Influence of immunoglobulin G-glycan and subclass variation on antibody effector functions
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FcγRIIb
Inhibition of FcγR-mediated phagocytosis by IVIg is independent of IgG-Fc sialylation and FcγRIIb in human macrophages

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ABSTRACT

In immune thrombocytopenia (ITP) and warm autoimmune hemolytic anemia (wAIHA), circulating immunoglobulin G (IgG)-opsonized blood cells are cleared from the circulation by macrophages. Administration of intravenous immunoglobulin (IVIg) can prevent uptake, but the exact working mechanism is not known. The prevailing theory from murine studies, which states that Fc-sialylated IgG alters the balance between activating and inhibitory Fc-gamma receptors (FcγRs) by inducing upregulation of the inhibitory FcγRIIb on effector macrophages, is currently debated.

We studied phagocytosis of IgG-opsonized blood cells in a human system, assessing the effect of IVIg and blocking anti-FcγR F(ab′)2 fragments on uptake by monocyte-derived macrophages (both M1 and M2 macrophages). Phagocytosis was remarkably sensitive to administration of IVIg but unexpectedly, recombinant Fc-sialylated IgG or sialic acid-enriched IVIg were equally active as unsialylated IgG fractions in mediating this inhibition, independent of FcγRIIb expression. Instead, IVIg inhibited phagocytosis by direct blockade of FcγRs. IgG fractions enriched for IgG dimers with enhanced avidity for FcγRs showed increased inhibition compared with monomeric IgG fractions.

Together, our data demonstrate that inhibition of IgG-mediated phagocytosis in human macrophages by IVIg is dependent on the capacity to directly bind FcγRs but independent of FcγRIIb or sialylation of the Fc fragment in the human setting.
Introduction

Human immunoglobulin G (IgG) antibodies directed against blood cells can cause destruction of these cells, leading to anemia or thrombocytopenia. This occurs, for instance, in warm antibody auto-immune hemolytic anemia (wAIHA) or immune thrombocytopenia (ITP), in which IgG auto-antibodies against erythrocytes or thrombocytes, respectively, are formed. These IgG-opsonised blood cells are cleared by macrophages in the spleen and liver, which recognize and destroy IgG-opsonized cells by Fc-gamma receptors (FcγRs). Human FcγRs are divided into high-affinity receptors (FcγRI [CD64], which has a high affinity for its ligand), and low-affinity receptors (the different isoforms of FcγRs II [CD32] and III [CD16], which have a lower affinity for IgG and bind monomeric IgG less efficiently than FcγRI, but do bind to dimeric and multimeric IgG). Five of these human FcγRs potentially play a role in the phagocytosis of IgG-opsonized blood cells by macrophages. The classical activating FcγRs (FcγRI, FcγRIIa and FcγRIIIa) are all independently capable of inducing phagocytosis by monocyte-derived macrophages when crosslinked, but the relative contributions of each of these receptors to phagocytosis of IgG-opsonized particles by different types of macrophages are not well known. FcγRIIc, an activating receptor that is expressed only in a minority of individuals, may also play a role, and its presence has been shown to contribute to ITP susceptibility. In contrast, FcγRIIb, the only inhibitory FcγR, has been reported to inhibit the pro-phagocytic signals.

Therapeutic strategies for the treatment of ITP and wAIHA include the administration of intravenous immunoglobulin (IVIg). IVIg is a first-line treatment of ITP in situations in which a rapid increase in the number of platelets is warranted. Although many different modes of action for the immune-modulating effect of IVIg have been proposed, the exact mechanism by which IVIg immediately alleviates the clearance of IgG-opsonized blood cells is still debated. The therapeutic effects of IVIg are somewhat surprising, considering that its main component, IgG, is normally present in plasma at high concentrations. Possibly, a specific fraction of the IgG present in IVIg preparations is responsible for the therapeutic effect. Because high doses of 1 to 2 g/kg of IVIg are needed to induce a good therapeutic response, more targeted therapies may potentially be developed. It is known that the effect of IVIg lies within the Fc fragments of the IgG, because purified Fc fragments alone were an effective treatment for ITP, whereas IVIg preparations depleted of Fc fragments were not effective. Direct blockade of the FcγRs by the Fc fragments of IgG molecules is one of the possible explanations, and IgG dimers and multimers (present at low concentrations in IVIg) have been implicated as an active fraction because of their capacity to more efficiently bind and block low-affinity FcγRs. Several murine studies have indeed suggested IgG dimers to be the most effective part of IVIg in ameliorating ITP, although a recent report showed that they are not absolutely required, as purely monomeric fractions were also able to ameliorate murine ITP.

