-1 and SHP-2 in DC (Figure 7C). Thus, ligation of DCIR is sufficient for triggering the intracellular signaling cascade; however.

Discussion

IgG is a crucial effector molecule in host defense, and is recognized for its role in inflammation both in pathogen elimination and in autoimmune disease. However, there are many clear examples of a duality of function, specifically, tolerance induction by IgG; intravenous immunoglobulin (IVIg) is frequently used as disease-modifying therapy for a large spectrum of autoimmune and inflammatory conditions. In our recent study, using a murine model of allergic airway disease, we reported that administration of high dose IVIg induces highly suppressive $Foxp3^+$ T_{reg} cells from $Foxp3^-CD4^+$ T cells which led to attenuation of allergen-induced AHR. We showed that the effect of IVIg is a result of a change in the phenotype of pulmonary $CD11c^+$ DC that exhibit tolerogenic properties (Massoud et al., 2012). Indeed, adoptive transfer of DC exposed to IVIg either *in vivo* or *ex vivo* led to attenuation of AHR and increases in $Foxp3^+$ T_{reg} cells. In this study we have convincing proof that the induction of T_{reg} by IVIg is mediated via a novel receptor on dendritic cells; the dendritic cell immunoreceptor (DCIR).

IVIg is known to modulate the activation of DC (Bayry et al., 2003a). Tolerogenic DC play an important role in maintaining peripheral tolerance through the induction, expansion or activation of T_{reg} populations (Delgado et al., 2006). The induction of T_{reg} can explain the protective effects of IVIg in a wide range of autoimmune and inflammatory disorders. In addition to our allergic airway disease model, the immune-regulatory actions of IVIg have been demonstrated to induce T_{reg} in experimental allergic encephalomyelitis (EAE) (Ephrem et al., 2008), in systemic lupus erythematosus (Costa et al., 2012), vasculitis (Tsurikisawa et al., 2012) in ITP (Aslam et al., 2012) and Kawasaki syndrome (Olivito et al., 2010).

IgG is a glycoprotein, with a protein backbone and variable glycosylation sites. The attached glycans are important for the structure-function relationships of the molecule; in fact, altered or diminished IgG glycosylation may be important in autoimmune and allergic diseases (Oefner et al., 2012). In host defense, the effector mechanism of IgG is compromised by deglycosylation (Jefferis, 2007; Krapp et al., 2003; Nimmerjahn and Ravetch, 2007b; van Kooten et al., 2011). Karsten et al. (Karsten et al., 2012) demonstrated that galactosylated IgG1 could specifically inhibit C5A induced inflammation, and sialylation of pathogenic cryoglobulins attenuates the nephotoxic action of these autoantibodies (Otani et al., 2012), indicating the importance of the glycan moieties for the effector function of IgG.

The glycosylation of the Fc region of IgG determines the relative affinity for inhibitory or activating Fcγ receptors (Takai, 2005). There has been considerable focus on sialic acid moieties on ASP^{297} of the Fc region of IgG (Anthony et al., 2008b). Sialylated-IgG molecules have reduced affinity for activating FcγR (Scallon et al., 2007), and are less efficient in induction of antibody-dependent cytotoxicity (ADCC) (Anthony et al., 2008a). The lectin receptor SIGN-R1, or its human ortholog DC-SIGN, has been identified as a binding site for sialylated-Fc, which initiates regulatory pathways involving splenic marginal zone macrophages (Anthony et al., 2008b). In a model of serum-induced arthritis, SIGN-R1-deficient mice do not exhibit improvement with IVIg therapy (Anthony et al., 2008b) and ligation of SIGN-R1 by IVIg appears to induce basophil-mediated IL-4 production in an IL-33 dependent manner (Anthony et al., 2011b).

In this light, in the current study we showed that sialic-acid enriched IVIg completely reproduced the action of unfractionated IVIg, at a 10-20 fold lower dilution than IVIg, in our model by

inhibition of OVA-induced AHR, induction of T_{reg} and resolution of inflammatory histological changes in the lungs. This was not the case for deglycosylated IVIg, which was ineffective in all parameters. Furthermore, treatment with IVIg inhibited production of the characteristic cytokines in allergic airways disease, including IL-4 and IL-17.

In allergic airways disease, Th2 cytokines including IL-4 and IL-13 and Th17 cytokines such as IL-17 act as an integral part of the inflammatory response, and we demonstrated that IVIg or SA-IVIg treatment inhibits IL-4 in bronchial alveolar lavage fluid in our model (Figure 1) and from cultured splenocytes (Kaufman et al., 2011). Thus the mechanism of action of IVIg in allergic airways disease, and potentially in other models which require active immune responses, does not mirror all of the features of the arthritis model, where IL-33, IL-4 and potentially other facets of Th2 immunity may be protective. Secondly, SIGN-R1 has not been reported to be expressed in pulmonary DC (van Die and Cummings, 2010), and we did not detect SIGN-R1 mRNA or protein on pulmonary $CD11^+$ DC isolated from C57BL/6 mice. We therefore examined other potential receptors with the capacity to bind sialylated-IgG.

We have determined that SA-IVIg can bind to DCIR. DCIR is a Type-II C-type lectin receptor, and a member of the Dectin-2 family of receptors, but is unique in that it has an extended intracellular chain that harbors an ITIM in its intracellular tail. DCIR also exhibits a single carbohydrate recognition domain (CRD) at the COOH terminal (Kanazawa, 2007). Members of this family of lectin receptors are widely expressed on DC, and other APCs (Lambert et al., 2011; Meyer-Wentrup et al., 2009). DCIR expression is down-regulated by signals that induce DC maturation, such as CD40 ligand, LPS, or TNF-α (Bates et al., 1999; Richard et al., 2002). Interestingly, DCIR-deficient mice spontaneously develop symptoms of autoimmunity (Fujikado

et al., 2008), and DCIR2^{+} DC subset is able to differentiate peripheral Foxp3^{+} T_{reg} through endogenous production of TGF-β (Yamazaki et al., 2008).

At present, the ligands recognized by DCIR are incompletely elucidated. DCIR is known as a potential receptor and portal for HIV (Lambert et al., 2008), and as with other lectin receptors, exhibits promiscuous binding of a variety of sugars, including mannose, fucose, N-acetlyglucosamine, and alpha 1-acid glycoprotein (AGP) (Hsu et al., 2009), which are enriched with sialic acids (Gunnarsson et al., 2010).

The primary function of C-type lectin receptors in the innate immunity is to trap pathogens via CDR, with subsequent internalization, antigen processing and presentation by phagocytic cells. Binding of the receptor is associated with activation signals or, in the case of ITIM-linked DCIR, with inhibitory signals via phosphatases. DC from SA-IVIg-treated mice exhibited increased DCIR expression than DC from mice treated with deglycosylated-IVIg (Figure 2). We have not determined if this increase in expression is directly due to IgG binding to DCIR, or as a part of decreased maturation observed in tolerogenic DC (Kaufman et al., 2011; Massoud et al., 2012). However, IVIg binding to DCIR initiated inhibitory signaling cascade that involves phosphatases SHP-2 and SHIP-1. Inhibition of expression of DCIR, using siRNA, completely inhibited binding of sialylated-IgG in the presence of FcγR blockade, and abrogated the enhancement of SHP-2/SHIP-1 phosphorylation, suggesting that DCIR ligation was crucial for phenotypic modification of DC by SA-IVIg.

We also demonstrated that binding of SA-IVIg to DCIR results in internalization of ligand/receptor complex within 1 hour. This internalization is independent of Fcγ receptors, and appeared to be crucial for the induction of T_{reg}. Proulx et al. (Aubin et al., 2011) (Proulx et al.,

2009) demonstrated the internalization of IVIg into APCs, independent of Fcγ receptors, which led to diminished T-cell activation, although no receptor was identified for IgG in their study.

The steps following internalization of IgG in our model remained to be elucidated. Meyer et al. (Meyer-Wentrup et al., 2008) demonstrated that antigens targeted to DC via DCIR result in efficient antigen presentation to T cells. De Groot et al. (De Groot et al., 2008) predicted several T_{reg} epitope sequences, named Tregitopes, which are highly conserved regions in both the Fc and $F(ab')^2$ regions of IgG and the produced peptides based on these epitopes appear to suppress immune responses in co-culture and animal models of inflammatory diseases via expansion of T_{reg} (Cousens et al., 2012a). In the context of their model, our data suggests that endocytosed IgG may be degraded and conserved epitopes presented by DC via MHC class-II to natural T_{reg} in an antigen dependent manner. This requires further examination.

Sialic acid residues are common on both the Fc and $F(ab')^2$ regions of IgG. Käsermann et al. (Kasermann et al., 2012) found that the effect of IVIg on monocytes is dependent on sialylated-$F(ab')^2$, rather than the Fc fragment. In either case, we have clearly identified a novel receptor for IVIg, which alters dendritic cell activity, and bridges the innate system and adaptive immune systems to enhance immune regulation.

