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Novel roles for the IgG Fc glycan

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IgG antibodies trigger leukocyte activation and inflammation by forming immune complexes that crosslink activating Fcγ receptors (FcγRs). This is essential to combat infection, but detrimental if antibodies target or cross-react with autoantigens. The high specificity and long serum half-life of IgG antibodies confers tremendous therapeutic potential. Indeed, antibodies have been successfully employed to target cancers, autoreactive B cells, and pro-inflammatory cytokines. Conversely, IgG antibodies can also initiate anti-inflammatory responses. In the form of intravenous immunoglobulin (IVIG), IgGs are routinely administered to treat inflammatory autoimmune diseases. Importantly, the N-linked glycans on the IgG Fc are absolutely required for initiating these IgG effector functions. In fact, the Fc glycan composition dictates IgG affinity to individual FcγRs, and in a broader sense, binding to different FcγRs classes: activating, inhibitory, and anti-inflammatory (dendritic cell-specific ICAM-3 grabbing nonintegrin, DC-SIGN). The Fc glycan requirements to initiate and suppress inflammation will be discussed herein.

Keywords: DC-SIGN; Fcγ receptor; inflammation; autoimmune disease; sialylation

Introduction

IgG antibodies are important mediators of inflammation. These molecules are responsible for the antitoxin activity described by von Behring and Kitasato in the late 19th century used to treat diptheria, for horror autotoxicus described by Ehrlich in the early 20th century, and are the basis for a number of therapeutics currently in use.[1,2] IgG antibodies have tremendous therapeutic potential because they are highly specific, have a long serum half-life, and are well tolerated by patients. In fact, monoclonal IgG antibody drugs are among the most successful therapeutics developed in the last 15 years. They have been used to effectively treat breast cancer and autoimmune disease by targeting surface antigens, leading to specific clearance of pathogenic cells, and attenuate inflammation by blocking inflammatory cytokines.[3–10]

IgG antibodies are the predominant antibody class in circulation and comprise two identical light chains and heavy chains, which couple to form a "Y-shaped" structure.[11] The two domains of IgG antibodies that are responsible for their *in vivo* properties are the Fab (antigen binding fragment) and the Fc (crystalizeable fragment) (Fig. 1A).[12] The Fab portion binds its targets with high affinity, leaving the Fc to interact with FcγRs expressed by leukocytes in a low affinity, high avidity interaction. Four distinct IgG classes, which differ in their heavy chains (and, consequentially, Fc), exist in humans (hIgG1-4) and in mice (mIgG1, 2a, 2b, 3). To initiate inflammation, IgG antibodies bind FcγRs, which are classically described as activating FcγRs or inhibitory FcγRs, signaling through immunoreceptor tyrosine activation motifs (ITAMs) or immunoreceptor tyrosine inhibitory motifs (ITIMs), respectively. The relative affinity of IgG Fcs for respective FcγRs, as well as the expression levels of activating and inhibitory FcγRs, ultimately dictates the ensuing inflammatory response type, which has been reviewed extensively.[13–17]

The Fc glycan

A single N-linked glycan is attached to each heavy chain in the Fc portion asparagine-297 (Asn297, Fig. 1A).[18] The glycan has a complex biantennary

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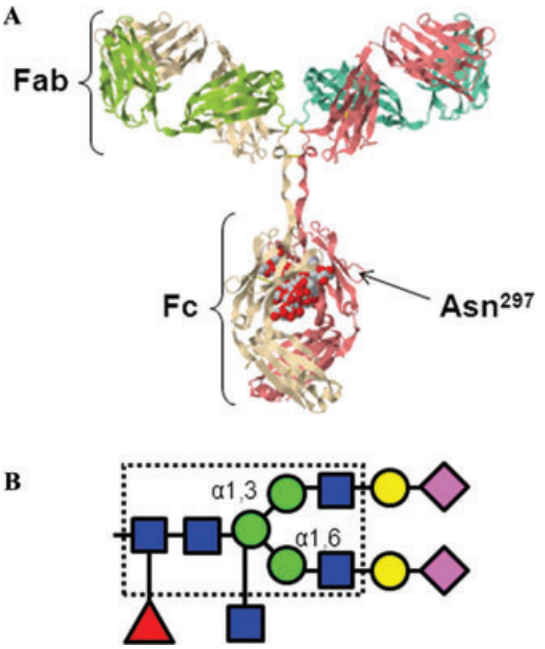