During the past decade, however, the focus of research on immune modulation by
Figure 1. FcyR expression on monocyte-derived macrophages

a) Histograms showing expression levels of different FcyRs in monocyte-derived macrophages cultured for 9 days with M-CSF. Gray shading: relevant isotype control. b-e) Comparison of expression levels of FcyRI, FcyRIIa,b, FcyRIIb and FcyRIII between M-CSF and GM-CSF macrophages. Data represent the mean of experiments with macrophages from 34 individuals, or 22 independent experiments in the case of FcyRII.
IVlg has shifted to the role of the inhibitory FcγRIIb and glycosylation of IgG molecules at the Fc tail. Upregulation of FcγRIIb on splenic macrophages was implicated as the working mechanism of IVlg in a mouse model of ITP\textsuperscript{18}. Since then, several reports have suggested that the minor IgG fraction with sialic acid-containing N-linked glycans at Asn297 in the Fc region is important for this immune-modulating effect of IVlg\textsuperscript{19-21}. This has led to a model for IVlg activity in ITP in which sialylated IgG binds to specific ICAM-3 grabbing nonintegrin-related 1 (SIGN-R1) or its human homolog dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) on macrophages, leading to increased expression of the inhibitory FcγRIIb on effector macrophages\textsuperscript{22-24}. However, sialylation did not affect efficacy of IVlg in two other reports using mouse models of ITP\textsuperscript{25-27}. Taken together, the studies in mouse models are inconclusive with regard to the working mechanism of specific fractions of IVlg.

In addition to these inconsistent results in murine models, human FcγRs are very different from those in mice. Therefore, we set out to directly compare the different components of IVlg in a fully human in vitro system. Using monocyte-derived macrophages, we studied the effect on the phagocytosis of human erythrocytes that were opsonized with a human polyclonal anti-RhD serum. The study revealed that IVlg had a direct blocking effect on phagocytosis, which was even stronger in the presence of dimeric and multimeric IgG preparations, whereas sialic acid-enriched fractions of IVlg or recombinant Asn297-α2,6-sialic acid-containing IgG did not show increased effects. Saturation of FcγRI and FcγRIIa were sufficient to explain the inhibitory effects of IVlg, whereas no inhibitory role could be ascribed to FcγRIIb in human macrophages.

**Materials and Methods**

**Human samples.** Heparinized blood samples were obtained from healthy volunteers. The study was approved by the Medical Ethics Committee of the Academic Medical Center and was performed in accordance with the Declaration of Helsinki.

**Culture of monocyte-derived macrophages.** Monocytes were isolated from peripheral blood mononuclear cells with a CD14 MACS isolation kit (Miltenyi Biotec). Cells were cultured in 6-well (1 x 10\textsuperscript{6} cells/well) or 24-well (0.2 x 10\textsuperscript{6} cells/well) plates in complete Iscove modified Dulbecco medium (Gibco) containing 10% fetal calf serum (Bodinco, Alkmaar, The Netherlands) and antibiotics, stimulated with either 50 ng/ml macrophage colony-stimulating factor (M-CSF; eBioscience) or 10 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF; Peprotech) for 9 days.

**Phagocytosis assay.** Erythrocytes of donors positive for the RhD blood group were isolated and stained with cell tracker carboxyfluorescein diacetate succinimidyl ester (CFSE; Life technologies)
Figure 2. Phagocytosis of anti-RhD opsonized erythrocytes by monocyte-derived macrophages

**a)** RhD-positive erythrocytes opsonized with anti-RhD after phagocytosis by monocyte-derived macrophages
cultured for 9 days, before (left) and after (right) lysis of erythrocytes. b) Representative images of the quantification of phagocytosis by flow cytometry. Unopsonized or anti-RhD opsonized CFSE-labeled RhD-positive erythrocytes were incubated with macrophages, in the presence or absence of cytochalasin B. Percentages indicated the percentage CFSE-positive macrophages relative to the total macrophage population. Data are representative of at least 6 (cytochalasin B) experiments. c) Time course of phagocytosis of anti-RhD opsonized erythrocytes by M-CSF and GM-CSF macrophages. Data are representative of 3 independent experiments, error bars represent standard error of the mean. d) Comparison of IgG-mediated phagocytosis (corrected for unopsonized phagocytosis) for M-CSF and GM-CSF macrophages (note different timepoints). Mean and dots represent experiments with macrophages of different individuals (n=58 for M-CSF, n=55 for GM-CSF). Some individuals were measured twice; the mean of the measurements is shown for these individuals. Statistics with Mann-Whitney test.

Preparation of monomeric and dimeric IgG. Normal IVIg was Nanogam 50 mg/ml (Sanquin, The Netherlands). Nanogam was fractionated into different size components in the following way: 2 ml Nanogam (containing 50 mg/ml polyclonal IgG) was fractionated with LC-SEC (AKTA system, GE Healthcare, Sweden) using Superdex200 column 16/60 and PBS at pH 7.4 as elution buffer. The fraction corresponding to dimeric IgG was pooled from several runs.

Dimeric idiotype-anti-idiotype immune complexes were generated starting from equimolar amounts of a human IgG1 monoclonal anti-Adalimumab antibody 2.6 (described previously28) and
adalimumab (Human IgG1, AbbVie). The composition of each preparation was analyzed by injecting 50 μl of 30 mg/ml (Nanogam), 3 mg/ml (dimer-enriched IVIg), or 100 μg/ml (Adalimumab/anti-adalimumab complexes) into LC_SEC using superdex200 column 10/30 with PBS at pH 7.4 as elution buffer.