Figures

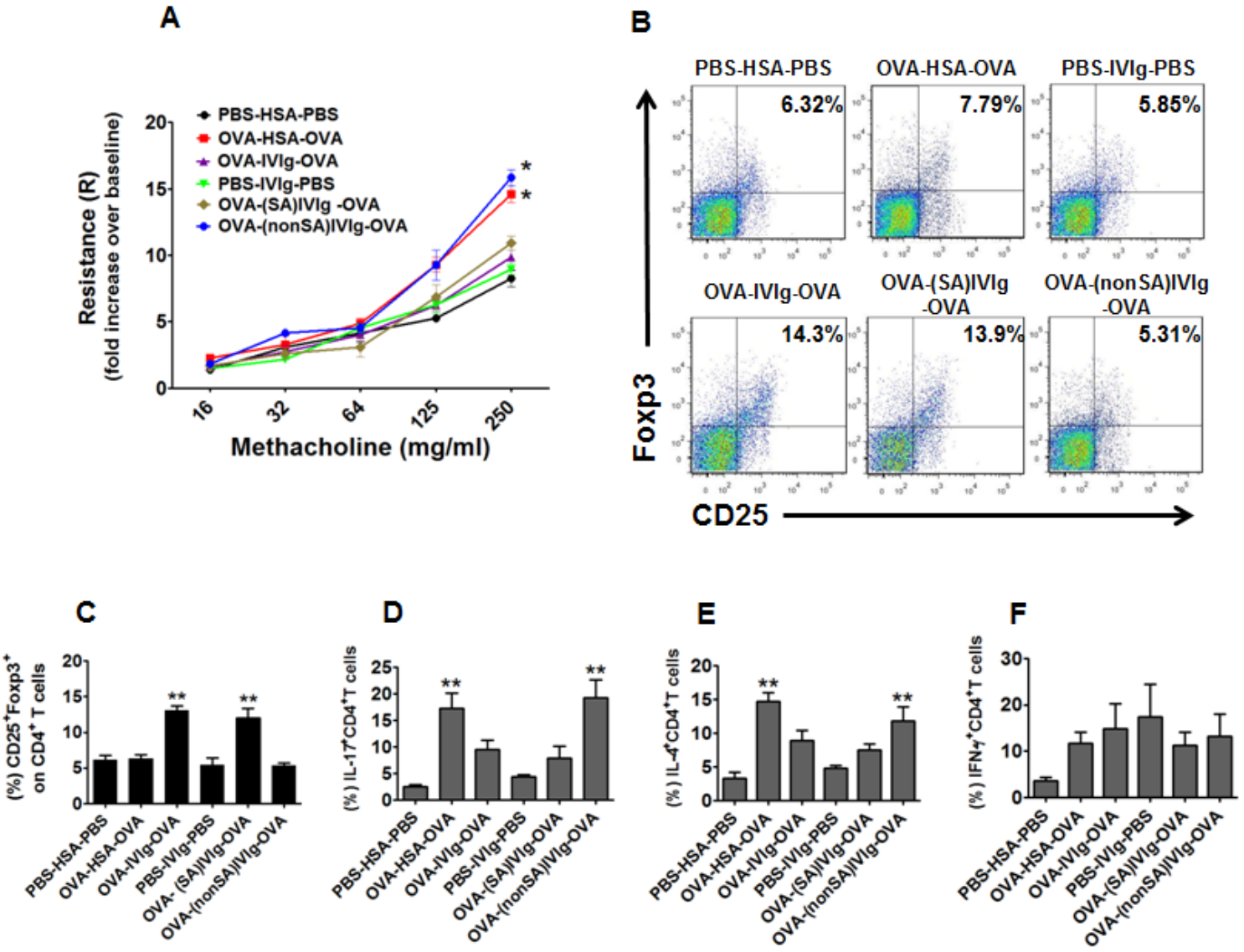

Figure 1. The sialylated fraction of IgG abrogrates allergic airways disease. A.

IVIg and SA-IVIg, but not nonSA-IVIg, treatment attenuated MCh-induced AHR in OVA-sensitized and challenged mice **=P<0.01 vs. Medium or OVA-IVIg/SA-IVIg for peak MCh response **B. C.** Representative flow cytometric analysis and Bar graph demonstrating the frequency of pulmonary $CD25^{+}Foxp3^{+}$ T_{reg} on gated $CD4^{+}$ T cells. IVIg and SA-IVIg, but not nonSA-IVIg, induced T_{reg} in lungs of OVA-challenged mice. **D, E, F.** Bar graphs

demonstrating that IVIg and SA-IVIg, but not nonSA-IVIg, significantly decreased the frequency of pulmonary IL-4 and IL-17 producing $CD4^+$ T cells. All graphs represent averages and mean±SD from 3 independent experiments. N=9 **=P < 0.01 vs. control groups.

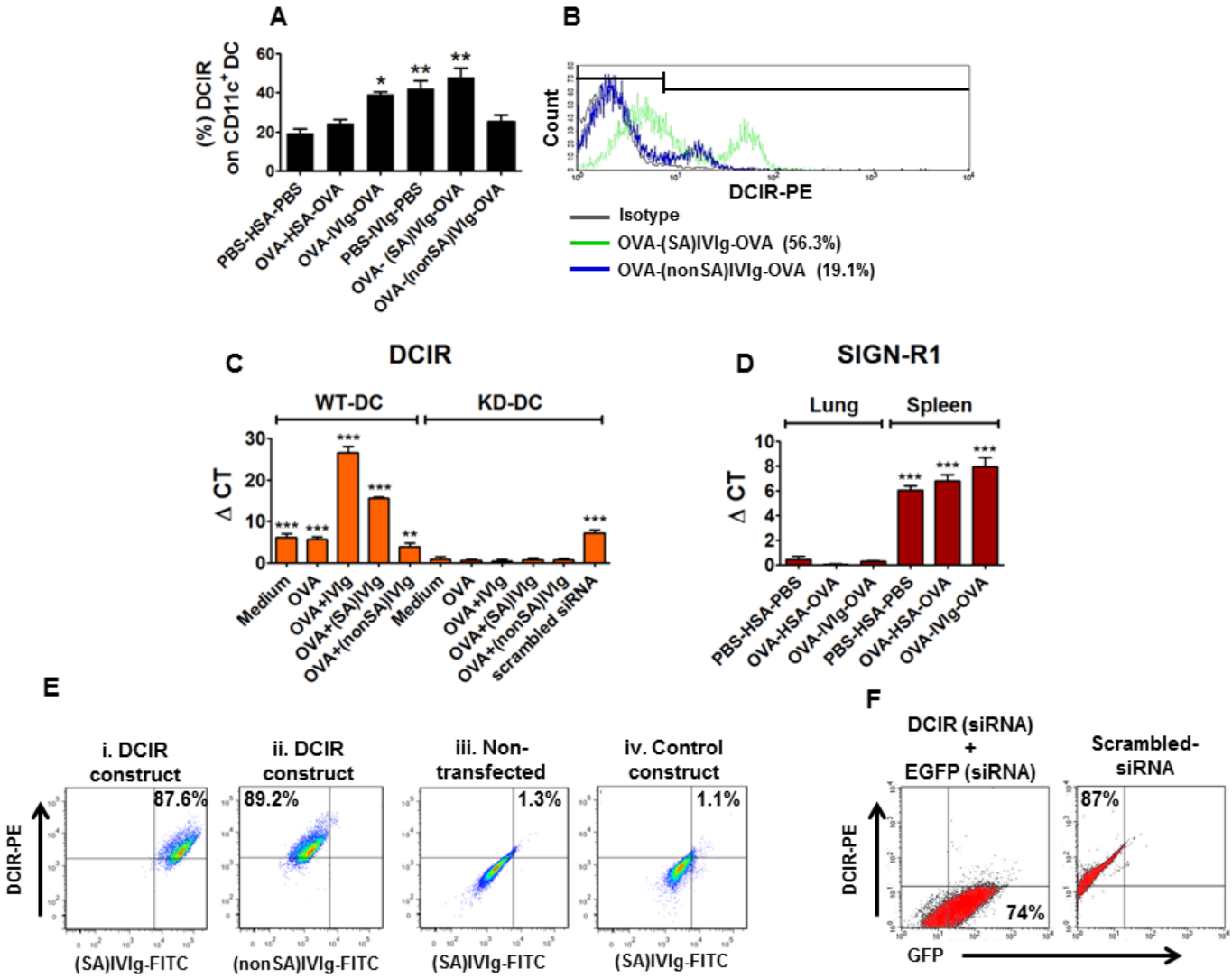

Figure 2. SA-IVIg interacts with CD11c$^+$ DC by binding to DCIR. A and B.

Bar graph and representative histogram illustration of DCIR expression on gated pulmonary CD11c$^+$ DC. IVIg and SA-IVIg induced upregulation of DCIR expression on pulmonary DC. **C, D.** DCIR and SIGN-R1 mRNA expression measured by qRT-PCR**.** Bar graphs demonstrating the mean in ΔCt values (normalized against GAPDH) of **C.** BM-DC conditioned with OVA-IVIg/SA-IVIg induced the upregulation of DCIR mRNA expression and **D.** SIGN-R1 mRNA expression in total lung vs. spleen from different experimental groups, demonstrating low expression of SIGN-R1 in lungs. **E.** Representative flow cytometric analysis of DCIR construct transfected CHO cells. The cells were co-stained with PE-anti-DCIR and FITC-IgG. Transfected cells co-localized DCIR

and SA-IVIg (i), but nonSA-IVIg did not interact with transfected cells (ii). Non-transfected cells neither interact with anti-DCIR nor SA-IVIg (iii). Scrambled-construct-transfected cells behaved similar to non-transfected cells (iv). **F.** Representative flow cytometric analysis of co-transfected BM-DC with DCIR-siRNA and EGFP (non-targeting) siRNA followed by staining with anti-DCIR Ab. EGFP-siRNA were incorporated into more than 70% of cells and DCIR-siRNA blocked the expression of DCIR on DC (left plot), whereas transfection with scrambled siRNA did not impact the DCIR expression on DC (right plot). Data represent averages and mean ± SD from 3 independent experiments with 2 replicates in each one. *=$P<0.05$, **=$P < 0.01$. (n=6).

