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IgG subclasses shape cytokine responses by human myeloid immune cells through differential metabolic reprogramming

Running title: **IgG subclasses shape cytokine responses**

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Abstract

IgG antibodies are crucial for various immune functions, including neutralization, phagocytosis, and ADCC. Here, we identified another function of IgG, by showing that IgG immune complexes elicit distinct cytokine profiles by human myeloid immune cells, which is dependent on Fc gamma receptor (FcγR) activation by the different IgG subclasses. Using monoclonal IgG subclasses with identical antigen specificity, our data demonstrate that the production of Th17-inducing cytokines such as TNF, IL-1β, and IL-23 is particularly dependent on IgG2, while type I IFN responses are controlled by IgG3, and IgG1 is able to regulate both. In addition, we identified that subclass-specific cytokine production is orchestrated at the post-transcriptional level through distinct glycolytic reprogramming of human myeloid immune cells. Combined, these data identify that IgG subclasses provide pathogen- and cell type-specific immunity through differential metabolic reprogramming by FcγRs. These findings may be relevant for future design of antibody-related therapies in the context of infectious diseases, chronic inflammation, and cancer.

Keypoints:

- Human IgG subclasses elicit distinct cytokine profiles by myeloid immune cells
- Subclass-specific effects are mediated by distinct metabolic reprogramming by FcyRs
- IgG subclasses provide pathogen- and cell type-specific immunity

Introduction

Antibodies of the IgG isotype are one of the most abundant proteins in human serum. IgG antibodies are crucial for various immune function such as phagocytosis, degranulation, complement activation, and antibody-dependent cellular cytotoxicity (ADCC) (1). Many of these effector functions are dependent on their interaction with Fc gamma receptors (FcγRs), a receptor for the Fc region of IgG (2). IgG antibodies can be further subdivided in four subclasses, IgG1, IgG2, IgG3, and IgG4, which are named in order of decreasing abundance in human serum (3). The production of different IgG subclasses is skewed depending on the type of antigen involved. For example, responses to bacterial capsular polysaccharide antigens are mostly restricted to IgG2 (4-7). In contrast, viral infections are generally characterized by antibodies of the IgG1 and IgG3 subclass, with IgG3 appearing first during the course of infection (4, 8). In addition, the four IgG subclasses display a different capacity to induce effector responses. In general, IgG3 is considered to have the most pronounced immune-activating effects (1, 9, 10), which is based on the potent ability of IgG3 to induce processes such as phagocytosis, complement activation, and ADCC (3). This pro-inflammatory reputation of IgG3 is strengthened by its short half-life compared to the other IgG subclasses, which may function to limit excessive inflammatory responses ignited by IgG3 (3).

While the different capacities of IgG subclasses to induce effector responses such as phagocytosis and complement activation have been well studied, still little is known about IgG subclass-specific differences in induction of cytokine production, another essential effector function of IgG antibodies. Although initially underappreciated, it has become clear that, particularly in humans, IgG plays a critical role in orchestrating inflammation by controlling the production of key pro-inflammatory cytokines through activation of FcγRs (11-14). This IgG-induced up- and down-regulation of cytokines serves an important function in host defense by tailoring immune responses to the encountered pathogen. For example, IgG opsonization of bacteria strongly amplifies the production of cytokines by dendritic cells (DCs) that promote T helper 17 (Th17) responses, which is required to efficiently counteract bacterial infections (12, 15). Alternatively, IgG opsonization of viruses selectively decreases the production type I and type III interferons (IFNs) by DCs, which may provide a negative feedback mechanism to prevent

collateral damage and promote anti-viral CD8 T cell responses (16, 17). However, undesired activation of IgG-induced cytokine production, e.g. by auto-antibodies, also drives excessive inflammation, as observed in chronic inflammatory disorders such as rheumatoid arthritis (RA) (11, 14) and systemic lupus erythematosus (SLE) (18-20).