**IgG preparations with altered levels of galactosylation and sialylation.** Fractions of IVIg enriched or depleted for sialic acid by Sambucus nigra agglutinin (SNA) were prepared as described previously\(^25\). Neuraminidase treatment of IVIg was performed essentially as described previously\(^{19,26}\) (see Supplementary Methods for details). Recombinant IgG was enriched for sialic acid-Fc by cotransfection of HEK-293F Freestyle cells with human IgG1 anti-2,4,6-trinitrophenol (TNP) and galactosyl- and sialyltransferases as described in detail in the Supplementary Methods.

**Flow cytometry, mass spectrometry, genotyping and quantitative mRNA analysis.** Flow cytometry for surface expression on monocyte-derived macrophages, mass spectrometry for IgG-Fc glycosylation, genotyping of healthy individuals for FCGR genetic variation and quantitative messenger RNA analysis of IVIg-stimulated macrophages were performed as described in the Supplementary Methods.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 6.02. For comparison of expression levels and the level of IgG-mediated phagocytosis a Kruskal-Wallis test or Mann-Whitney test was used. For comparison of blocking studies, analysis of variance (ANOVA) was used. When this revealed a P value lower than 0.05 (Bonferroni corrected per figure, uncorrected P values are shown), subsequent testing was performed with unpaired t-tests.

**Results**

**FcγRI, FcγRIIa, FcγRIIb and FcγRIII are expressed on M-CSF and GM-CSF cultured macrophages**

Culturing of peripheral blood monocytes with M-CSF is known to induce differentiation towards a general anti-inflammatory (M2) macrophage phenotype, whereas GM-CSF induces macrophages with a pro-inflammatory (M1) phenotype\(^{29}\) (**Supplementary Fig. 1**). When analyzing the expression pattern of the different FcγRs after differentiation with these stimulants for 9 days, we detected FcγRI, FcγRIIa, FcγRIIb and FcγRIII on the cell surface (**Fig. 1a-e**). Staining with MoAb AT10, recognizing FcγRIIa but also FcγRIIb and FcγRIIc, resulted in the highest fluorescence intensities, especially in the M-CSF macrophages (**Fig. 1c**). To evaluate expression of FcγRIIb, we stained with MoAb 2B6, which actually binds the identical extracellular domains of both FcγRIIb and FcγRIIC\(^{30}\). To ensure specificity for FcγRIIb, we excluded individuals carrying the open reading frame allele of FCGR2C from this analysis. FcγRIIb expression was indistinguishable for M-CSF and GM-CSF macrophages (**Fig. 1d**), indicating that the observed increased FcγRII expression originated from FcγRIIa. Whereas FcγRI and FcγRII isoforms were always present on the whole population of macrophages, FcγRIIIa showed a bimodal distribution (**Fig. 1a**), with
very low or absent expression on the majority of cells, but higher expression on a subset of the macrophages (Fig. 1f). A time course of the expression levels during differentiation into macrophages revealed that FcγRI was re-expressed on the macrophages after an initial decrease compared with levels on monocytes, whereas expression levels of the other FcγRs steadily increased (Supplementary Fig. 2).

**Macrophages phagocytose erythrocytes opsonized by human anti-RhD IgG**

To assess the capacity of the macrophages to phagocytose IgG-opsonized blood cells we used a phagocytosis assay with RhD-positive human erythrocytes opsonized with polyclonal human anti-RhD antibodies as the phagocytic target. Incubation of IgG-opsonized erythrocytes with the macrophages at 37 °C led to binding and phagocytosis (Fig. 2a). To distinguish binding from phagocytosis, noningested extracellular erythrocytes were lysed (Fig. 2a). This efficiently lysed all noningested erythrocytes, as no erythrocytes could be detected after lysis in macrophages pretreated with cytochalasin B, which inhibits phagocytosis by interfering with actin polymerization (Fig. 2b). Phagocytosis was quantified by CFSE-labeling of the erythrocytes and detection of macrophages that have taken up 1 or more erythrocytes by flow cytometry (Fig. 2b). A time course showed that phagocytosis was faster and more extensive in M-CSF macrophages than in GM-CSF macrophages (Fig. 2c). Overnight incubation did not increase the percentage positive macrophages in GM-CSF

![Figure 3](image-url)  
**Figure 3. M-CSF macrophages phagocytose mainly through FcγRIIa, whereas GM-CSF macrophages phagocytose mainly through FcγRI**

Effect of FcγR receptor blocking F(ab′)2 (α-FcγRI, α-FcγRIIa,b, α-FcγRIIib, α-FcγRIII) or Fab (α-FcγRIIb) fragments on the phagocytosis of anti-RhD IgG-opsonized erythrocytes. Data are normalized against phagocytosis of unblocked macrophages. For experiments with Fab α-FcγRIIb individuals with an FCGR2C-open reading frame are excluded. Data represent means and standard error of the mean from 5 to 12 independent experiments. P values indicate results of 1-way ANOVA, subsequent unpaired t-tests were performed with results depicted as follows: * P < 0.05, ** P < 0.01, *** P < 0.001 (Bonferroni corrected P values).
cultures (data not shown). Incubation times of 20 minutes (M-CSF) and 2 hours (GM-CSF) were chosen as optimal times for read-out and were used in all subsequent experiments. An overview of the range of phagocytosis at these points is given in Fig. 2d. The extent of phagocytosis was not significantly correlated to FcγR expression levels (data not shown) or to single nucleotide polymorphisms in the low-affinity FcγRs (Supplementary Fig. 3).