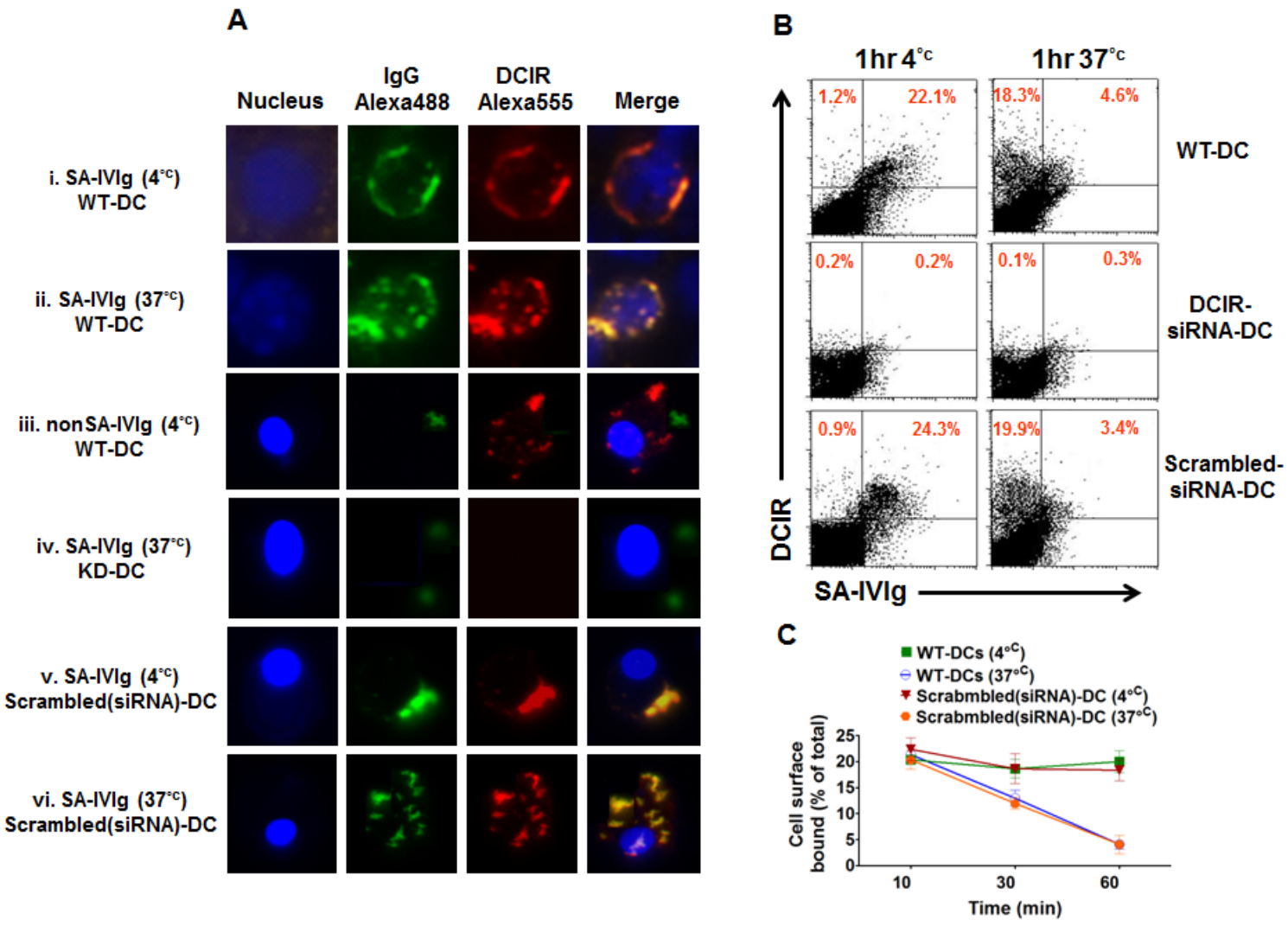

Figure 3. DCIR mediates binding and internalization of IgG into DC. A.

Fluorecent microscopy demonstrating co-localization of DCIR and IgG. FcγR-blocked BM-DC were stained with Alexa555-anti-DCIR (red) and Alexa488-SA-IVIg or Alexa488-nonSA-IVIg (green). Nuclear staining was carried out with DAPI (blue). DCIR co-localized with SA-IVIg, but not nonSA-IVIg on WT-DC at 4°C incubated for 1hr (i and iii). DCIR and SA-IVIg internalized into DC at 37° over 1 hr (ii). SA-IVIg neither bound nor internalized into DCIR-siRNA treated (KD-DC) (iv). DC treated with scrambled siRNA prior to staining had the same cellular behavior as WT at both 4°C and 37°C (v, vi). Stained cells were visualized at 400X magnification. Figures are representative images of 3 independent experiments (n=50 to 100 cells counted for each condition). **B.** Representative flow cytometry and **C.** Survival graph

demonstrating cell surface bound SA-IVIg. WT or KD-DC incubated with PE-anti-DCIR, and biotinylated SA-IVIg at 4°C or 37 °C (for 10 min, 30 min and 1 hr), followed by incubation with FITC-strapavidin at 4°C for another 30 min. Incubation at 37°C reduced cell surface bound SA-IVIg by 80% over 1 hr, compared to DC incubated at 4°C. KD-DC did not bind anti-DCIR at either 4°C or 37°C. Control cells with scrambled-siRNA-treated DC were similar to WT. Data represent averages and mean ± SD from 2 independent experiments**.**

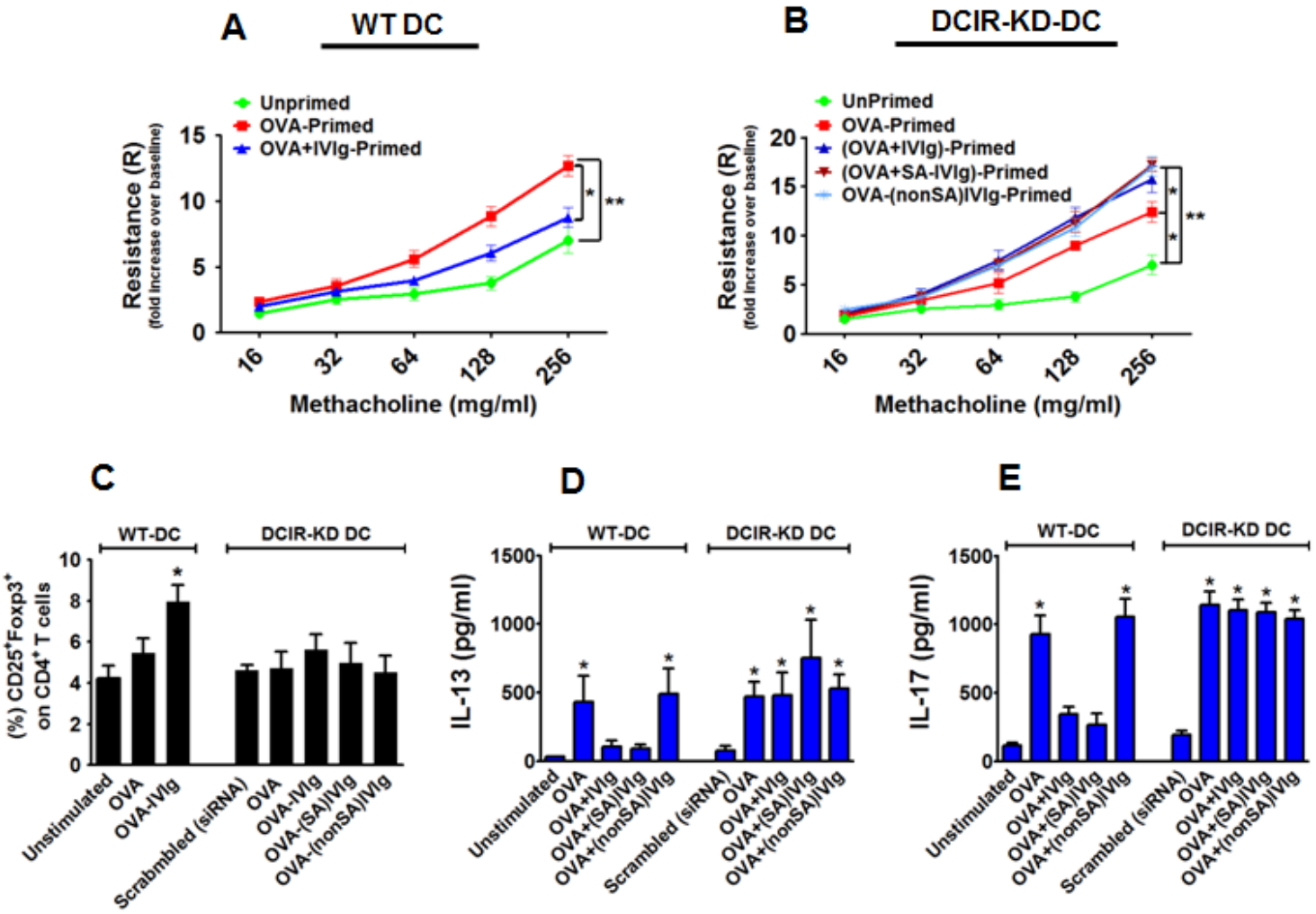

Figure 4. DCIR is critically involved in the inhibition of AHR by IVIg/SA-IVIg.