IgG antibodies can elicit cytokine production by a variety of (human) cell types in different tissues, ranging from immune cells such as DCs and macrophages to non-immune cells such as nasal and lung epithelium (21). Although FcγRIIa is the main cytokine-inducing receptor on myeloid immune cells such as DCs (12, 15), also other members of the FcγR family have been described to elicit cytokine production, including FcγRI (14) and FcγRIII (21). Importantly, the recognition of (unbound) IgG by FcγRs in itself is not sufficient to induce cytokine production. Instead, this only occurs after two additional steps. First, IgG antibodies need to bind to their antigen, e.g. pathogens or autoantigens, resulting in the formation of IgG immune complexes (IgG-IC), which activate FcγRs by binding with increased avidity to provide FcγR-cross linking and subsequent signaling (22). Second, FcγRs induce very little cytokine production upon individual stimulation, and have to collaborate with other receptors to amplify or inhibit the production of specific cytokines (13). FcγRs can engage in 'cross-talk' with a variety of different receptors, which include pathogen-sensing receptors such Toll-like receptors (TLRs), NOD-like receptors (NLRs), and cytosolic DNA sensors (CDS) (12, 16), but also cytokine receptors such as IL-1R and IFNγR (23). Notably, the ultimate cytokine cocktail that is induced by IgG-ICs is not uniform, but instead is shaped by the immunological context, e.g. by the pathogen involved or the cell type on which FcγRs are triggered. However, how IgG antibodies are able to generate these pathogen- and cell type-specific responses is still largely unknown.

In this study, we set out to identify the relative role of IgG subclasses in their capacity to induce cytokine production. Strikingly, we identified that the production of pro-inflammatory cytokines such as TNF, IL-1β, and IL-23 by human myeloid immune cells is dependent on IgG1 and particularly IgG2, while IgG3 had very little effect. In contrast, the suppression of type I IFN was mainly induced by IgG1 and IgG3, indicating that IgG induces subclass-specific cytokine profiles. We further demonstrate that the cytokine profile induced by IgG subclasses differs between myeloid cells, indicating IgG subclasses induce cell type-specific immunity. Moreover, we identified that IgG subclass-specific cytokine

responses are not regulated at the level of gene transcription or inflammasome activation, but result from distinct glycolytic reprogramming of myeloid immune cells. Combined, these data identified that human IgG subclasses tailor cytokine responses to the immunological context through differential metabolic reprogramming by FcγRs.

Material and Methods

Cells

Cells were isolated from buffy coats (Sanquin Blood Supply). Buffy coats obtained after blood donation are not subjected to informed consent, which is according to the Medical Research Involving Human Subjects Act and AMC medical Ethics Review Committee. All samples were handled anonymously. Ethical review and approval was not required for this study in accordance with the local legislations. Dendritic cells and macrophages were obtained by isolating monocytes from buffy coats, using density gradient centrifugation on Lymphoprep (Nycomed) and Percoll (Pharmacia). Then cells were generated by culturing for 6 days in IMDM (Lonza) containing 5% FBS (Biowest) and 86µg/ml gentamicin (Gibco) with supplemented cytokines. At day 2 or 3 half of the medium was replaced for new medium and cytokines. DCs were differentiated with 20ng/ml GM-CSF (Invitrogen) and 2ng/ml IL-4 (Miltenyi Biotec) and macrophages with 50ng/ml recombinant M-CSF (ImmunoTools). Monocytes were directly isolated from PBMCs, after Lymphoprep, by MACS isolation using CD14 microbeads (Miltenyi Biotec) and stimulated directly afterwards. For silencing cells were harvested at day 3 and microporated with 500nM IRF5 si-RNA or control si-RNA (Dharmacon) and cultured for 3 more days in the indicated cultured medium except gentamycin.

Antibodies

For the opsonization of *S.pneumoniae*, IgG-subclass antibodies against polysaccharide 6A were used, produced as described before (24, 25). The different IgG subclasses had no effect on the opsonization capacity of *S.pneumoniae.* For the other experiments, IgG was coated on plates to mimic IgG immune complexes. IgG subclasses 1-4 and allotypes (IGHG3*01/*04/*16/*18) with either an specificity for anti-RhD or anti-TNP were used. Anti-RhD and anti-TNP antibodies showed similar observations and were described before (26).