Differential role of FcγRs in M-CSF and GM-CSF macrophages

To determine the relative contribution of the different FcγRs to the phagocytosis of IgG-opsonized erythrocytes, we used FcγR-specific blocking F(ab')2 fragments. The combination of F(ab')2 fragments against all FcγRs induced an almost complete inhibition of phagocytosis, especially in GM-CSF macrophages (Fig. 3). The blocking F(ab')2 against FcγRII in M-CSF macrophages was strongest, whereas F(ab')2 against FcγRI had the most effect in inhibiting phagocytosis with GM-CSF macrophages. Blocking Fab fragments of
MoAb 2B6 (against FcγRIIb) and F(ab’)_2 fragments against FcγRIII had only a minor effect in both types of macrophages. Whereas the MoAbs against FcγRII (clone 7.3), FcγRIIb (clone 2B6) and FcγRIII (clone 3G8) that we used recognize the IgG binding-site of the FcγRs and block IgG binding as F(ab’)_2 fragments\textsuperscript{31-33}, blocking experiments with F(ab’)_2 fragments of MoAb 10.1 were more difficult to interpret because the epitope of FcγRI recognized by this antibody is not located at the IgG-binding site itself, although close enough to interfere with binding\textsuperscript{34}. To compare the blocking capacity of 10.1 F(ab’)_2 fragments to intact 10.1, we performed a rosetting assay with FCGR1A1-transfected 293T cells (Supplementary Fig. 4a-c). This revealed that intact 10.1 was able to completely inhibit binding of IgG-opsonized erythrocytes to FcγRI at the concentration used in our phagocytosis assay, but F(ab’)_2 fragments could not inhibit binding completely, even at high concentrations. In fact, when intact 10.1 was used in the phagocytosis assay, inhibition was much more striking in both types of macrophages (Supplementary Fig. 4d), and phagocytosis could be completely inhibited with a combination of intact antibodies against all 3 FcγRs. However, with the use of intact antibodies, blocking of other FcγRs through the Fc fragment (Kurlander phenomenon\textsuperscript{35}) cannot be excluded.

**IVIg directly inhibits phagocytosis of IgG-opsonized erythrocytes**

IVIg has previously been shown to be able to inhibit macrophage phagocytosis of IgG-opsonized erythrocytes in experimental setups in which macrophages were incubated for 1 hour with IVIg, after which the IVIg was washed away before the start of phagocytosis\textsuperscript{14,36}. To test whether IVIg also had the capacity to directly inhibit IgG-mediated phagocytosis in our in vitro system, we added IVIg to our macrophages just before starting the phagocytosis. IVIg inhibited phagocytosis in a dose-dependent manner, with doses as low as 1 μg/ml still showing some effect in both types of macrophages (Fig. 4a). IVIg was equally effective when added 2 days before the phagocytosis was initiated (Fig. 4b).

**Inhibition of phagocytosis by IVIg is independent of Fc-sialylation and does not require an increase of FcγRIIb expression**

The inhibitory effect of sialylated IVIg was first reported using an enrichment by Sambucus nigra agglutinin (SNA) lectin fractionation\textsuperscript{19}. When we used preparations that were enriched or depleted for sialic acid in this way, we could not detect any differences in capacity to inhibit phagocytosis (Fig. 5a). However, enrichment of IVIg for sialic acid by SNA lectin fractionation only results in an enrichment of Fab-sialylated, but not Fc-sialylated, IgG\textsuperscript{25,37}. Because the presumed effects of sialylated IgG would be Fc sialic acid specific\textsuperscript{21}, we subsequently used neuraminidase-treated IVIg to determine whether a preparation depleted of Fc sialic acid was still capable of inhibiting phagocytosis. Despite the fact that
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**Figures:**

- **Figure 5a:**
  - M-CSF macrophages
  - GM-CSF macrophages
  - IVIg (µg/ml) vs. positive macrophages (% of anti-D control)
  - Data points and error bars indicate p-values of 0.20 for both M-CSF and GM-CSF macrophages.

- **Figure 5b:**
  - M-CSF macrophages
  - GM-CSF macrophages
  - IVIg (µg/ml) vs. positive macrophages (% of anti-D control)
  - Data points and error bars indicate p-values of 0.09 for M-CSF and 0.71 for GM-CSF macrophages.

- **Figure 5c:**
  - IgG1 Fc Sialylation -
  - IgG1 Fc Sialylation +
  - Sialic acid Fc: 0.4%
  - Sialic acid Fc: 14.5%
  - Mass spectrometry data showing peaks at different m/z values.