Figure 1. IgG and the Fc glycan structure. (A) The Y-shaped structure of human IgG1 antibody b12 (PDB number 1HZH displayed using FirstGlance in Jmol), with the protein backbone displayed in ribbon, with space filling depiction of the glycan.[96] The IgG protein heavy and light chains combine to form the antigen-binding Fab portion, and the heavy chains extend to the Fc portion, which is responsible for initiating effector functions. (B) The fully processed Fc glycan has a complex, biantennary structure. The core structure within the box is composed of *N*-acetylglucosamine (blue squares) and mannose (green circles). The core glycan can be modified by the addition of fucose (red triangle), bisecting *N*-acetylglucosamine, and the two arms, defined by α1,3 and α1,6 mannose linkages, can be extended by the addition of galactose (yellow circles) and sialic acid (pink diamonds).

structure (Fig. 1B), and can vary by the addition of sugar residues to specific parts of the core structure. There is tremendous heterogeneity in the IgG Fc glycan, with over 30 distinct glycans detected on circulated IgG antibodies in healthy individuals.[19] In addition, approximately 20% of IgG Fabs are glycosylated, which results from introduction of a glycosylation site during somatic hypermutation.[18] Glycosylation of the Fab can be important for the binding to antigens, as selected by affinity maturation, and the presence of a Fab glycan can confound analysis of the Fc glycan composition, because the Fab glycan is more sterically accessible.

The Fc glycans are positioned facing toward the center of the IgG molecule, with the α1,3 arm protruding into the cavity between heavy chains, and the α1,6 arm extending along the heavy chain backbone.[15,18] The Fc glycan is an absolute requirement for binding of wild type IgG to FcγRs, as the interaction is lost by deglycosylating IgG.[18] The Fc glycan is thought to maintain an open confirmation of the Fc heavy chains required for interactions with FcγRs. The structure of aglycosylated Fcs supports this, as the two heavy chains form a closed conformation, preventing formation of the FcγR binding pocket.[20] However, mutations in the Fc backbone can be introduced that enable aglycosylated Fcs to bind FcγRs, which presumably mimic the appropriate conformation folding.[21] This indicates the Fc glycan primarily affects protein–protein interactions by altering IgG backbone conformation.

In the lumen of the endoplasmic reticulum, a 14 monosacchride glycan (glucose3 mannose9N-acetylglucosamine2, (Glc3Man9GlcNAc2)) is transferred to aspargine-297 (Asn297, Fig. 1A) on each IgG heavy chain by the enzyme oligosaccharyltransferase (Fig. 2).[22] Next, the glycans are trimmed to a high-mannose structure (Man8–9GlcNAc2) by exoglycosidases, the IgG heavy and light chains are assembled together, and the intact IgG molecule is transported to the Golgi. The glycan structure is further processed throughout the secretory pathway. In the cis-Golgi, the mannose residues are trimmed by α1,2 mannosidase-I to yield Man5GlcNAc2. In the medial-Golgi, *N*-acetylglucosamine is added by β1,2-*N*-acetylglucosaminyltransferase-I forming GlcNAc1Man5GlcNAc2, and α1,2 mannosidase-II further removes mannose residues forming the hybrid glycan structure GlcNAc1Man3GlcNAc2. Next, the core IgG glycan (GlcNAc3Man3GlcNAc2) is generated by the transfer of *N*-acetylglucosasmine by β1,2-*N*-acetylglucosaminyltransferase-II. Here, the core *N*-acetylglucosamine is available for fucosylation by α1,6-fucosyltransferase. Bisecting *N*-acetylglucosamine is attached to the core by *N*-acetylglucosaminyltransferase-III. As the IgG antibody progresses along the secretory pathway, the glycan can be further modified in the trans-Golgi by the addition of galactose and sialic acid to the arms by β1,4 galactosyltransferase and α2,6 sialyltransferase, respectively.