Stimulation

DCs and macrophages were harvested at day 6 by respectively using ice for 30 minutes or TrypLE Select (Invitrogen). 2µg/ml IgG subclasses or IgG allotypes were coated on a 96-well high-affinity plate (Nunc) or a 48-wells plate (Costar). High affinity plates were blocked with 10% FBS in PBS at 37°C, 1h prior to use. Cells were stimulated with 10µg/ml Pam3CSK4 (Invivogen) or 20µg/ml Poly I:C (Sigma-Aldrich). To block glycolysis, 10mM 2-Deoxy-D-glucose (2DG, Sigma Aldrich) was added to the cells simultaneously with the stimuli. For the bacteria experiment, *S.pneumoniae* were pre-opsonized with 250 µg/ml IgG in PBS for 45 minutes. After washing, bacteria were diluted in X-vivo media supplemented with gentamycin (Gibco) and stimulated with DCs afterwards (MOI 1:10) in a round bottom plate (Costar) for 24h.

ELISA

To determine cytokine production, supernatants were harvested after 24h of stimulation and stored at -20°C. Cytokines levels were measured using antibody pairs for TNF (eBioscience), IL-1β, IL-6, IL-23 (U-CyTech Biosciences), and IL-10 (BD Bioscience). IFN-β was detected using an IFN-β ELISA kit (PBL Assay Science).

Quantitative RT-PCR.

Cells were stimulated and lysed at indicated time points and mRNA was extracted using RNeasy Mini Kit (Qiagen). cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative RT-PCR was performed (StepOnePlus Real-Time PCR System; Applied Biosystems) using the following mix and TaqMan primers (Thermo Fisher Scientific): GAPDH (4310884E), TNF (Hs00174128_m1), IL-1β (Hs00174097_m1), IFN-β (Hs01077958_s1), IFN-λ1 (Hs00601677_g1), and IRF5 (Hs00158114_m1). mRNA levels were normalized to the geometric mean of the Ct-values of housekeeping gene GAPDH [$2^{Ct(housekeeping)-Ct(target)}$], and folds were calculated.

Caspase-1 activation.

DCs (120.000-150.000/well) were stimulated for indicated time points. Caspase-1 activity was determined according to manufactures protocol, using caspase-1 binding compound FAM-YVAD-FMK (FAM-FLICA Caspase-1 Assay Kit; ImmunoChemistry Technologies).

Metabolism assays

Glycolysis in DCs was determined by real-time analysis of the extracellular acidification rate (ECAR)(XF-96e Extracellular Flux Analyzer; Seahorse Bioscience). A XF-96 culture plates was coated with 4ug/ml IgG subclasses and 30,000 DCs were plated per well in unbuffered RPMI-1640 (Sigma), which was filtered through a 0.22 µ filter system (Corning, Amsterdam, The Netherlands), the pH was set to 7.4 using 37% HCl, and was supplement with 5% FCS. Cells were and rested at 37°C in 0% CO_2 for 1 hour. ECAR was monitored in response to glucose (10 mM; port A; Sigma) and 30 minutes later to 10ug/ml Pam3CSK or 20µg/ml Poly I:C (Port B). ECAR values as shown in the graphs were represent the increased in ECAR as determined 45 minutes after injection of port B over the baseline ECAR values before port A injection.

Results

IgG-induced pro-inflammatory cytokine production is dependent on IgG1 and IgG2

An important function of human IgG antibodies is to direct immune responses through the induction of pro-inflammatory cytokines. Here, we set out to identify which of the IgG subclasses are responsible for this antibody-induced inflammation. Previous studies revealed that IgG-induced inflammation critically depends on two events. First, IgG needs to form immune complexes (IgG-IC), as occurs by binding to (auto)antigens, which enables binding to low-affinity FcγRs (27). Second, IgG-IC activation of FcγRs requires concomitant stimulation with other receptors such as TLRs, leading to a strong and synergistic amplification of particular pro-inflammatory cytokines (11-14). To assess the relative role of IgG subclasses we used human monocyte-derived dendritic cells (DCs), which we previously showed have stable FcγR expression with little donor-to-donor differences (12, 23). We stimulated cells for 24h with IgG-IC in combination with TLR2 ligand Pam3CKS4 (Pam3), a bacterial mimic, and determined pro-inflammatory cytokine production in the supernatant. As IgG-IC we used plate-bound IgG, which we have previously optimized to generate a standardized and highly reproducible stimulus that is comparable to IgG-IC generated in other ways, including opsonized bacteria and viruses, coated beads, and heat-aggregated IgG (12, 16).