- **Figure 5d:**
  - M-CSF macrophages
  - GM-CSF macrophages
  - IVIg (µg/ml) vs. positive macrophages (% of anti-D control)
  - Data points and error bars indicate p-values of 0.78 for M-CSF and 0.12 for GM-CSF macrophages.
neuraminidase removed almost 85% of the sialic acid residues at the N297-linked glycan, the neuraminidase-treated IVIg was equally as effective as untreated IVIg (Fig. 5b). Finally, we tried an IgG preparation enriched or depleted for sialic acid Fc by using a non-Fab-glycosylated recombinant anti-2,4,6-trinitrophenyl MoAb, enriched for Fc sialylation by expressing the antibody with or without galactosyltransferases and sialyltransferases. This resulted in an α2,6-sialic acid content of 14.5% in the Fc-sialylated IgG and 0.4% in non Fc-sialylated IgG (Fig. 5c). These were equally capable of inhibiting phagocytosis, showing that the inhibition of phagocytosis was completely independent of IgG-Fc sialylation status (Fig. 5d).

To determine whether the observed inhibitory effects were the result of an upregulation of FcγRIIb, as previously proposed, we determined FcγRIIb expression levels by staining macrophages preincubated with IVIg for 2 days with MoAb 2B6. This revealed no increase in expression levels of FcγRIIb in either M-CSF or GM-CSF macrophages (Fig. 6a). To rule out the possibility that we could not detect an increased expression of FcγRIIb because of competition of the IgG in IVIg for the binding site of MoAb 2B6, we also measured mRNA levels of FCGR2B in macrophages incubated with IVIg. This revealed no increase in FCGR2B mRNA (Fig. 6b). Together, these results indicate that IVIg could inhibit phagocytosis directly, independent of changes in expression of FcγRIIb.

Dimeric IgG most effectively blocks phagocytosis of IgG-opsonized erythrocytes

We then tested whether preparations of IVIg rich in dimeric IgG, with a higher avidity for FcγRs, were more capable of inhibiting phagocytosis. We first prepared a fraction of IVIg enriched for IgG dimers by HPLC fractionation of IVIg, which led to an increase of dimeric IgG from ~5% to ~63% (Fig. 7a). This fraction was even more effective than normal IVIg in inhibiting phagocytosis in the M-CSF, but not the GM-CSF macrophages (Fig. 7b). Subsequently, to test IgG preparations with even higher contents of dimeric IgG, we made use of the ability of a human anti-idiotype MoAb against the TNF-blocking MoAb adalimumab.
Monoclonal fractions of these two antibodies consist of IgG monomers, but a combination of the two results in the formation of dimers, as well as some multimers (Fig. 7c). When using either of the 2 monoclonal antibodies alone, phagocytosis was still inhibited to the same extent as inhibition with normal IVIg, showing that purely monomeric IgG also has an inhibiting effect on phagocytosis. However, the combination of the 2 antibodies, having formed dimers, was much more effective than either of the 2 monoclonal antibodies alone, even in the GM-CSF macrophages (Fig. 7d).
Discussion

Our data demonstrate that the inhibiting effect of IVIg on the phagocytosis of IgG-opsonized blood cells\textsuperscript{14,36} is a very direct effect in our fully human \textit{in vitro} system, as IVIg was effective after a very short incubation time of only 5 minutes. The addition of IVIg to cultured macrophages resulted in a dose-dependent inhibition of phagocytosis of IgG-opsonized erythrocytes, which could be enhanced with preparations containing more IgG dimers and multimers, but was independent of IgG-Fc and IgG-Fab sialylation. Because IgG was effective after a very short incubation, we presume that the most likely working mechanism is a direct blockade of FcγRs.

After the discovery that IVIg induces a steep increase in the circulating platelet counts in ITP\textsuperscript{7}, many theories regarding the working mechanism have been proposed, including inhibition of phagocytosis as a result of blockade of activating FcγRs. Despite the fact that many observations support this mechanism\textsuperscript{1,12,38}, the current paradigm is that IVIg induces upregulation of inhibitory FcγRIIb on effector macrophages\textsuperscript{18,24}, although this was only shown in mice. However, we did not detect any upregulation of this receptor even after 2 days of incubation with IVIg in our macrophages, clearly showing that IVIg has very potent effects that are independent of an increased expression of FcγRIIb. Although we cannot completely rule out upregulation of FcγRIIb in response to IVIg \textit{in vivo}, it was recently postulated that this receptor may not actually be required for the protective effect of IVIg in murine models in all circumstances\textsuperscript{39}. In any case, our findings do not support any role for FcγRIIb in the working mechanism of IVIg in preventing clearance of opsonized blood cells by human macrophages. Neither could we detect a direct effect of sialylation of the N-linked Fc-glycan at position 297 on the clearance of IgG-opsonized blood cells. Our findings, in conjunction with the conflicting results obtained in murine studies of ITP comparing sialic acid-enriched versus sialic acid-depleted IVIg\textsuperscript{22,25,26}, do not support a role for the sialylation of IgG-Fc in the working mechanism of IVIg preventing uptake of IgG-opsonized blood cells.