KD-DC or WT-DC were by OVA +/- IVIg, and then administered intratracheally to syngeneic animals, followed by OVA challenge. **A.** Transferring of OVA WT-DC enhanced AHR to MCh in OVA-challenged mice, whereas WT-DC conditioned with OVA+ IVIg did not confer increased AHR. **=P<0.01 vs. Medium WT-DC, *=P<0.05 vs. OVA-IVIg WT-DC for peak MCh response **B.** Transferring of OVA-IVIg or OVA-SA-IVIg KD-DC did not inhibit AHR to MCh. **=P<0.01 vs. Medium KD-DC *=P<0.05 OVA-HSA vs. OVA-IVIg/SA-IVIg KD-DC for peak MCh response **C.** Frequency of $CD25^{+}Foxp3^{+}$ T_{reg} on gated $CD4^{+}$ T cells in total lung

digests demonstrated no increase in T_{reg} in mice transferred with OVA-IVIg/SA-IVIg KD-DC compared to similarly conditioned WT-DC. **D,E.** Lung digests of mice received WT-DC primed with OVA-IVIg and OVA-SA-IVIg demonstrated decreased levels of IL-13 and IL-17 production to OVA-stimulation compared to the cells from OVA WT-DC recipients, determined by ELISA in supernatants, whereas adoptively transferring of OVA-IVIg/SA-IVIg KD-DC did not have the same effect. Results are representative of 2 studies (n=6 for each condition). *=$P<0.05$ **=$P < 0.01$.

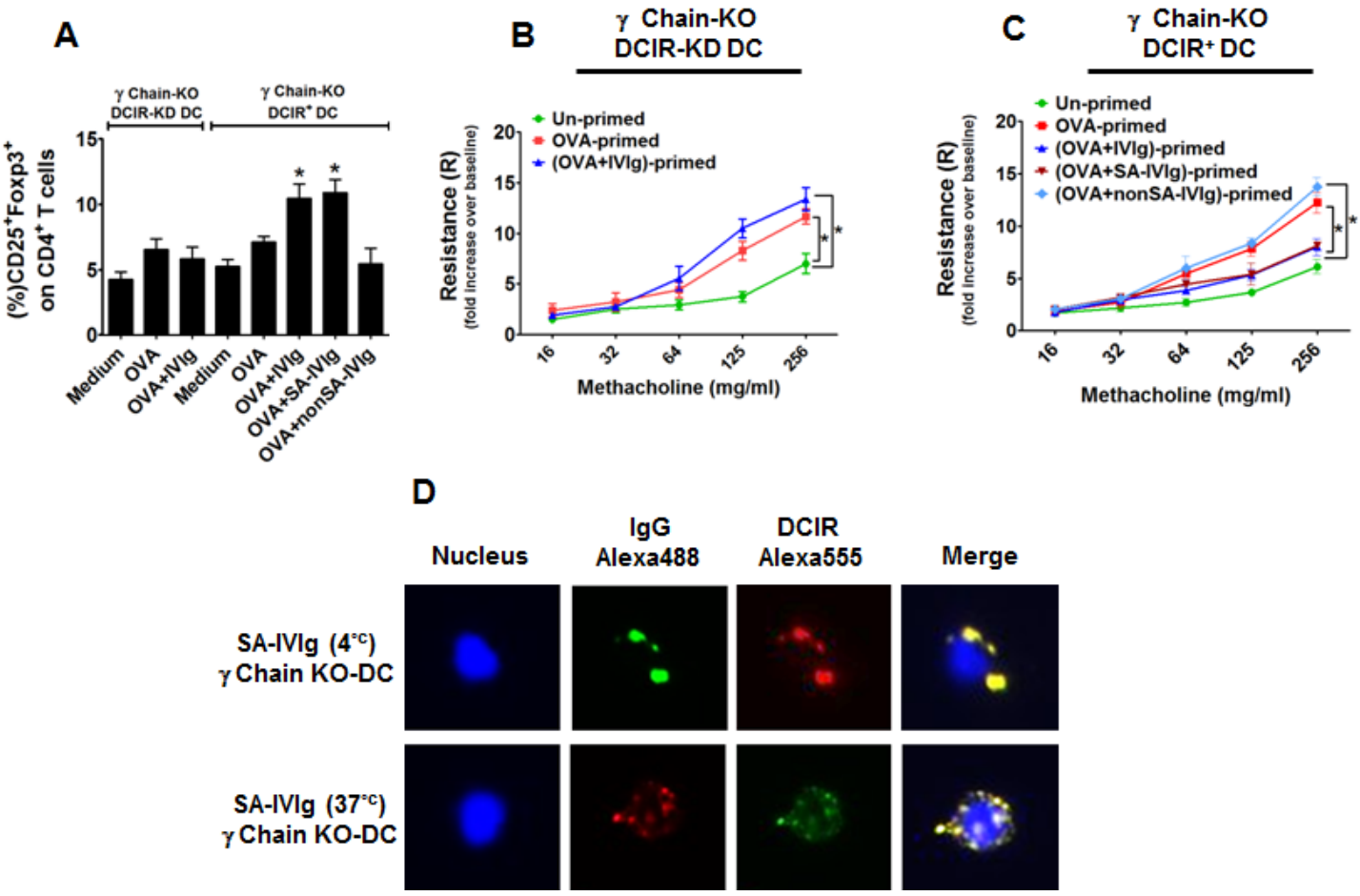

Figure 5. The effect of SA-IVIg is not dependent on Fcγ receptors.

BM-DC from Fcγ-chain-KO (KO-DC) mice were conditioned in the presence or absence of DCIR-siRNA *in vitro*, and administered i.t. to WT mice, followed by OVA challenge. **A.** Transferring of OVA+IVIg or OVA+SA-IVIg KO-DC, but not KO-DC pretreated with DCIR-siRNA prior to conditioning, induced $T_{reg.}$ in lungs of OVA challenged mice. **B, C.** OVA-IVIg or OVA-SA-IVIg KO-DC did not induce AHR, measured by FlexiVent, following OVA challenge, while transferring of KO-DC pretreated with DCIR siRNA prior to conditioning with OVA+ IVIg did not inhibit AHR to MCh. *=P<0.05. Results are representative of 2 identical studies (n=6 per condition). **D.** KO-DC were stained and visualized by fluorescent microscopy as explained in **(Figure 3)**. DCIR and SA-IVIg co-localized and remained on the cell surface when the cells stained at 4°C for 60 min (upper panel). Incubation at 37°C induced DCIR/IgG

internalization into KO-DC comparable to WT (lower panel). Figures are representative images from 3 independent observations (50 to 100 observations for each condition).