Strikingly, TLR co-stimulation with IgG1 and IgG2 strongly amplified the production of pro-inflammatory cytokines TNF, IL-1β, and IL-23, while IgG3 had very little effect on these cytokines (representative donor Suppl. Fig. 1A, multiple donors Fig. 1A). Corroborating previous findings, IgG-IC co-stimulation had little effect on IL-6 production (Suppl. Fig. 1A and Fig. 1A). These data demonstrate that IgG-induced pro-inflammatory cytokine production is mainly amplified by IgG1 and IgG2, and to a far lesser extent by IgG3. TLR co-stimulation with IgG4, which generally has lowered interaction with FcγRs (3), did induce TNF production but not to the same extend as IgG1 and IgG2 (Suppl. Fig. 1B).

IgG subclasses can be further divided into different allotypes, of which particularly for IgG3 there are many variations (3). To determine whether the lack of cytokine induction by IgG3 is dependent on

the allotype, we stimulated DCs with four different IgG3 allotypes that have major differences in hinge length (IGHG3*01=IGHG3*16 > IGHG3*18> IGHG3*04) and in their affinity for FcγRIIa (IGHG3*01 = IGHG3*04 > IGHG3*16 > IGHG3*18), the main cytokine-inducing FcγR on human DCs (3). Although we observed some differences between IgG3 allotypes in their capacity to promote pro-inflammatory cytokine production, all IgG3 allotypes induced substantially less TNF compared to IgG1 and IgG2 (Fig. 1B). These data indicate that the capacity to induce pro-inflammatory cytokine is mainly dependent on the IgG subclass, and not the IgG allotypes.

To further confirm that the IgG isotypes differ in their capacity to promote cytokine production in a more physiological manner, we opsonized *S. pneumoniae* bacteria with an identical monoclonal IgG antibody against polysaccharide 6A that only differed in subclass (24, 25). Similar to the IgG-IC subclasses, opsonization of *S. pneumoniae* with IgG1 or IgG2 amplified TNF production, while IgG3 did not (Fig. 1C).

Combined, these data indicate that IgG-induced pro-inflammatory cytokine production is dependent on the isotype, and is mainly induced by IgG1 and IgG2, but not IgG3.

IgG subclasses induce cell type-specific cytokine responses by human myeloid immune cells

In addition to DCs, IgG-IC (generated from purified pooled serum) have been shown to promote cytokine production by various other human myeloid immune cells, including monocytes (23) and macrophages (11, 14). Yet, while the amplification of TNF and IL-1β is universal, other cytokines such as IL-6 and IL-10 are modulated by IgG-IC in a cell type-specific manner. For example, while IgG-IC increase IL-10 production by DCs and macrophages, IgG-IC suppress IL-10 production by human monocytes (23). Therefore, we here set out to determine whether IgG subclasses also contribute to induction of cell type-specific inflammatory responses. Similar to DCs, in human monocytes TNF and IL-1β was mainly amplified by IgG1 and IgG2, while IgG3 had little effect (Suppl. Fig. 1C for representative donor, Fig. 2A for combined data of multiple donors). However, in monocytes we observed that IL-6 production was specifically amplified by IgG3, but not by IgG1 and IgG2 (Suppl. Fig. 1C and Fig. 2A). In turn, the suppression of IL-10 in monocytes was mainly dependent on IgG1 and IgG2 (Suppl. Fig. 1C and Fig. 2A). In macrophages, the cytokine profile induced by the IgG

subclasses was comparable to that in DCs (Suppl. Fig. 1D for representative donor, Fig. 2B for combined data of multiple donors). Combined, these data indicate that IgG subclasses also contribute to the induction of cell type-specific cytokine responses.

Suppression of type I IFNs is dependent on IgG1 and IgG3

While IgG-IC amplify pro-inflammatory cytokines induced by bacteria-sensing pattern recognition receptors (PRRs) such as TLR2, TLR4, and TLR5, IgG-IC (from pooled IgG) has recently been shown to suppress the production of type I and type III IFNs upon co-stimulation with virus-sensing PRRs such as TLR3, RIG-I-like receptors (RLRs), and cytosolic DNA sensors (CDS) (16, 20, 28). We therefore set out to determine whether IgG subclasses also differently control type I IFN suppression. Interestingly, while Poly I:C (TLR3)-induced production of IFN-β by human DCs was suppressed upon co-stimulation with IgG1 and IgG3, co-stimulation with IgG2 had very little effect on IFN-β production (representative example Suppl. Fig. 2A, multiple donors Suppl. Fig. 2B). These data indicate that, in contrast to pro-inflammatory cytokines, the modulation of type I IFN is mainly dependent on IgG1 and IgG3.