Being such a potent direct inhibitor of FcγR-mediated phagocytosis \textit{in vitro}, we assume that IVIg will also be able to block this process in macrophages \textit{in vivo}. The notion that FcγRs can be blocked by IgG \textit{in vivo} may seem surprising given that IgG is normally present in the circulation at ~7–16 mg/ml, which would be expected to block FcγRs \textit{in vivo} already under steady state conditions. Monomeric IgG has been shown to bind FcγRIIa\textsuperscript{40}. Therefore, monomeric IgG will probably form an equilibrium with low-affinity FcγRs \textit{in vivo}, with a proportion of the low-affinity FcγRs bound by IgG and a proportion freely available. The administration of IVIg could shift this balance towards a higher proportion of FcγRs being occupied, leaving too little FcγRs freely available for binding to IgG-opsonized blood cells. IgG dimers and polymers in IVIg will even more efficiently block the low-affinity FcγRs, as they have a higher avidity.

We indeed found an increased effect of IgG dimers and multimers in our phagocytosis...
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**Fig. 7**

(a) 

- **normal IVIg**
- IgG Dimers (5%)
- IgG Monomers (95%)

(b) 

- M-CSF macrophages
- IVIg
- Dimer-enriched IVIg
- p<0.0002*
- p<0.01**
- p<0.01**

(c) 

- IgG Dimers (63%) 
- IgG Monomers (37%)

(d) 

- Adalimumab
- anti-Adalimumab
- IgG Monomers (98.3%)
- IgG Dimers (1.7%)

- M-CSF macrophages
- Adalimumab monomer
- anti-adalimumab monomer
- Adalimumab-anti-adalimumab complexes
- p=0.008*
- p<0.001**
- p<0.0001*
- p<0.05**
studies, although the presence was not absolutely necessary, and in M1 macrophages it could only be detected with preparations with a high IgG dimer and multimer content. This differential sensitivity reflects the differences in FcγR usage between M1 and M2 macrophages. Phagocytosis by M-CSF macrophages relies greatly on the low-affinity FcγRII which is much more effectively blocked by IgG dimers than monomers, whereas GM-CSF macrophages rely more exclusively on FcγRI which is already fully occupied by IgG monomers alone. Our experiments with F(ab')2 fragments indicate that FcγRI is relevant to the phagocytosis by monocyte-derived macrophages, especially when cultured with GM-CSF. The effects with the F(ab')2 fragments will be an underrepresentation of the actual role of FcγRI, as MoAb 10.1 (nor any other available MoAb against FcγRI) does not block efficiently as a F(ab')2 fragment. However, the effects of the intact 10.1 MoAb may, rather, be an overrepresentation of the role of FcγRI, as the Fc-tail can block other adjacent FcγRs, as described by Kurlander35.

Macrophages of the spleen and liver are responsible for the clearance of IgG-opsonized blood cells in vivo, but remarkably little is known about expression levels of FcγRs on these macrophages. It has been proposed that mainly the low-affinity FcγRs are involved4, based on circumstantial evidence such as association studies with genetic polymorphisms in the low-affinity FcγRs5,41 and in vivo blocking studies with specific FcγR MoAbs in limited series of patients42,43. If it is indeed the case that these macrophages have a predominant expression of low-affinity FcγRs, we may expect the difference between dimeric and monomeric IgG to be even greater than the difference we have found in monocyte-derived macrophages.

In conclusion, the IVlg-induced inhibition of phagocytosis of IgG-opsonized blood cells by human macrophages, in contrast to the current prevailing theory24, is not dependent on Fc sialylation or FcγRIIb upregulation. The lack of support for this theory from our data in human macrophages is in line with multiple recent studies challenging the concept of Fc sialylation and/or FcγRIIb being important for IVlg efficacy in various diseases, as studied in murine models25,26,39,44 or with human material17,45,46. Therefore, the importance of IgG Fc-
sialylation and upregulation of FcγRIIb in IVIg treatment needs to be reconsidered. Instead, the capacity of IgG molecules to bind and block FcγRs seems to be more important, at least when inhibiting phagocytosis of IgG-opsonized blood cells. These findings can help to improve the treatment of ITP or wAIHA with IVIg, as preparations with increased binding to low-affinity FcγRs, such as IgG dimers or recombinant engineered IgGs with an increased affinity of the Fc-tail for FcγR may form suitable alternatives requiring a lower dosage than normal IVIg.

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Author Contributions

Contribution: S.Q.N. performed experiments, analyzed data and wrote the manuscript; G.D., I.K., F.S.v.d.B., J.G. and R.P. performed experiments and analyzed data; M.W., G.V., T.R. and T.K.v.d.B. designed experiments, interpreted and discussed data; T.W.K. discussed data, designed the study and wrote the manuscript.

Conflict-of-interest Disclosure

The authors declare no competing financial interest.
References

17. Tremblay, T., Pare, I. & Bazin, R. Immunoglobulin G dimers and immune complexes are dispensable for the therapeutic efficacy of intravenous immune globulin in murine immune...