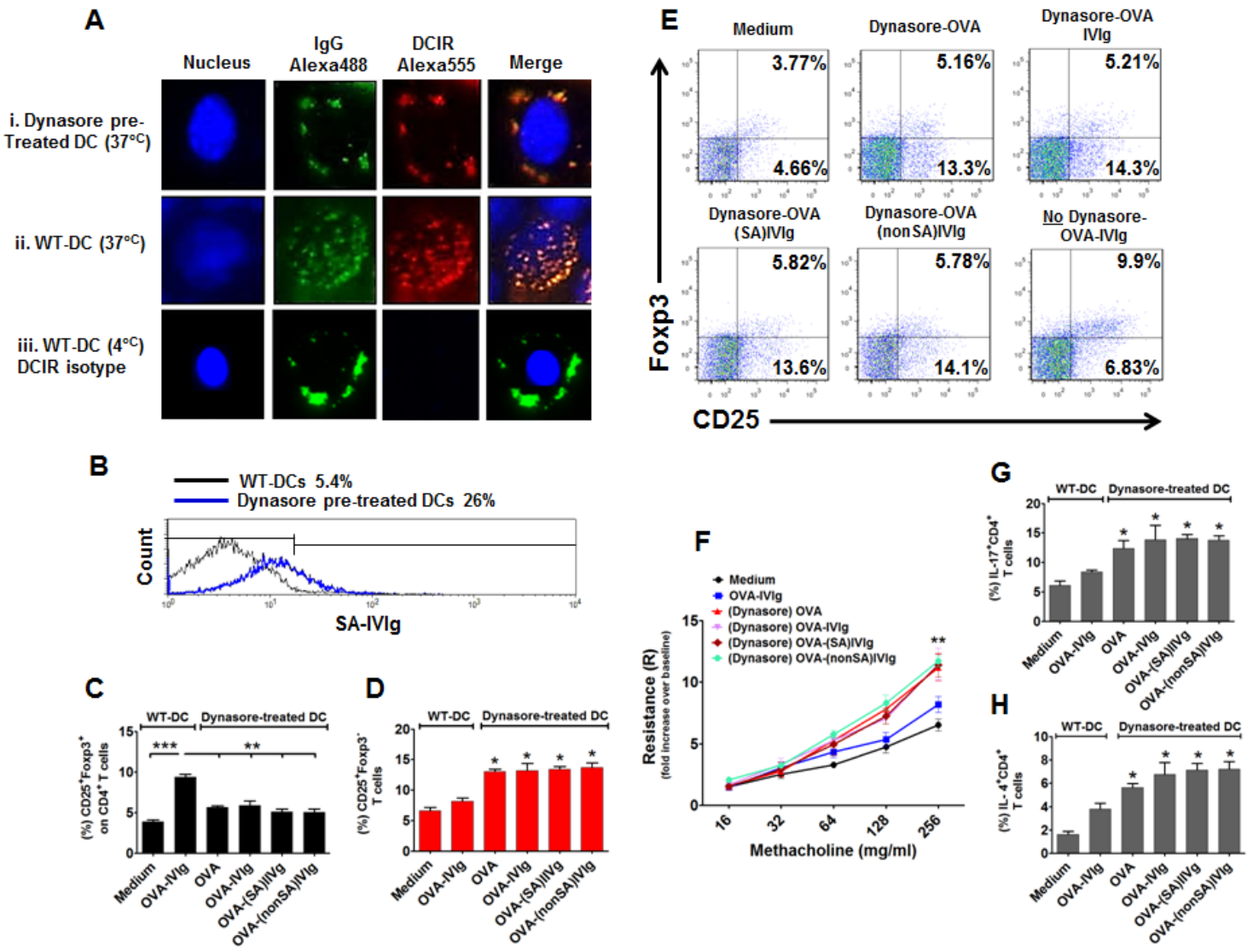

Figure 6. Inhibition of IgG internalization abrogates the modulatory effects of IVIg/SA-IVIg on DC. A.

Fcγ-blocked BM-DC were pretreated with dynasore, stained and visualized under fluorescent microscopy as explained in **(Figure 3)**. Dynasore Pretreatment abrogated internalization of SA-IVIg at 37°C (i), DC without dynasore pretreatment internalized SA-IVIg at 37°C (ii). Figures are representative images from 3 independent observations (50 to 100 observations for each condition). **B.** Representative histogram analysis of SA-IVIg binding on the cell surface of dynasore pretreated DCIR^{+} BM-DC. Internalization assay was carried out as explained in (Figure 3). Dynasore pretreatment (blue) inhibited internalization of SA-IVIg into DC. **C.** Frequency of

$CD25^+Foxp3^+$ T_{reg} cells within pulmonary $CD4^+$ T cells. i.t. administration of OVA-IVIg WT-DC increased the frequency of T_{reg} in lung, but Dynasore-pretreatment abrogated this effect. **D.** Transferring of OVA-IVIg WT-DC decreased the frequency of activated T cells in OVA challenge mice, comparing to OVA WT-DC recipients, determined by flow cytometry, whereas dynasore pretreatment of DC abrogated this effect. **E.** Representative flow cytometric analysis of $CD25^+Foxp3^+$ T_{reg} on gated $CD4^+$ population within the lungs of each treatment group. ***=$P < 0.001$, **=$P < 0.005$ OVA-IVIg or OVA-SA-IVIg DC recipient vs. other control groups. **F.** Transfer of OVA-IVIg and OVA-SA-IVIg-DC attenuated MCh-induced AHR to OVA challenge. Dynasore pretreated OVA-IVIg and OVA-SA-IVIg DC did not attenuate AHR. **=$P<0.01$ vs. Medium or OVA-IVIg WT-DC for peak MCh response. **G, H.** Bar graphs demonstrating IL-4 and IL-17 producing $CD4^+$ T cells within lung. Whole lung digests were stimulated with PMA/ionomycin, stained with appropriate antibodies and the frequency of each population was monitored by Flow Cytometry. *=$P < 0.05$ vs. medium and OVA-IVIg-DC recipients. All results are representative of 2 experiments (n=6 mice per group).

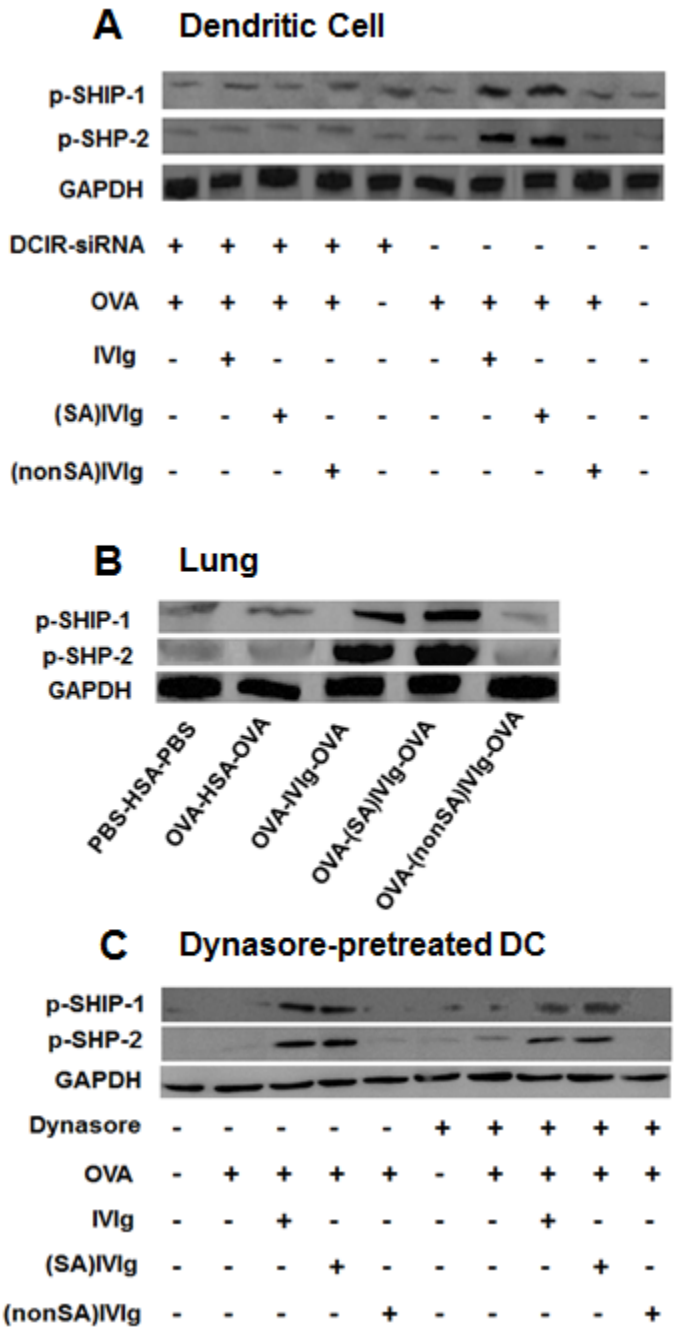

Figure 7. Ligation of DCIR by IVIg/SA-IVIg induces phosphorylation of SHIP-1 and SHP-2. A and B.

Representative Western blots of p-SHIP-1 and p-SHP-2 in protein extracts from conditioned BM-DC and total lung from treated mice. OVA-IVIg and OVA-SA-IVIg treatment increased

pSHIP-1 and pSHP-2 levels in **A.** conditioned BM-DC or **B.** Total protein extracts from treated mice, whereas **A.** BM-DC pretreated with DCIR-siRNA did not exhibit increased phosphorylation to conditioning with OVA-IVIg/SA-IVIg **C.** Dynasore pretreatment of DC did not change the pattern of phosphorylation of these proteins of conditioned BM-DC. GAPDH was used as the internal control and was in constant levels in all the tested groups. Representative of 3 similar experiments.

Chapter III

General discussion

IVIg is a polyclonal immunoglobulin preparation obtained from pooled plasma of thousands of blood donors. Clinical findings have demonstrated the beneficial effects of high dose IVIg in the treatment of a wide range of autoimmune and inflammatory diseases (Kazatchkine and Kaveri, 2001; Nimmerjahn and Ravetch, 2008). Despite intensive efforts over last two decades, no comprehensive mechanism encompassing all the conditions where IVIg has a demonstrable beneficial effect have been yet described. The immune-regulatory function of IVIg is attributed to the modulation of Fc-γ receptors on APCs, regulation of cytokine network, interference of neutralizing antibodies with inflammatory mediators, regulation of activation and cell growth of dendritic cells (DC), macrophages (MΦ), natural killer (NK) cells, and T and B cells (Gold et al., 2007; Kazatchkine and Kaveri, 2001; Nimmerjahn and Ravetch, 2008), which were extensively discussed in the introduction section.

In addition to be widely used in treatment of autoimmune disorders, a number of studies have reported the efficacy of IVIg in allergic diseases with relatively few side effects. These include chronic sinusitis (Ramesh et al., 1997), severe atopic dermatitis (Kwon and Kim, 2012; Turner et al., 2012), urticaria (Watkins et al., 2012) and refractory or severe asthma (Boznanski and Widerska, 2002; Gelfand et al., 1996; Mazer and Gelfand, 1991; Mazer et al., 1989). The inhibitory effect of IVIg in allergic states is proposed to be achieved by regulation of cytokines, mainly by increasing the levels of inhibitory cytokines and decreasing the levels of pro-inflammatory cytokines. Nevertheless the precise mechanism of action of IVIg in these allergic conditions has not been well understood.

