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Circulating Normal IgG as Stimulator of Regulatory T Cells: Lessons from Intravenous Immunoglobulin

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Abstract

Intravenous immunoglobulin (IVIG), a pooled normal IgG formulation prepared from the thousands of healthy donors' plasma, is extensively used for the immunotherapy of autoimmune and inflammatory disorders. Recent reports demonstrate that IVIG exerts anti-inflammatory actions by stimulating the activation and expansion of regulatory T (Treg) cells by multiple mechanisms via antigen presenting cells (APCs).

CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) have a pivotal function in maintaining immune tolerance and lessening the inflammation-induced tissue damage. Tregs can be generated in the thymus (tTregs cells or natural Tregs) or derived in the periphery (pTregs, previously known as induced Tregs) from naive CD4⁺ T cells. As impaired generation and function of Tregs leads to severe autoimmune and/or inflammatory diseases, various approaches that improve Treg number and function are being explored to treat autoimmune diseases.

Regulation of autoimmune and inflammatory responses by circulating normal IgG: knowledge gained from IVIG

Immunoglobulin G (IgG) is a predominant class of antibodies in the circulation and one of the major players of defense against invading pathogens. Accordingly, deficiency of IgG, as in the case of common variable immunodeficiency (CVID) or X-linked agammaglobulinemia, leads to increased predisposition to recurrent infections. Conversely, immunodeficiencies are also associated with autoimmunity and inflammatory conditions,

suggestive of a dysregulated immune status. Intriguingly, replacement therapy with low-dose intravenous immunoglobulin (IVIg, 400 mg/kg) consisting of intact IgG molecules purified from the pooled plasma of several thousand healthy blood donors, not only prevents recurrence of infectious diseases but also suppresses autoimmune and inflammatory responses. These observations underline the crucial regulatory role of circulating normal IgG in the maintenance of immune homeostasis.

Although initially conceived for the IgG replacement therapy of primary and secondary immunodeficiencies, high-dose IVIG (1-2 g/kg) is now used for the immunotherapy of many autoimmune and inflammatory diseases including Guillain-Barré syndrome (GBS), Kawasaki disease, myositis, immune thrombocytopenic purpura, chronic inflammatory demyelinating polyradiculoneuropathy and others [1]. Several non-exclusive mechanisms have been proposed to explain the therapeutic effects of IVIG in these diseases, including the inhibition of activation of innate immune cells like dendritic cells (DC), macrophages and monocytes, the suppression of effector T cells such as Th1 and Th17, and the inhibition of inflammatory cytokines, complement pathway and autoantibody production.

IVIg targets Tregs to exercise anti-inflammatory actions in autoimmune diseases

Several recent reports demonstrate that high-dose IVIG therapy also stimulates the activation and expansion of Tregs, and enhances their functions to inhibit autoimmune and inflammatory responses [2, 3]. This action of IVIG on Tregs correlates with its therapeutic benefits both in autoimmune disease patients and experimental models [2, 4]. High-dose IVIG therapy exerts sustained effect on Tregs and despite a gradual decline; this effect goes beyond the half-life of infused IgG in majority of the patients who respond to this therapy [4]. As IVIG is nothing but pooled IgG from normal donors, the effect of IVIG on Tregs likely represents a primordial function of circulating IgG in regulating immune homeostasis.

Treg expansion by IVIG: How many mechanisms after all?

Dissection of underlying mechanisms by which high-dose IVIG expands Tregs has led to identification of multiple mechanisms (Figure 1).

Cyclooxygenase (COX)-2-dependent-Treg expansion by IVIG: Our *in vitro* data demonstrate that by signaling through DC-SIGN, IVIG and its F(ab')₂ fragments at concentrations corresponding to amount of IgG received by the patients under high-dose IVIG therapy, expand human Tregs by inducing COX-2-dependent prostaglandin E₂ (PGE₂) secretion in DC [5]. Furthermore, Treg expansion and disease ameliorative ability of IVIG *in vivo* were compromised in experimental autoimmune encephalomyelitis (EAE) mouse model upon COX-2 inhibition. Investigations in autoimmune patients also confirmed the induction of PGE₂ following high-dose IVIG therapy [6].

Dendritic cell immunoreceptor (DCIR)-dependent induction of Tregs: In a mouse model of airway hyperresponsiveness, IVIG at 2 g/kg dose significantly enhanced pTregs *in vivo* in the lungs [7]. Mechanistically, pTreg induction by IVIG required interaction of $\alpha(2,6)$ -sialic acid linkages of IgG (either at Fc or Fab region) with DCIR on CD11c⁺ DC. SIGN-R1 (murine ortholog of DC-SIGN) on the other hand was dispensable. Interference with DCIR-binding or DCIR-signaling abrogated IVIG-mediated Treg induction by CD11c⁺ DC upon adoptive transfer and attenuation of airway inflammation.