The Fc glycan in inflammation

As mentioned previously, the Fc glycan provides tremendous heterogeneity to IgG antibodies, with the variable addition of the bisecting

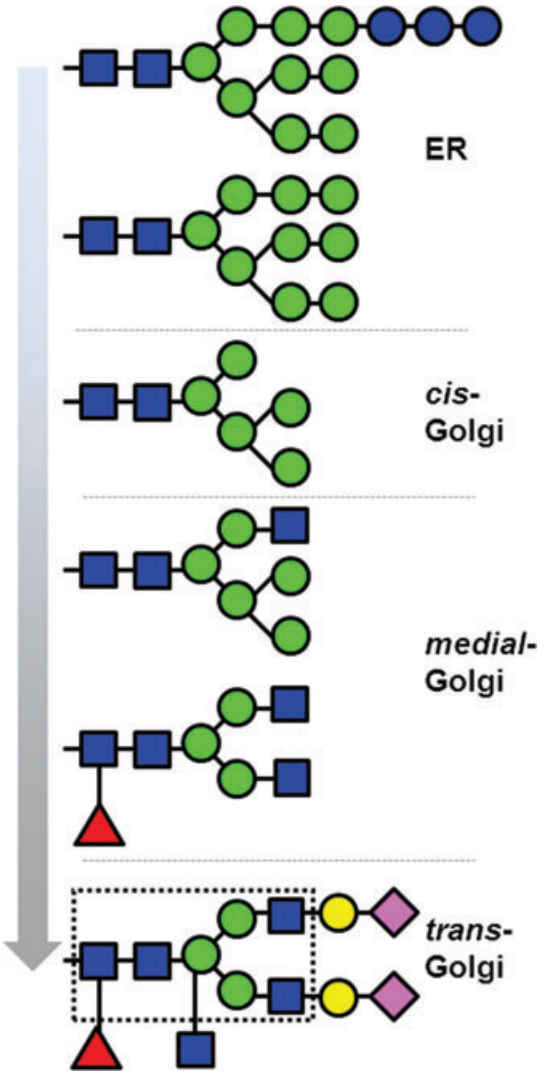

Figure 2. Processing of the Fc glycan. The precursory N-linked glycan (Glc3Man9GlcNAc2) is transferred to Asn-297 on the IgG heavy chain by oligosaccharyltransferase in the ER. Next, the glucose residues are trimmed to form a high-mannose structure (Man8-9GlcNAc2), the IgG heavy and light chains are assembled, and the complex is transported to the Golgi. In the cis-Golgi, the mannose residues are trimmed to yield Man5GlcNAc2. In the medial-Golgi, *N*-acetylglucosamine is added forming GlcNAc1Man5GlcNAc2, and more mannose residues are removed forming the hybrid glycan structure GlcNAc1Man3GlcNAc2. Next, the core structure is formed by transfer of *N*-acetylglucosasmine. The core *N*-acetylglucosamine is available for fucosylation, and bisecting *N*-acetylglucosamine is added. The glycan can be further modified in the trans-Golgi by the addition of galactose and sialic acid to the arms. Glucose (blue circles), *N*-acetylglucosamine (blue squares), mannose (green circles), fucose (red triangles), galactose (yellow circles), sialic acid (pink diamonds).

N-acetylglucosamine, fucose to the core, as well as galactose and sialic acid to the arms of the biantennary structure (Fig. 1B). Interestingly, the composition of the IgG Fc glycan appears to be regulated by the immunological mileu, which feeds back by contributing to either maintenance of homeostasis or by enhancing inflammation (Fig. 3).

Increased levels of fucosylation have been observed in rheumatoid arthritis patients, follow analysis of all circulating proteins, as well as specifically on the IgG heavy chain.[23,24] Murine experiments have demonstrated that repeated immunizations resulted in increased fucosylation of antigen-specific IgG, however, no changes in fucosyltransferase expression in antigen-specific B cells were observed.[25] Attachment of fucose to the IgG core glycan negatively affects antibody effector functions, and afucosylated IgG1 antibodies have a 50- to 100-fold increase in affinity to FcγRIIIa, thought to be the result of interactions between the Fc glycan and the FcγR glycan (Fig. 3).[26–31]