In our study we utilized an antigen-driven murine model of allergic airway disease wherein therapeutic administration of IVIg inhibited the airway inflammation by attenuating perivascular and peribronchial cell influx, and decreasing airway hyperreactivity. This was accompanied by inhibition of inflammatory cytokine production. Treg-targeted therapies would primarily diminish inflammation via modulation of cytokines (Chaudhry and Rudensky, 2013); therefore we sought to investigate if IVIg therapy was associated with activated or increased Treg in our model. IVIg has been reportedly shown to restore reduced levels of circulating $Foxp3^+$ Treg and enhance their functional activities in clinical trials (Kwekkeboom, 2012). Notably the deficiency of $Foxp3^+$ Treg in patients with Kawasaki disease is associated with resistance to IVIg therapy (Hirabayashi et al., 2013), indicating the essential role of Treg in mediating the immune-modulatory effects of polyclonal IgG. The relevance of these observations also is supported by recent studies in experimental animal models of autoimmune and inflammatory diseases.

Tregs, specified by the expression of transcription factor *Foxp3*, are dedicated to maintaining immune tolerance via suppressing dysregulated innate and adaptive immune responses (Chaudhry and Rudensky, 2013). Deletion or loss-of-function mutations in the *Foxp3* gene result in a multiorgan autoimmune disorder in both humans and mice known as IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome (Fontenot et al., 2003), supporting the non-redundant integral role of Tregs in immune homeostasis. Foxp3 is viewed as the master regulator of Treg cells. Ectopic expression of Foxp3 in conventional CD4+ T cells recapitulates the phenotype and function of Treg cells (Fontenot et al., 2003)

Naturally occurring Treg (nTreg) acquire Foxp3 expression during development in the thymus. Foxp3 expression can also be induced in the periphery in a fraction of naive $CD4^+Foxp3^-$ T cells

following T cell receptor (TCR) stimulation in a variety of inflammatory settings (Curotto de Lafaille and Lafaille, 2009). The total peripheral Treg cell pool comprises both nTreg and induced Treg (iTreg) cells. A fraction of both Treg subsets can lose Foxp3 in specific inflammatory microenvironments and have the potential to differentiate either into T helper 1 (TH1), TH17 or TH2 effector cells that contribute to inflammation (da Silva Martins and Piccirillo, 2012).

iTreg and nTreg share many features in their functional activities, and thus far no definite marker has been determined to distinguish these two subsets. However iTreg cells have been shown to be an essential regulatory subset in maintaining immune tolerance and supplement nTreg cells, in part by expanding TCR diversity within regulatory responses (Haribhai et al., 2011). Peripheral induction of Treg during induction of mucosal tolerance can efficiently alleviate antigen-induced airway inflammation (Curotto de Lafaille et al., 2008; Curotto de Lafaille and Lafaille, 2009), as iTreg display the same antigen specificity as effector T cells, and develop in response to exogenous antigens (Mucida et al., 2005). In line with that, Josefowicz et al (Josefowicz et al., 2012) demonstrated that selective blockade in differentiation of iTreg cells in mice spontaneously induced the development of pronounced TH2-type pathologies at mucosal sites -- in the gastrointestinal tract and lungs-- with hallmarks of allergic inflammation and asthma, although this did not lead to extensive multi-organ autoimmunity, exacerbation of induced tissue-specific autoimmune pathology, or increased pro-inflammatory responses of TH-1 and TH-17 cells. All these suggest that Tregs generated in the thymus or extrathymic sites might serve distinct functions in the control of inflammatory responses.

Emerging evidence implicates innate cell populations, such as dendritic cells (DCs), in regulation of immune tolerance via peripheral induction of iTreg (Kornete and Piccirillo, 2012). DCs represent a heterogeneous population of bone marrow-derived cells and are the most potent antigen presenting cells (APCs) (Banchereau and Steinman, 1998). Upon initiation of an immune response DCs receive maturation signals through pathogen associated molecular patterns (PAMPs) and damage associated molecular pattern (DAMPs) receptors that detect microbial or tissue damage signals *via* activation of nuclear factor-κB (NF-κB) and interferon regulatory factors (IRF) families (Maldonado and von Andrian, 2010). Activated DCs up-regulate a wide variety of gene products involved in antigen presentation and co-stimulation such as MHCII, CD86, CD80, OX40-L, inducible co-stimulator (ICOS) ligand as well cytokines involved in the modulation of effector function such as IL-1β, IL-2, IL-6, IL-8, IL-12, and IL-18 (Maldonado and von Andrian, 2010). Under certain conditions DCs acquire tolerogenic properties and might involve in maintaining of immune tolerance.

Tolerogenic DCs are characterized by extensive antigen uptake and processing capabilities in order to present antigen to antigen-specific T cells, but fail to deliver proper co-stimulatory signals for effector T (Teff) cell activation and proliferation (Steinman, 2003) due to decreased expression of co-stimulatory molecules (Mahnke et al., 2002). This may result in T cell death, anergy, or extrathymic induction of $Foxp3^+$ Treg cells in an antigen-specific fashion (Morelli and Thomson, 2007). Specific markers capable of discriminating tolerogenic from inflammatory DCs are still poorly defined. However, $CD8^+$ DCs expressing CD95L and DEC205 often possess tolerogenic properties (Yamazaki et al., 2008).

In our study, we demonstrated that IVIg promotes iTreg from non-regulatory precursors in inflamed organs, in a mechanism dependent on DC and the presence of antigens. Moreover by assessing the functionality of the Treg we demonstrated that the IVIg-induced Treg have very high capacity to suppress the proliferation of responder T cells. Consistent with our work, it has been postulated by others that IVIg enforces immune-suppression by modifying DC phenotype via down-regulation of DC maturation and functional activation, which subsequently control T cell-mediated responses (Aubin et al., 2010; Bayry et al., 2003a). DC are thought to be the primary target of IVIg in different models (Crow et al., 2009). We demonstrated that IVIg-primed DCs derive differentiation of Treg in the presence of antigens, suggesting the indispensable role of T cell receptor (TCR) stimulation in the generation of iTreg (Massoud et al., 2012). Priming of purified naïve $CD4^+$ T cells with IVIg did not enhance the expression of Foxp3 in those cells, which ruled out the direct action of IVIg on effector T cell in generation of Treg. Collectively these data led us to conclude that IVIg resolve airway inflammation via a multi-step process whereby IVIg conditions DC to promote the induction of Treg in an antigen-specific manner and subsequently suppress T-cell mediated inflammatory responses in inflamed organs. On the other hand, the production and activity of the immunomodulatory cytokine IL-10 derived from Treg might reciprocally inhibit multiple aspects of DC function, which remains to be elucidated.

Despite the principle role of IVIg-primed DCs in promotion of Treg, one report claimed that IVIg directly stimulates activation of pre-established nTreg. Tha-In et al. (Tha-In et al., 2010) demonstrated that *in vitro* IVIg treatment of human Treg cells result in activation of those cells, as evidenced by increased expression of surface activation markers and enhanced suppressor

activity *in vitro*. Hence the activation and development of Treg by IVIg can be attributed to the direct and indirect effect of IVIg on Treg compartments.

Tregs employ distinct suppression mechanisms depending on the context as well as the location of the inflammatory response (Chaudhry and Rudensky, 2013). Diverse and heterogeneous patterns of lymphocyte homing receptors expression by Treg facilitate their homing and enhance their ability to modulate organ-specific autoimmunity (Wing and Sakaguchi, 2012). In our study we hypothesized that IVIg-derived Treg must localize to and function within pulmonary tissues in order to prevent or diminish allergic inflammation. Induced Treg cells in lymphoid tissue are a relatively homogenous population of $CD25^{hi}$ $CD62L^{+}$ $CCR7^{+}$ cells that resemble conventional naive T cells. However, upon homing to peripheral tissues, Treg rapidly acquire phenotypic features of effector or memory T cells, and change the pattern of homing receptors that allow them to access non-lymphoid sites (Lee et al., 2007; Sather et al., 2007). The up-regulation of CCR4 expression that we observed in the pool of pulmonary Treg in IVIg-treated mice indicated the capacity of IVIg-derived iTreg to be recruited to the site of inflammation.

In the next step of our study we sought to determine the molecular events downstream of the interaction of IgG to DC. The sialylated fraction of immunoglobulin (SA-IVIg) has recently been postulated to be responsible for a portion of the anti-inflammatory effects of IVIg, using an experimental model of serum-induced arthritis; this involves a population of marginal zone macrophages expressing the C-type lectin receptor SIGN-R1 (Anthony et al., 2008b). Wwe confirmed that application of low dose SA-IVIg replicates the actions of intact IVIg in our allergic airways model. However, we did not detect SIGN-R1 in pulmonary tissues; therefore we focused our attention on other receptors with the potential capacity to bind SA-IVIg within

pulmonary organs. Dendritic Cell Immunoreceptor (DCIR) is a newly described lectin receptor which is unique among other CLRs in DCs, in its association with ITIM in its intracellular tail. We showed the specific binding capacity of DCIR to SA-IVIg, but not nonSA-IVIg, and subsequently demonstrated the crucial role of DCIR in mediating the immune-regulatory effect of IVIg in our model by showing that specific blocking of DCIR expression on DC by siRNA led to abrogation of the protective function of IVIg-primed DCs upon transfer to sensitized mice.