IL-33-dependent induction of Tregs by IVIG: A more recent study in EAE reports IL-33-dependent activation and expansion of pTregs by high-dose IVIG (1 g/kg) that inhibit

pathogenic CD4⁺ T cell responses *in vivo* [8]. This model further suggests that IVIG induces IL-33 in SIGN-R1⁺ marginal zone macrophages via interaction of $\alpha(2,6)$ -sialic acid linkages of Fc fragment with SIGN-R1 (Box 1). During cognate interaction between APC (like DC) and Tregs, IL-33 in the microenvironment activates Tregs by signaling through ST2 receptor. Although IVIG therapy in autoimmune patients induces IL-33, as discussed later, the proposed model of IL-33 induction was not reproduced in human (Box 1).

Activation of Tregs by processing and presentation of IgG-derived 'Tregitopes': Data from bioinformatics, *in vitro* stimulation of human peripheral blood mononuclear cells and house dust mite allergy model in HLA-DR4 transgenic mice suggest the existence of highly promiscuous MHC class II T-cell epitopes in the Fc and Fab regions of IgG (and hence in IVIG) with regulatory features called “Tregitopes” [9]. These Tregitopes are processed and presented by APC to activate tTregs. It is not yet clear however whether such mechanism is directly implicated in the amelioration of autoimmune response and Treg stimulation in IVIG-treated patients.

Thus, irrespective of aforementioned mechanisms, APC such as DC and C-type lectin receptors on them appear to be chief architects of IVIG-mediated Treg responses both in humans and animal models. While human data advocate mechanisms for tTreg expansion, murine data provide insight on pTregs. Implication of both tTregs and pTregs in the inhibition of autoimmune response and inflammation, favors the existence of multiple mechanisms of Treg induction by IVIG. Findings in human further echo this possibility as IVIG therapy in a small cohort of GBS patients significantly induced both PGE₂ and IL-33 [6]. It is also important to note that IVIG could directly stimulate human Treg expansion *in vitro* under the influence of co-stimulatory signals.

Mouse data suggest that at least two pathways of pTreg induction are under operation: a direct pathway involving the interaction of sialylated IgG with DCIR on DC and an indirect pathway dependent on IL-33 via interaction of sialylated Fc with SIGN-R1 on marginal zone macrophages [7, 8]. As SIGN-R1 is not expressed by mouse DC, DCIR seems to be the major receptor for IVIG on murine DC. The mechanism(s) by which DCIR⁺ DC induce Tregs is open for further exploration.

However, unlike in mouse, *in vitro* differentiated human DC express both DC-SIGN and DCIR. Therefore, which receptor has a dominant role remains unclear, although current data favors a dominant role of DC-SIGN in mediating Treg expansion via COX-2-PGE₂ pathway [5]. We propose that either DCIR has a redundant role in human Treg expansion in the context of inflammatory DC or that the relative contribution of these two lectin receptors is determined by subsets of DC and tissues, as it is important to note here that DC *ex vivo* express mainly DCIR.

Why does Treg expansion by IVIG mimic the function of circulating IgG?

IVIG symbolizes a complete repertoire of normal circulating IgG. The distribution of IgG subclasses and IgG glycosylation patterns in IVIG generally overlaps with normal human plasma/serum. Whereas a single donor might lack certain individual IgG specificities, that is likely to be compensated in IVIG because of pooling of plasma.

The immunodeficient patients either lack or have reduced amount of IgG in the circulation. As a corollary, such conditions are also associated with reduced frequency of Tregs and/or Foxp3 expression [10]. Of note, replacement therapy with IVIG (400 mg/kg) aiming at bringing up the IgG level in immunodeficient patients close to that of healthy individuals, transiently (and temporarily) enhances Tregs immediately after therapy [11]. These results

point out that circulating IgG are indeed critical for maintaining Tregs. But to achieve this, it is important to maintain normal levels of IgG constantly. This paradigm might explain why long-term repetitive IVIG replacement therapy in immunodeficient patients did not reveal consistent effect on Tregs [12].

The most debated question is, if IgG are capable of inducing Tregs, then despite having them in the blood at normal levels, why do autoimmune patients require high-dose IVIG therapy to ameliorate autoimmune condition and to expand Tregs?

It appears that the high-dose requirement for IVIG to treat autoimmunity rests in the composition of IVIG itself. In autoimmunity and inflammation, both innate and adaptive immune cells are hyper-activated, while regulatory mechanisms are either dysfunctional or reduced in their ability to control these pathogenic responses. Natural antibodies and natural autoantibodies with low to medium affinity are likely to be the major active components of IVIG, but these specificities are not in high frequencies. Thus, given the altered physiology in autoimmune patients, it is conceivable that these natural autoantibodies are needed at higher amounts than those present in the normal circulation of a donor. Moreover, IgG also lose sialic acid content during affinity maturation and inflammation. These requirements likely underlie the requirement for high (1-2 g/kg) doses of IVIG to treat autoimmune conditions, while in healthy individuals, physiological levels of IgG with a normal frequency of natural autoantibodies and sialylation, are sufficient to maintain homeostasis.

Conclusion

In summary, emerging evidences have revealed that multiple pathways are targeted by IVIG (or circulating normal IgG) to propagate Treg expansion. Further work is necessary to translate the experimental findings into the clinic and to harness the pathways of Treg

expansion as potential biomarkers of IVIG response, as, other than PGE₂ and IL-33, the other mechanisms are not yet investigated in the patients.