IgG subclass-specific cytokine production is not regulated by gene transcription and caspase-1 activation

IgG-IC co-stimulation is known to change immune responses by modulating cytokine production by human myeloid immune cells at three different levels. These are (1) altered cytokine gene transcription (12); (2) activation of caspase-1, which promotes IL-1β production by cleaving pro-IL-1β into its functional form (12, 14); and (3) increased cytokine gene translation which is dependent on glycolytic reprogramming (29, 30). To determine at which level IgG subclasses regulate their distinct cytokine profiles, we first assessed cytokine gene transcription upon co-stimulation with IgG1, IgG2, or IgG3. Notably, all subclasses amplified gene transcription of pro-inflammatory cytokines *TNF* and *IL1B* in a similar manner (Fig. 3A). Similarly, all subclasses also equally suppressed the transcription of *IFNB* and *IFNL* (encoding IFN-λ1) (Fig. 3B). Next, we set out to investigate whether IgG subclasses display a different capacity to activate caspase-1. However, no differences in caspase-1 activation were

observed upon co-stimulation with the different IgG subclasses (Fig. 3C). Combined, these data show that cytokine gene transcription and caspase-1 activation are modulated by IgG subclasses in a similar manner, suggesting that a mechanism other than gene transcription or caspase-1 activation drives the differences in pro-inflammatory cytokine and type I/III IFN production by IgG subclasses.

IgG subclasses induce distinct metabolic reprogramming

Since no differences between IgG subclasses were observed in cytokine gene transcription and caspase-1 activation, we next studied IgG-IC-induced metabolic reprogramming. IgG-IC (from pooled IgG) induces metabolic reprogramming by increasing the glycolytic rate, which is essential for post-translational amplification of (particularly) pro-inflammatory cytokine production (30). To determine whether the subclass-specific cytokine production is dependent on differences in glycolytic reprogramming, we stimulated human DCs and analyzed them for changes in rates of extracellular acidification (ECAR), as a measure of lactate production (a proxy for the glycolytic rate). Strikingly, while co-stimulation of Pam3 with IgG1 and IgG2 amplified the glycolytic rate, co-stimulation with IgG3 had very little effect on glycolysis (Fig. 4A).

Metabolic reprogramming of myeloid immune cells by IgG-IC has been described to be dependent on the transcription factor interferon regulatory factor 5 (IRF5) (30, 31). Yet, since these prior experiments have been performed using pooled IgG that mostly contains IgG1, it is still unclear whether IgG2, which we here identified is the most potent inducer of pro-inflammatory cytokines, also induces metabolic reprogramming through IRF5. We therefore silenced IRF5 (silencing efficiency of 70%) and assessed the glycolytic rate upon stimulation with Pam3 and/or IgG2. As shown in Fig. 4B, co-stimulation with IgG2 strongly increased the glycolysis, which was prevented by silencing of IRF5, indicating that IgG2-induced glycolytic reprogramming is indeed dependent on IRF5. To ascertain that the production of pro-inflammatory cytokines by IgG subclasses is dependent on glycolysis, we used glycolytic inhibitor 2-deoxyglucose (2DG), which inhibits hexokinase activity (32). Blocking of glycolysis indeed abolished the amplification of TNF, IL-1β, and IL-23 production by all subclasses, including the very moderate amplification by IgG3 (Suppl. Fig. 3).

Interestingly, the nature of modulation of cytokine production by IgG-IC is dependent on the co-receptor involved. In general, IgG-IC co-stimulation with bacteria-sensing receptors such as TLR2, 4, 5, or NLRs leads to amplification of pro-inflammatory cytokines TNF, IL-1β, and IL-23 (12, 14), while co-stimulation with virus-sensing receptors such as TLR3, RLRs, and CDS particularly suppresses type I IFNs, type III IFNs, and ISGs (16, 20). To determine whether IgG-IC also induce subclass-specific glycolytic changes upon co-stimulation with virus-sensing receptors, we measured the ECAR upon co-stimulation with TLR3 ligand Poly I:C. TLR3 stimulation, similar to TLR2 stimulation, increased the glycolysis (Fig. 4C). Yet, surprisingly, co-stimulation with IgG1 and IgG2 lowered TLR3-induced glycolysis, while IgG3 had little effect (Fig. 4C). These data indicate that the nature of metabolic reprogramming by IgG-IC is strongly dependent on the co-receptor involved.