This data contributes to the emerging body of evidence implicating glycosylation of IgG in the regulation of both pro- and anti-inflammatory activities. In a serum–induced nephritis model (Kaneko et al., 2006), it was shown that induction of kidney disease in mice results in reduction of total IgG sialylation within serum IgG on average by 40% compared to the naïve controls. A recent pilot study also showed that successful specific immunotherapy in allergic patients induced sialylated allergen-specific IgGs (Oefner et al., 2012). In fact the extent of IgG glycosylation determines their distinctive binding capacities to various classes of activating or inhibitory receptors (Anthony et al., 2012), such as siglecs or C-type lectin receptors.

It is still unclear if IgG has multiple sites of cell interaction that can lead to immune regulatory events. A recognized paradigm for the therapeutic action of IVIg in experimental models of immune thrombocytopenia (ITP) and nephrotoxic nephritis involves up-regulation of the inhibitory FcγRIIB in splenic macrophages. The inhibitory low-affinity receptor for immunoglobulin G (IgG), FcγRIIB, is widely expressed by hematopoietic cells such as B cells, mast cells, monocytes and dendritic cells. Yamamoto et al. (Yamamoto et al., 2010) have demonstrated that in an OVA-driven model of airway inflammation FcγIIB deficient mice are

not responsive to IVIg therapy, which is restored by transferring of DCs from FcγIIB WT mice to KO animals. However, they did not assess the direct interaction of IgG with this receptor.

Moreover, we and others have shown that there is no direct interaction between IVIg preparations and FcγRIIB on B cells or DCs. In addition (Crow et al., 2003) have shown that FcγRIIB signaling pathways are not triggered by IVIg in amelioration of murine ITP. This and other data led to the conclusion that although the activity of IVIg may be dependent on upreregulation of FcγRIIB, this receptor is not likely a target of IVIg, but rather involves in maintaining a balance with activating FcγRs on APCs or likely plays an intermediate role in mediating of IVIg's protective effects.

Recent studies characterize the nature of innate receptors, with the binding capability for glycoproteins, and their associated signaling pathways, in programing DC. Among these receptors C-type lectin receptors (CLRs) can induce DC, and in a broader sense APCs, to stimulate the differentiation of Th1, Th2, or Th17 or Treg (Geijtenbeek and Gringhuis, 2009), depending on the nature of receptor (Fig. 1)

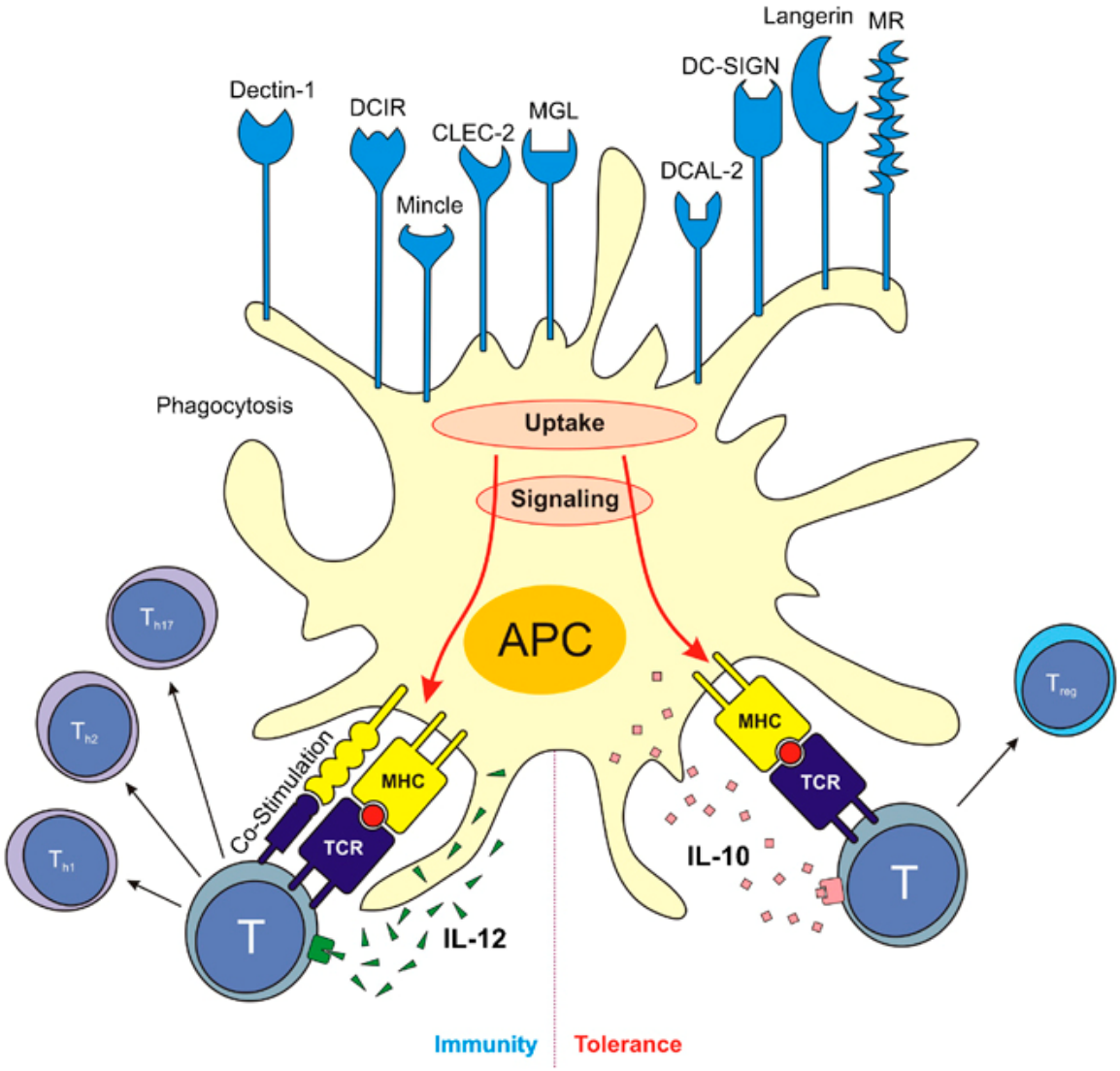

Figure 1. Innate function of C-type lectin receptors (CLRs) in antigen uptake and signaling processes in antigen-presenting cells. Adapted from (van Vliet et al., 2008).

Antigen-presenting cells can express various CLRs that interact with specific glycan structures. Upon binding CLRs internalize antigen for presentation onto MHC class I or II molecules for presentation to T cells. CLRs can also function as signaling molecules that trigger specific cytokines.

CLRs belong to a superfamily of trans-membrane and soluble proteins that sense carbohydrate components of pathogens as well as self-glycoproteins (Manicassamy and Pulendran, 2011). CLRs contain single or several carbohydrate recognition domains (CRDs) (Figdor et al., 2002) which are important in binding and uptake of glycosylated antigens. Ligation of these receptors by their cognate antigens can initiate intracellular signaling that induces or inhibits the activation of the ligated cells (Fig. 1).

DCIR is among the CLRs that are expressed on immature monocyte-derived DC, and shows specificity for mannosylated and fucosylated proteoglycans (Bates et al., 1999; Lee et al., 2011). DCIR contains a single carbohydrate recognition domain (CRD) in its extracellular domain, and a consensus ITIM domain in its intracellular tail (Kaden et al., 2009). Expression of DCIR is differentially regulated depending on the DC maturation/activation state. For instance, DCIR expression is down-regulated in response to signals inducing DC activation, such as CD40 ligand, LPS, TNF-α, or TLR9 (Bates et al., 1999; Meyer-Wentrup et al., 2008). A possible contribution of DCIR to the maintenance of peripheral tolerance by induction and expansion of Treg has been demonstrated, although not in the context of IVIg therapy. Yamazaki et al. (Yamazaki et al., 2008) demonstrated that $CD8^{-}DCIR2^{+}$ DC may differentiate peripheral $Foxp3^{+}$ Treg, in part through the endogenous formation of TGF-β.

Another feature of most of CLRs, including DCIR, is the capability to facilitate internalization of their cognate ligand into the cytoplasm. We showed that DCIR-mediated internalization of IgG molecules is crucial for Treg induction in our model. IgG can be internalized into DC incubated with therapeutic concentration of IVIg (Trepanier et al., 2012). The endocytosed IgG may interferes with MHC-II-dependent antigen presentation and potentially reduce the ability of

APCs to present antigens to T cells which may contribute to induction of Tregs (Bilate and Lafaille, 2012). Moreover, DCIR activation can promote inhibitory signaling, leading to inhibition of cell maturation and function. Targeting of such signaling pathways regulates transcription factors such as NF-kB that function to induce gene expression of inflammatory cytokines (Fig.1). Further investigation is required to unveil the exact role of DCIR-associated cellular events in immune-regulatory action of IVIg.