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BOX 1

Is Fc-sialylation critical for IVIG-mediated anti-inflammatory actions and Treg expansion?

Structurally, IgG is divided into F(ab')₂ fragment that binds to specific antigen, and Fc region that binds to diverse FcγRs present on innate immune cells and B cells to mediate effector functions. Being glycoprotein, all IgG are glycosylated at Asn297 of Fc region (in the CH2 domain).

Mechanistically, both Fc and F(ab')₂-mediated anti-inflammatory mechanisms of IVIG have been reported. Recently, Fc-dependent action of IVIG was proposed to be mediated mainly by a fraction of IgG that contains terminal α(2,6)-sialic acid linkages at Asn297. These α(2,6)-sialic acid linkages interact with C-type lectin receptors such as DC-SIGN (SIGN-R1), DCIR or Siglecs like CD22. However, 15-20% of IgG are also glycosylated at Fab region (in V_H and V_L regions) and few reports show that α(2,6)-sialic acid linkages of Fab but not Fc fragments are important for anti-inflammatory effects of IVIG. Current evidences clearly indicate that Fc-sialylation-mediated action represents 'one of the' pleiotropic anti-inflammatory effects of IVIG rather than being 'only' the mechanism. Several groups have reported that sialylation is not a pre-requisite and IVIG devoid of Fc-sialylation also mediate anti-inflammatory actions. This is not surprising as IVIG is not a preparation with single IgG specificity rather it is a heterogeneous IgG preparation and hence acts on almost all compartments of the immune system.

Is Fc-sialylation critical for IVIG-mediated Treg expansion in humans? The answer remains unclear. SIGN-R1 that is identified as one of the lectin receptors for the recognition Fc-α(2,6)-sialic acid linkages, is expressed mainly by splenic marginal zone macrophages. On the contrary, DC-SIGN in humans is expressed predominantly on DC. Distinct expression

pattern of DC-SIGN and SIGN-R1, and differences in the splenic architecture of human and mouse are the major hurdles in translating Fc-sialylation data of mouse to human.

In vitro data from human monocyte-derived DC unequivocally support interaction of IVIG with DC-SIGN for Treg expansion. But it might be mediated via either sialylated glycans of Fab region or interaction of DC-SIGN-specific natural autoantibodies in the IVIG. Furthermore, unlike mouse, DC-SIGN⁺ DC (either monocyte-derived or from the spleen) did not secrete IL-33 upon IVIG exposure *in vitro*. But analyses of autoimmune patients' plasma following high-dose IVIG therapy confirmed that IL-33 is induced. Therefore, it is likely that endothelial or epithelial cells but not DC-SIGN⁺ cells might have contributed to IL-33 increase seen in the patients. Taken together, Fc-sialylation might have a role in Treg expansion via IL-33 induction, but the proposed pathway is not true in humans.

Figure 1. Proposed mechanisms of induction and expansion of Tregs by IVIG

Intravenous immunoglobulin (IVIG) is a pooled normal IgG preparation. Several reports have now provided evidence that interaction of IgG molecules with antigen presenting cells (APC) such dendritic cells (DC) and macrophages via C-type lectin receptors (CLRs), constitutes first step of regulatory T cell (Treg) induction and expansion by IVIG. Internalized IVIG then targets multiple pathways to modulate the phenotype and function of APC and to induce and expand Tregs. Thus, underlying mechanisms by which high-dose IVIG expands Tregs diverge depending on the experimental models used: autoimmune vs inflammatory conditions; and model species, i.e., human vs mouse. (a) Interaction of IVIG with DC-SIGN through F(ab')₂ fragments (but not Fc fragments) signals cyclooxygenase (COX)-2-dependent prostaglandin E₂ (PGE₂) secretion in DC to expand tTregs. Inhibition of PGE₂ synthesis by COX-2 inhibitors or blocking IVIG interaction with DC-SIGN prevented IVIG-triggered Treg expansion by human DC. (b) Cross-talk between $\alpha(2,6)$ -sialic acid linkages of IgG (either at Fc or Fab region) with DCIR on murine CD11c⁺ DC initiates downstream signaling through Src homology domain 2-containing protein tyrosine phosphatase 2 (SHP-2) to induce tolerogenic status of DC and *de novo* generation of pTregs with enhanced suppressive function. (c) IVIG induces IL-33 in SIGN-R1⁺ innate cells via interaction of $\alpha(2,6)$ -sialic acid linkages at Asn297 of Fc fragment with SIGN-R1, and IL-33 in turn mediates the *de novo* generation of pTregs in EAE model. (d) Activation and expansion of tTregs by Tregitopes present on IgG molecules. APC such as DC endocytose IgG (probably via C-type lectin receptors), process IgG in the intracellular MHC-II compartment and present Tregitopes in the context of MHC class II that specifically activate Tregs. In addition to activation and expansion of Tregs, IVIG also modulates APC and can act at the T-cell level to interfere with polarization and function of pathogenic Th cell subsets (Th1, Th2 and Th17 cells) to exert therapeutic beneficial effects.