The bisecting *N*-acetylglucosamine residue also affects IgG interactions with FcγRs, as the presence of this residue increases affinity to FcγRIII (Fig. 3).[32] Consequentially, IgGs with bisecting *N*-acetylglucosamine display more potent ADCC than *N*-acetylglucosaminated controls. Increases in addition to bisecting *N*-acetylglucosamine have been reported in Lambert–Eaton myasthenic symdrome, but were unchanged in Myasthenia gravis patients.[33]

The presence of terminal sialic acid on the Fc glycan reduces FcγR affinity 10-fold, and results in less potent IgG antibodies *in vivo* (Fig. 3).[34] Consistent with this, sialylated IgGs were significantly less efficient at antibody-dependent cytotoxicity (ADCC) compared to asialylated control IgGs.[35] The reduction in FcγR affinity caused by sialylation is independent of sialic acid linkage, as seen in both α2,3 and α2,6 sialic acid attachments.[34] Importantly, reductions in circulating siaylated IgGs are reported during inflammation. Following immunization, sialylation was markedly reduced on Fc glycans in mice.[19,36] Consistent with this notion, rheumatoid arthritis patients and Wegener's granulomatosis patients are reported to produce decreased levels of antigen-specific sialylated IgG for citrillated proteins and proteinase-3, respectively.[37,38] Interestingly, an increase in sialylated autoantigen-specific IgG has been observed during remission of these