Supplementary Figure 1. Differentiation of human monocyte-derived macrophages with GM-CSF leads to macrophages with an M1 (pro-inflammatory) profile, whereas differentiation with M-CSF leads to an M2 (anti-inflammatory) profile.

a) Cytokine secretion by M-CSF and GM-CSF macrophages after stimulation with Lipopolysaccharide (LPS) and LPS-binding protein overnight, showing that GM-CSF macrophages produce the pro-inflammatory cytokine TNFα (left), whereas M-CSF macrophages produce more of the anti-inflammatory cytokine IL-10 (right). Data represent 3 independent experiments, statistics with student’s t test. No TNF or IL10 was detected in non LPS/LBP-stimulated M-CSF or GM-CSF macrophage culture supernatants (data not shown).

b) Flow cytometry with surface staining for CD80 and CD200R on M-CSF and GM-CSF macrophages shows predominant expression of CD80 (M1 marker) on GM-CSF macrophages and predominant expression of CD200R (M2 marker) on M-CSF macrophages. Data represent 3 independent experiments, statistics with student’s t test.
Supplementary Figure 2.
Time course of expression levels of the different FcγRs on monocyte-derived macrophages cultured with M-CSF or GM-CSF. FcγRI – clone 10.1; FcγRIIa,b – clone AT10; FcγRIIb – clone 2B6; FcγRIII – clone 3G8, in this case including both FcγRIII-low and FcγRIII-high cells. Data represent mean and s.e.m. For FcγRI, FcγRIIa,b and FcγRIII: day 0: n=15; day 1: n=12; day 3 n=16; day 7 n=16; day 9 n=34. For FcγRIIb (including only donors without an FCGR2C-ORF): Day 0 n=8; day 1 n= 6; day 3 n=9; day 7 n=10; day 9 n=22.
Chapter 4. IVIg fractions Supl. Fig. 3

a) M-CSF macrophages

Phagocytosis (% positive macrophages)

FCGR2C-stop
FCGR2C-ORF (1 copy)
FCGR2C-ORF (2 copies)

p = 0.43**

b) M-CSF macrophages

Phagocytosis (% positive macrophages)

FCGR2B-232 II
FCGR2B-232 IT
FCGR2B-232 TT

p = 0.44*

c) M-CSF macrophages

Phagocytosis (% positive macrophages)

FCGR2A-131 HH
FCGR2A-131HR
FCGR2A-131R

p = 0.23*

d) M-CSF macrophages

Phagocytosis (% positive macrophages)

FCGR3A-158 VV
FCGR3A-158 VF
FCGR3A-158 FF

p = 0.57*

GM-CSF macrophages

Phagocytosis (% positive macrophages)

GM-CSF macrophages

p = 0.44**

GM-CSF macrophages

p = 0.44**

GM-CSF macrophages

p = 0.44**

GM-CSF macrophages

p = 0.44**

GM-CSF macrophages

p = 0.14*

GM-CSF macrophages

p = 0.23*

GM-CSF macrophages

p = 0.57*

GM-CSF macrophages

p = 0.93*
Supplementary Figure 3. Single nucleotide polymorphisms in the genes encoding FcγRII and FcγRIII isoforms are not associated with the extent of phagocytosis

Comparison of phagocytosis (expressed as percentage positive macrophages corrected for phagocytosis of unopsonized cells) by monocyte-derived macrophages cultured with M-CSF (left) or GM-CSF (right) from donors with a) FCGR2C-stop vs FCGR2C-ORF genotypes, b) FCGR2B-232I/I vs FCGR2B-232I/T vs FCGR2B-232T/T genotypes, c) FCGR2A-131H/H vs FCGR2A-131H/R vs FCGR2A-232R/R genotypes and d) FCGR3A-158V/V vs FCGR3A-158V/F vs FCGR3A-158F/F genotypes. Some individuals were measured twice, means of the two measurements are shown for these. * Kruskal Wallis test ** Mann-Whitney test.
Supplementary Figure 4. F(ab')2 fragments of MoAb 10.1 are not capable of blocking binding of IgG to FcγRI, whereas the intact MoAb completely blocks this binding

Rosetting assay with 293T cells (having no endogenous FcyR expression) transfected with an FCGR1A1 construct, resulting in expression of FcγRI on the cell surface, or mock transfection, and RhD positive erythrocytes unopsonized or opsonized with human anti-RhD IgG. a) Rosetting occurs when IgG-opsonized erythrocytes are incubated with FCGR1A1 transfected 293T cells, but not when erythrocytes are not opsonized or 293T cells are mock transfected. b) Inhibition of rosetting of IgG opsonized red cells to FCGR1A1 transfected 293T cells by different concentrations of intact MoAb 10.1. c) Inhibition of rosetting of IgG-opsonized red cells to FCGR1A1 transfected 293T cells by different concentrations of F(ab')2 fragments of MoAb 10.1. d) Different effects of blocking F(ab')2, fragments and MoAbs of 10.1 on the phagocytosis of anti-RhD IgG-opsonized erythrocytes (experiments as in Figure 3 of the main article). Data are shown as percentage relative to the percentage positive macrophages of the unblocked phagocytosis of anti-RhD IgG-opsonized erythrocytes. Mean and s.e.m. are shown for each blocking F(ab')2 fragment and intact MoAb and summarize at least 6 experiments.
**Supplementary methods**