Most recently we have assessed the relative contributions of IVIg-induced $Foxp3^+$ Treg cell in resolution of airway inflammation, by utilizing DEREG mice carrying a DTR-EGFP transgene under the control of the Foxp3 promoter, allowing selective depletion of $Foxp3^+$ Treg by application of diphtheria toxin (DT). IVIg failed to attenuate AHR and alleviate airway inflammation in mice following complete Treg depletion (i.e. prior to and after IVIg treatment). However, the action of IVIg on modification of DC maturation remained intact despite the absence of Treg. When pre-existing Treg cells were depleted before bot not following IVIg treatment, IVIg could induce Treg and these Treg attenuated AHR and histologic airway inflammation following allergen-challenge. This population of IVIg-induced Treg cells was aslo able to block allergic airway hyper-responsiveness when adoptively transferred to allergen-sensitized mice, in an antigen-specific manner (data not shown). Our study demonstrated that induction of $Foxp3^+$ Treg by IVIg is a critical step in inhibition of allergic response, and provides a better mechanistic insight into the therapeutic action of polyclonal IgG in a variety of settings.

Concluding remarks and future directions

Since immunoglobulin replacement therapy was first used for the treatment of X-linked agammaglobulinaemia (XLA) (Bruton, 1952), the therapeutic use of immunoglobulins has widely expanded. This not only includes many primary and secondary immunodeficiencies, but also numerous autoimmune and inflammatory disorders. IVIg is considered as an alternative treatment for a wide range of refractory diseases where conventional therapies result in unacceptable side effects. However, IVIg use is hampered by the high costs and shortage of human plasma sources. Therefore, research into optimization of IVIg use, or even replacement by recombinant products, has become inevitable.

The complexity of immunoglobulin as a medicine is increasingly recognized. Antibodies are multi-faceted, multi-functional molecules that play important and varied roles both in conferring protection against pathogens and maintenance of immune tolerance. An improved understanding about the mechanism of action of IVIg in various conditions can lead to better efficacy of the therapeutic formulations. In this regard, elucidation of the molecular interactions between polyclonal IgG and the immune cells as well as identification of the active fraction of IVIg that modulate the immune system could result in the development of new treatments. For instance it may be possible to reduce the amount of immunoglobulin needed for the treatment of selected inflammatory conditions if an enriched sialylated IgG could be provided. Thus determination of different specificities and glycosylation states of antibodies seems necessary. Separation of IVIg into two products, an immunosuppressive fraction to be used for immunosuppressive therapy, and the non-immunosuppressive residual IgG for replacement therapy in IgG-deficiencies, will be useful in economizing the use of IVIg, and reducing the cost and adverse effects. The

variability among individual lots of IVIg also makes it difficult to compare the results obtained by different medical centers. In the future, it may be necessary to evaluate IVIg preparations for the presence of specific antibodies, and characterize the beneficial effect of each subset individually. This might lead to prepare monoclonal human antibodies with specific desirable characteristics. These targeted antibodies could then be used to supplement intravenous immunoglobulin preparations for the treatment of specific disorders.

We have shown the anti-inflammatory effect of IVIg is mediated through induction of Treg by IVIg affected DC. However, how DC can regulate Treg and which receptors or soluble molecules are involved in this process remained to be elucidated. Recently, the effect of notch receptor and ligand interaction in differentiation of T cells has been shown. These molecules might be involved in the peripheral induction of Treg as well. Understanding the molecular and signaling pathways that are implicating in IVIg mediated and DC-dependent induction of Treg might raise an interest for the therapeutic approaches specifically targeting this axis of the immune system (Figure 2).

In addition to the beneficial effects of human polyclonal IgG mentioned above, the use of IgM and IgA as immunomodulators is another area of intensive scientific research that could potentially alleviate some of the demand on therapeutic IgG. IgA has inhibitory effects in several inflammatory disease models, including asthma and glomerulonephritis (Pasquier et al., 2005; Wolf et al., 1994). Similarly, the use of IgM for immunomodulation seems promising as it is known to interact with potentially self-reactive IgG in healthy individuals (Rossi et al., 1990).

Furthermore, research into new routes of administration, such as nebulized immunoglobulin and local subcutaneous use, may in the future allow expansion of non-intravenous administration into

other therapeutic areas. In a murine model of allergen-driven airway inflammation, sublingual administration of IVIg exhibited an anti-inflammatory activity. Thus the targeted delivery of IgG may also reduce the high dose requirement of IVIg to achieve immune-regulatory effects.

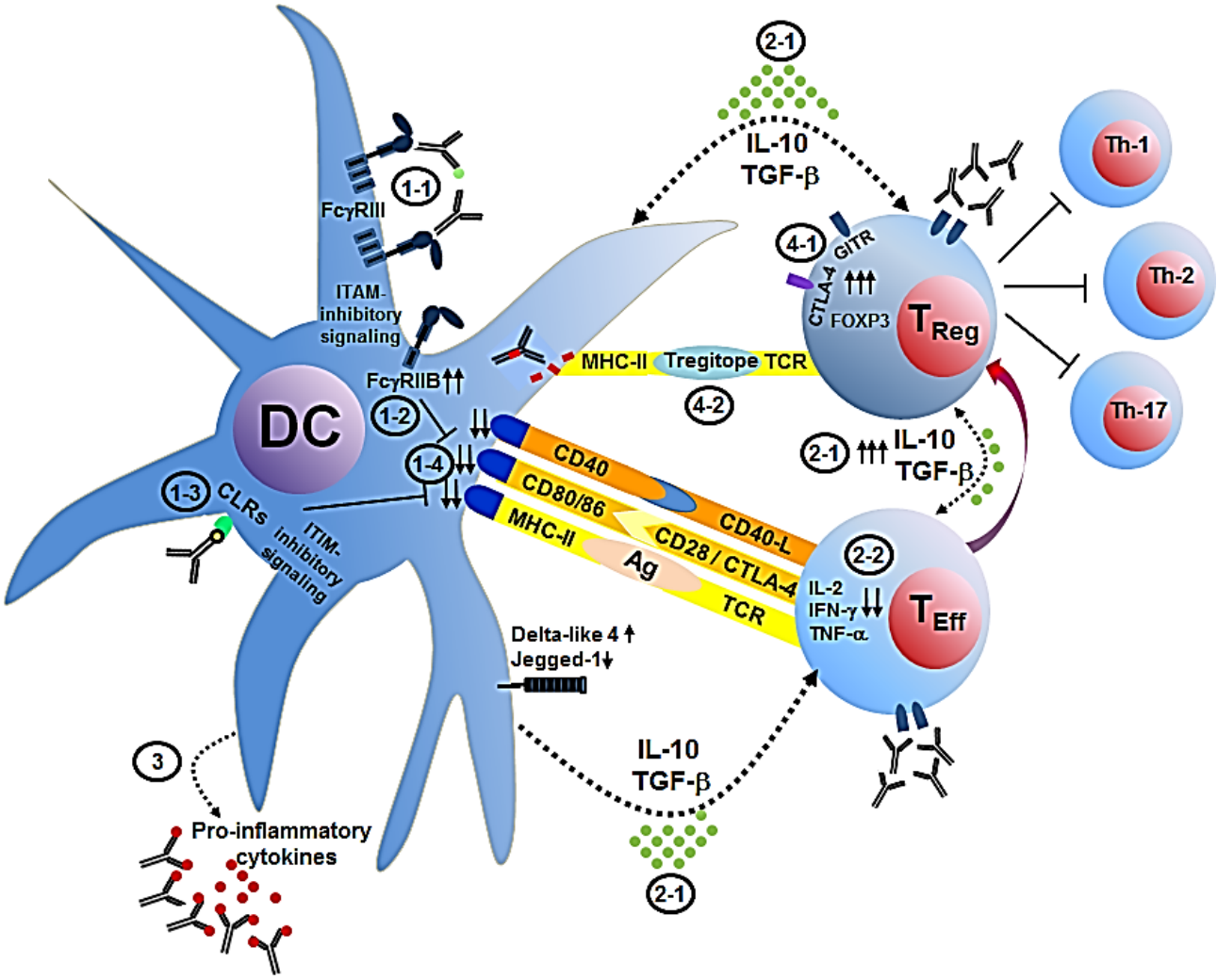

Figure 2. The known effects of IVIg on T_{reg} compartment. AHM ©.

1- IVIg contribute to the induction of T_{reg} via modulation of DC activation mainly through 3 different mechanisms;

1-1- Binding of immune-complex within IVIg to activating Fcg receptor and induction of ITAM inhibitory signaling pathway (ITAMi)*.

1-2- Enhancing the expression of inhibitory FcγIIB on DCs

1-3- Targeting of CLRs and the subsequent induction of ITIM-dependent pathway, as well as internalization of IgG molecules.

1-4- Downregulation of MHC and co-stimulatory molecules expression on DCs.

2- IVIg modulate the production of cytokine by DCs and effector T cells (T_{eff}), mainly by;

2-1- Increasing the inhibitory.

2-2- Suppressing the pro-inflammatory cytokine production.

3- Natural Abs within IVIg preparation, block and neutralize pro-inflammatory mediators.

4- IVIg stimulate expansion and functional activation of Treg by;

4-1- Upregulation of FOXP3, CTLA-4, GITR and other genes expression, associated to Treg activation, by binding to unknown receptor(s).

4-2- DC-mediated presentation of specific peptides derived from their Fc-parts (Tregitopes) to Treg.

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* The concept of inhibitory immunoreceptor tyrosine-based activation motif (ITAMi) has emerged as recent studies have demonstrated that ITAM can initiate inhibitory signaling pathways, in addition to the classical concept that it mainly involves in activating signaling toward heterologous receptors. The active inhibitory signaling by ITAM-bearing receptors was named inhibitory ITAM (ITAMi) (Blank et al., 2009; Hamerman and Lanier, 2006).

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