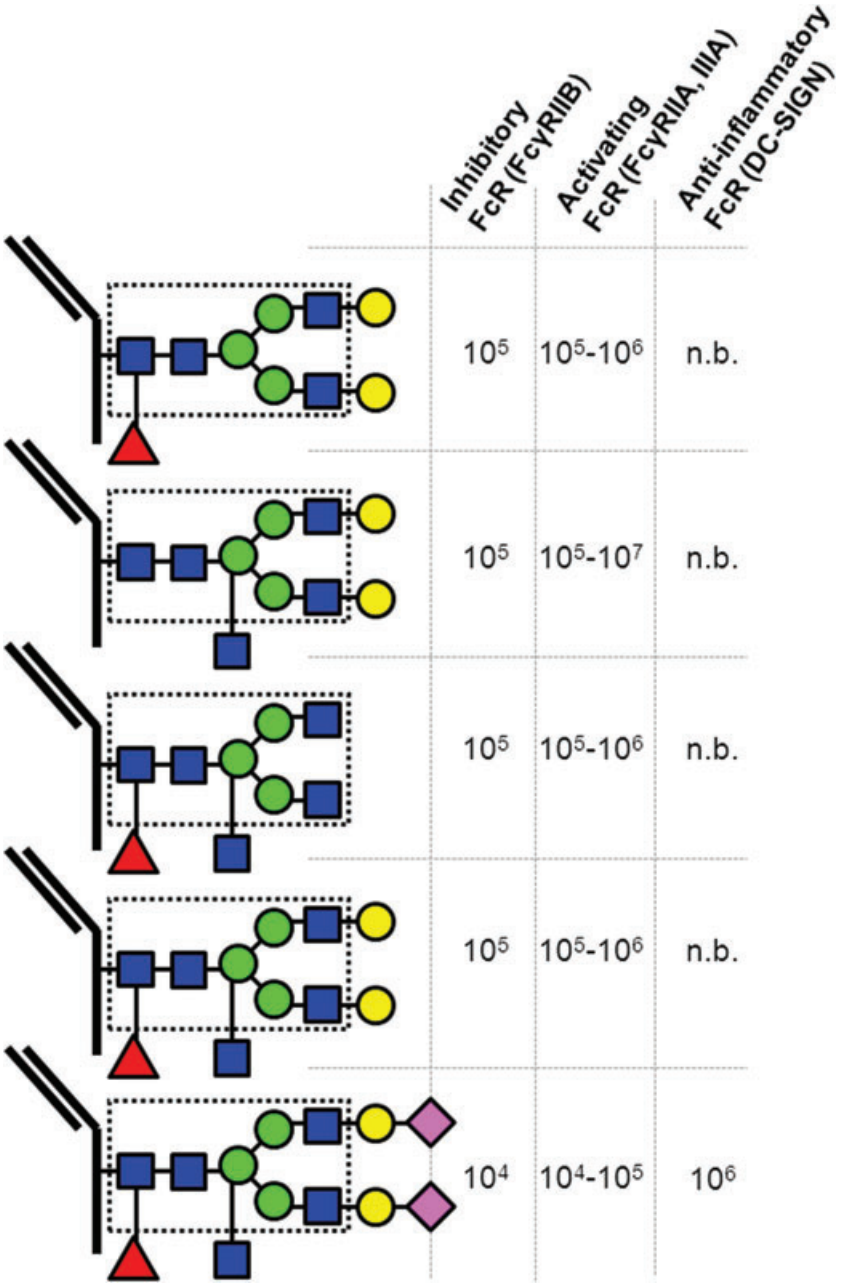

Figure 3. Fc glycan composition dictates FcγR affinity. Approximate association constant ranges (K_a in M-1) of activating, inhibitory, anti-inflammatory FcγRs for various human IgG1 glycoforms. *N*-acetylglucosamine (blue squares), mannose (green circles), fucose (red triangles), galactose (yellow circles), sialic acid (pink diamonds).

diseases, consistent with tight regulation of α2,6 sialyltransferase.[37,38]

Initial reports described reduction in terminal galactose moieties on IgG in patients suffering from various inflammatory diseases, including rheumatoid arthritis, and suggested that galactose might play a regulatory role. However, enzymatic removal of terminal galactose has no effect on FcγR affinity, serum half-life, or induction of inflammation in induced arthritis and ITP models.[35,39] These

studies suggest that galactose itself does not directly attribute to IgG effector properties. Therefore, the enhanced inflammatory activity of these antibodies is likely explained by the reduced levels of sialylated IgG Fc glycans, of which a consequence is exposure of terminal galactose residues.

Anti-inflammatory IgG

The immunoregulatory potential of IgG antibodies was first described in 1981 following successful treatment of pedritatric immune-mediated thrombocytopenia (ITP), an autoimmune disease in which platelets are targeted by autoantibodies.[40] Having exhausted other treatment avenues, patients were administered high doses of IVIG out of desperation, which had surprising results. Platelet numbers rebounded in 10 days after treatment. A further insight described in this manuscript indicated the F(ab)2 fragments generated by pepsin digestion of IVIG were ineffective as rescuing platelet numbers. These results were supported by another study that successfully treated pediatric ITP by infusion of IVIG-derived Fcs.[41] These studies confirmed that the general anti-inflammatory activity of IgG antibodies was a function of the Fc, consistent with all effector functions of antibodies.

To explain the observations that high doses of IgG antibodies can act to suppress inflammation, three hypotheses have been put forth.[42,43] One suggests the high dose of administered antibodies saturate the neonatal Fc receptor (FcRn), promoting increased catabolism of pathogen autoantibodies. Another suggests that high doses of IgG antibodies bind to activating Fcγ Rs on inflammatory cells, and prevent binding of pathogenic antibodies to Fcγ Rs and subsequent autoantibody induced inflammation. A third proposes that the high dose of IgG antibodies alters the ratio of Fcγ R types on inflammatory cells by increasing expression of the inhibitory Fcγ RIIB, thereby forcing inhibitory signaling following interaction of inflammatory cells and anti-antibody immune complexes.

A number of experimental systems have shed light of these hypotheses. These include the passive transfer of K/BxN serum inducing the effector stage of rheumatoid arthritis, passive transfer of antiplatelet antibodies modeling ITP, and an active immunization nephrotoxic nephritis model.[36,44–47] Importantly, these models mimic human antibody-mediated autoimmune disease, which are prevented by the clinical dose of IVIG. Studies from these models have suggested that IVIG anti-inflammatory activity is resulting from the Fc portion, requires the Fc glycan, and the inhibitory Fc receptor (Fcγ RIIB) (reviewed in Refs.[15,42,43,48], and.[49] FcRn interactions with IgG antibodies are independent of the Fc glycan, and the glycan requirement of IVIG indicates that FcRn is dispensable for IVIG anti-inflammatory activity. Furthermore, Fcγ R binding has been well described as a low affinity, high avidity interaction.[13,14,16,17] Therefore, it stands to reason that monomeric IgG antibodies, with low affinity and single valency, will not prevent immune complexes with multiple Fc from the binding to Fcγ Rs.