**Flow cytometry.** Macrophages were detached from the surface of culture plates after a 15 minute incubation with a PBS buffer containing 125 mM lidocaine (Sigma Aldrich) and 10 mM EDTA (Merck). Cells were costained in a buffer containing 2.5 mM CaCl₂ for 20 minutes on ice, with Annexin-V APC (BD biosciences), mouse anti-human CD14-Pacific Blue (BD Pharmingen) and a MoAb recognizing human FcγR. The following MoAbs were used: mouse anti-human CD64-FITC clone 10.1 (BD Pharmingen), mouse anti-human CD32-FITC clone AT10 (AbD Serotec), mouse/human anti-human CD32b,c-AF488 clone 2B6 (a generous gift from Macrogenics), mouse anti-human CD16-FITC clone 3G8 (BD Pharmingen), mouse anti human CD80-FITC (BD Pharmingen), mouse anti human CD200R-AF647 (AbD Serotec). Cells were measured on a FACS CANTO II (BD). Median fluorescence intensity of the Annexin Vneg, CD14pos population was calculated after subtraction of the median fluorescence intensity of the proper isotype control, giving the Δ MFI.

**IgG preparations with altered levels of galactosylation and sialylation.** Neuraminidase treatment was performed essentially as described by Kaneko et al.¹. Nanogam was dialyzed against a 50 mM sodium citrate buffer (pH 6.0) and incubated for 20 hours at 37 °C with 700 Units/ml of neuraminidase (20 Units/mg IgG) (neuraminidase = acetyl-neuraminyl hydrolase from Clostridium perfringens, New England BioLabs). Nanogam in the same buffer incubated for 20 hours at 37 °C without neuraminidase was used as control IVIg for these experiments.

For recombinant human IgG, the variable regions of the heavy and light chains (VH, VL) of the mouse IgG1 anti-2,4,6-trinitrophenol (TNP) hapten antibodies were cloned onto human IgG1 and κ backbones, respectively, as described previously²,³ and produced in the HEK-293F FreeStyle cell line expression system (Life Technologies, Paisley, UK) with co-transfection of vectors encoding p21, p27 and pSVLT genes as described⁴ to increase protein production. Codon-optimized genes encoding Beta-1,4-galactosyltransferase 2 (B4GALT2) and Beta-galactoside alpha-2,6-sialyltransferase 1 (ST6GALT1) including 5′-HindIII and 3′-EcoRI restriction sites and Kozak sequence, were designed and ordered from Geneart® (Life Technologies). The HindIII-EcoRI fragment for human ST6GALT1 was ligated into pEE14.4 (Lonza), and the HindIII-EcoRI fragment for human B4GALT2 was ligated into pEE6.4 (Lonza), and co-expressed with the IgG as described above to generate increased galactosylation and sialylation antibodies. Antibodies were purified on a proteinA (WTIgG1) HiTrapHP column (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) using the Acta Prime Plus system (GE Healthcare Life Sciences) and dialyzed against PBS overnight. IgG-Fc glycosylation was determined by mass spectrometry.

**Mass Spectrometry.** The glycosylation profile of the antibodies was determined by performing proteolytic digesting with trypsin (sequencing grade modified trypsin, Promega, Madison, WI) and analyzing the resulting glycopeptides using nanoLC-ESI-QTOF-MS, as described previously⁵. On the basis of the normalized intensities of IgG1-Fc glycopeptides the levels of sialylation was calculated according to the following formula: Sialylation = (G1FS + G2FS + G1FNS + H5N3S1_1 + H6N3S1_1 + H4N3S1F1_1 + H5N3S1F1_1 + H6N3S1F1_1) * 0.5 + G2FS2.

**Genotyping.** Copy number variation and single nucleotide polymorphisms (SNPs) in FCGR2A,
FCGR2B, FCGR2C, FCGR3A, and FCGR3B were determined on genomic DNA with an FCGR-specific multiplex ligation-dependent probe amplification (MLPA) assay (MRC-Holland, Amsterdam, The Netherlands) as described previously.

Quantitative mRNA analysis. mRNA was isolated from 2 × 10⁶ macrophages with the QIAamp® RNA blood mini kit (Qiagen, Hilden, Germany). cDNA synthesis and quantification of GUS and FCGR2B2 expression levels were performed as described previously. cDNA from M-CSF macrophages was used as a standard curve, with serial 10-fold dilutions of this cDNA quantified with the method described in Technical Note No. LC 13/2001 (Roche Applied Science). Expression levels were compared to this standard curve.

Macrophage stimulation assay and ELISA. Macrophages were incubated overnight with 20 ng/ml LPS from E. coli O55:B5 (Sigma) and 50 ng/ml recombinant human LPS binding protein (R&D systems). Culture supernatants were harvested and TNFα and IL-10 concentration was determined by ELISA (PeliPair™, Sanquin Reagents, Amsterdam, The Netherlands) according to the manufacturer’s protocol.
Supplementary references