Importantly, increased Fcγ RIIB expression in patients suffering from chronic inflammatory demyelinating polyneuropathy (CIDP) was associated with improved responsiveness to IVIG treatment.[50,51] Further, increased expression of Fcγ RIIB following IVIG administration has been observed in a number of animal models, and mice lacking this receptor are unresponsive to IVIG.[19,36,43,45,46,52] These observations strongly support a role for increased expression of Fcγ RIIB in the anti-inflammatory activity of IVIG.

Anti-inflammatory activity of the IgG Fc glycan

The high dose requirement of IVIG to initiate the anti-inflammatory activity of IgG antibodies suggested that a minor component of polyclonal IVIG preparations was responsible for this activity. As mentioned above, deglycosylated IVIG lost all anti-inflammatory activity, confirming an essential role of the Fc glycan.[19] Next, IVIG preparations were treated with neuraminidase to remove terminal sialic acid from the Fc glycan.[19] Similar to the deglycosylated IVIG, asialylated IVIG exhibited no anti-inflammatory activity *in vivo*. Further, enrichment for sialylated IgG antibodies yielded 10% of the IgG antibodies in an IVIG preparation. This sialylated IVIG preparation was effective at suppressing induced arthritis and nephrotoxic nephritis at a 10-fold lower dose than whole IVIG[19] (Ravetch and Kaneko, unpublished data). Furthermore, sialylated IgG Fcs (sFcs), generated either by sialic acid-specific lectin enrichment or *in vitro* sialylation, suppressed inflammation at a 30-fold lower dose than IVIG.[19,35] This anti-inflammatory activity required the attachment of sialic acid in

an α2,6 linkage, as α2,3 sialylated Fcs were unable to suppress induced arthritis.[35] These results confirmed an essential role for sialylated IgG Fc glycans in the anti-inflammatory activities of IgG antibodies.

As mentioned previously, the addition of sialic acid to the Fc glycan results in reduced affinity for FcγRs (Fig. 3). Therefore, IgGs with sialylated glycans in IVIG would not bind FcγRs, indicating that high dose IVIG would not suppress inflammation by saturating FcγRs, as the lower affinity IgG antibodies would not replace high affinity, high avidity autoimmunecomplexes. Further, the interaction of IgG antibodies with the FcRn is independent of the Fc glycan, and the glycan requirement of IVIG further supports no direct role for FcRn in this anti-inflammatory pathway.

Anti-inflammatory activity of SIGN receptors

Upon the description of the anti-inflammatory activity of sialylated IgG Fcs, studies were commenced to identify the cellular receptor responsible for their anti-inflammatory activity. Sialylation of the Fc glycan results in reduced affinity for FcγRs, indicating an additional receptor triggered by this ligand induced a novel pathway. A screen of genetically modified mice with defined defects in the immune system shed light on the localization of targeted cells. Mice deficient in $CD4^+$ T cells or B cells were protected by IVIG from induced arthritis.[53] However, op/op mice, deficient in CSF and consequentially specific macrophage populations, $Rag1^{-/-}$ mice, and splenectomized mice were not protected.[52,53] These results indicated a splenic population that required anatomical architecture of secondary lymphoid organs (which is severely disrupted in $Rag1^{-/-}$ mice, which lack both T and B cells[54]) was required by IVIG, strongly implicated macrophages in the splenic marginal zone.

In the mouse, two populations of splenic marginal zone macrophages (MZMΦs) are defined by expression of Siglec-1 or MARCO.[55–57] Siglec-1^+ metallophillic MZMΦs encircle the white pulp, and are themselves encircled by a ring of $MARCO^+$ MZMΦs.[58–66] Blocking antibodies to Siglec-1 or MARCO had no effect on the anti-inflammatory activity of IVIG.[53] However, blockade of another receptor expressed by $MARCO^+$ MZMΦs, specific ICAM3-grabbing non-integrin, related 1 (SIGN-R1), completely ablated the ability of IVIG to suppress autoantibody induced arthritis.[67–73] Similarly, SIGN-R1–deficient mice ($SIGN\text{-}R1^{-/-}$) were not protected from induced arthritis by IVIG or sialylated Fc fragments (sFc).[53,74]

The human orthologue of SIGN-R1 is DC-SIGN (hDC-SIGN), a well-documented lectin recognizing high-mannose glycans.[75–84] Indeed, α2,6 sialylation of Fcs resulted in the ability to bind SIGN-R1 and hDC-SIGN, in addition to reducing FcγR affinity.[53] SIGN receptor binding was not observed with α2,3 sialylated Fcs, consistent with the linkage requirements of the anti-inflammatory activity.[35] These results suggest α2,6 sialylation of the Fc results in a conformation change in the protein backbone that conveys SIGN receptor binding. $SIGN\text{-}R1^{-/-}$ mice engineered to express hDC-SIGN were responsive to IVIG and sFc, indicating that functionally, human DC-SIGN could replace SIGN-R1 in the IVIG induced anti-inflammatory pathway.[85,86] Furthermore, macrophages cultured from the bone marrow of $hDC\text{-}SIGN^+$ mice, when pulsed *in vitro* with sFc, could transfer anti-inflammatory activity and suppress induced arthritis.

Characterization of this response demonstrated that an innate Th2 response was triggered by sFc through DC-SIGN. Induction of the cytokine IL-33 in the spleen was observed after administration of sFc or IVIG, and blockade of the IL-33 receptor ST2 prevented sFc suppression of induced arthritis. Furthermore, exogenous IL-33 was sufficient to suppress the inflammation. IL-33 has been reported to induce an innate Th2 response, triggering IL-13 production by nuocytes, and the anti-inflammatory activity of IL-33 suggested Th2 cytokines might be involved in this response.[87]

Indeed, $IL\text{-}4^{-/-}$, $IL\text{-}4R^{-/-}$, and $Stat6^{-/-}$ mice were all unresponsive to sFc, confirming a role for a Th2 response in the anti-inflammatory activity of IVIG. However, these results suggested that IL-13, a cytokine closely related to IL-4, could not act to replace IL-4 in this pathway. However, administration of IL-4 or IL-13 was effective at inducing the pathway, indicating signaling through the IL-4 receptor was the important step triggered. Further, these cytokines potentially upregulated surface expression of FcγRIIB, consistent with the *in vivo* requirements of IVIG (Fig. 4).

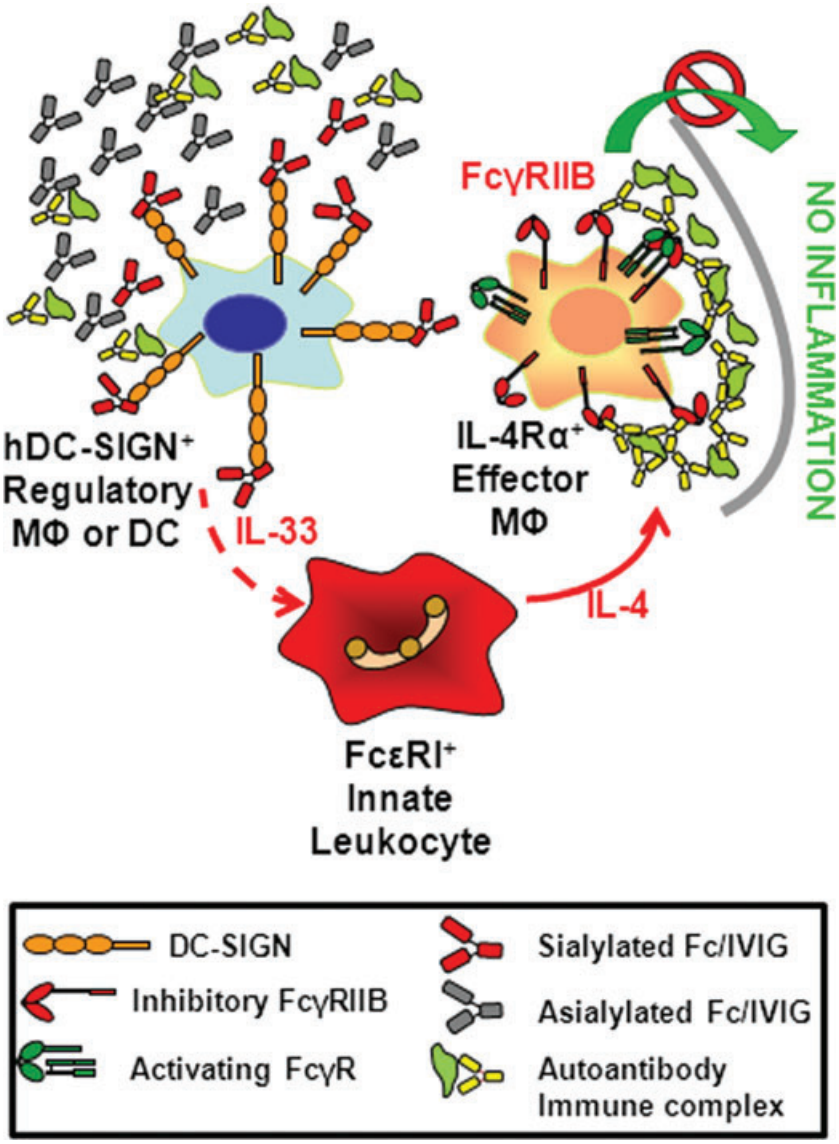

Figure 4. sFc suppresses autoantibody inflammation by inducing an innate Th2 response. Autoantibody immune complexes crosslink activating FcγRs promoting activation of macrophages, and inflammation associated with autoantibody-mediated autoimmune disease. Following administration of IVIG, antibodies with sialylated IgG Fcs bind DC-SIGN$^+$ macrophages (MΦs) or dendritic cells (DCs), promoting IL-33 expression, which activated FcεRI$^+$ innate leukocytes to produce IL-4. This cytokine promotes upregulation of FcγRIIB on macrophages, thereby increasing the activation threshold required to trigger inflammation.

Conclusions and perspectives

The IgG Fc glycan is essential for the structural integrity of IgG, an absolute requirement for FcγR interactions, which dictates the type of Fc receptor that is ligated. This level of contribution to a protein's biology is rather unusual for a glycan. While variations on the IgG Fc glycans directly contribute to the effector functions of antibodies, and specific glycan forms are associated with immunological status, little is known about the regulation of glycosyltransferases, and more investigation is required. As noted, increases in fucosylation and decreased sialylation and galactosylation on the Fc glycan are observed during inflammatory conditions. However,

it is not clear whether the expression of glycosyltransferases is regulated or whether other regulatory mechanisms are involved. Further, the specific inflammatory cytokines or coreceptor interactions that contribute to Fc glycan regulation are only beginning to be understood. IL-21 has been shown to increase galactosylation and sialylation on IgG produced by cultured B cells, and Th2 cytokines have been demonstrated to influence IgA glycosylation.[88,89]

The immunoregulatory potential of the IgG Fc glycan demonstrated by IVIG treatment, suggests that this anti-inflammatory pathway, triggered by an endogenous ligand, sFc, through a pattern-recognition receptor is important for the maintenance of homeostasis. Consistent with this notion, sialylation levels are decreased during inflammation, indicating this regulatory pathway is not triggered in patients suffering from chronic inflammatory diseases, and hence the effectiveness of increased sialylated Fc levels by high dose IVIG. Further characterization of the interaction of sFc and DC-SIGN, and examination of the downstream signaling events may help in the development of more effective anti-inflammatory therapies. The effectiveness of IVIG in Alzheimer's disease is currently being explored.[90–95] While preliminary studies have reported encouraging results, it is not clear whether sialylation of the Fc glycan is required for this activity.

Lastly, the Fc glycan composition is an important consideration for rational design of therapeutic antibodies. To potently trigger inflammation and ADCC, IgG Fc glycans should be afucosylated and asialyated, but contain bisecting *N*-acetylglucosamine. In contrast, anti-inflammatory antibodies should ideally be sialylated and fucosylated, and lacking *N*-acetylglucosamine. Further studies are necessary to dissect the regulation of glycosylation *in vivo* and to ensure immunizations and vaccinations result in the most efficient and appropriate IgG effector functions.

Conflicts of interest

The authors declare no conflicts of interest.

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