Combined, these data indicate that IgG subclass-specific cytokine production is associated with distinct metabolic reprogramming of myeloid immune cells, which is diversified even further depending on the co-receptor involved.

Discussion

IgG antibodies are crucial for various immune functions, including neutralization, phagocytosis, and ADCC. Here, we identified another function of IgG, i.e. that human IgG tailors responses to the immunological context by orchestrating subclass-specific pro-inflammatory cytokine profiles by human myeloid immune cells (schematically summarized in Fig. 5). While IgG2 strongly promoted the production of Th17-inducing pro-inflammatory cytokines such as IL-1β, IL-23, and TNF, IgG3 suppressed the production of type I and III IFNs, and IgG1 mediated both. Moreover, we identified that this isotype-specific cytokine production is particularly controlled at the post-transcriptional level, which was associated with distinct metabolic reprogramming of immune cells.

Although IgG subclasses are more than 90% identical at the amino acid level, each subclass has a unique profile with respect to its capacity to induce different effector responses. The prevailing concept regarding human IgG subclasses is that IgG3 is the most pro-inflammatory subclass (1), while IgG2 is sometimes even referred to as anti-inflammatory (10). However, this categorization into pro- or anti-inflammatory subclasses is mainly based on a selection of antibody effector functions, such as antibody-dependent phagocytosis and complement activation. In contrast, the capacity of IgG subclasses to elicit cytokines and chemokines, which is important for both host defense and (chronic) inflammation (13, 33), has long remained unexplored. In stark contrast to other effector functions, our data here suggest that IgG2 is the most potent inducer of key pro-inflammatory cytokines such as TNF, IL-1β, and IL-23, while IgG3 has very little effect on these cytokines. These data suggest that the current paradigm of pro- and anti-inflammatory IgG isotypes is likely to be an over-simplification. Instead, overall assessment of the different properties supports the concept of a ‘division of labor’ by human IgG subclasses (Fig. 5, top panel). While IgG3 antibodies are equipped to strongly induce phagocytosis, ADCC, and complement activation, IgG2 is the main subclass that controls pro-inflammatory cytokine production. Interestingly, IgG1 appears to be in the middle, by being able to induce both the IgG2 (cytokine production) and IgG3 (phagocytosis/ADCC/complement) effector functions, although this may come at the cost of being able to induce all these effector functions at a slightly lower level. Somewhat surprisingly, we also observed cytokine induction by IgG4, the IgG subclass that is generally

considered to have the least functional potency and little interaction with FcγRs (1, 10). Yet, a direct comparison in our assay demonstrated that IgG4 induces more pro-inflammatory cytokine production by myeloid immune cells than IgG3. This further underlines the concept of subclass-specific effector functions of human IgG antibodies, instead of the current paradigm that divides IgG in either pro- or anti-inflammatory subclasses.

The main physiological function of IgG-induced cytokine production is to tailor inflammatory responses to the immunological context, such as the pathogen and the tissue involved (13, 33). For example, IgG opsonization of bacteria promotes the production of anti-bacterial Th17-inducing cytokines by DCs (12, 15), while IgG opsonization of viruses has very little effect of Th17-inducing cytokines and mainly affects type I IFN production (16, 20). Yet, how IgG orchestrates such diverse responses has long remained enigmatic. Here, our data indicate that IgG subclasses play a role in generating these pathogen-specific immune responses. IgG2 is the main subclass that is produced in response to bacterial antigens (3), which we now show is also the most potent inducer of human Th17-inducing cytokines IL-1β, IL-23, and TNF. Alternatively, IgG3 is the first subclass that is produced in response to viruses (3), which we here identified to suppress type I IFN production, which is important to prevent collateral damage induced by prolonged IFN production and to counteract viral infections by promoting anti-viral CD8 T cell and B cell responses (16, 34-36).

In addition to pathogen-specific immunity, IgG subclasses also induced cell type-specific responses, as illustrated by differences in cytokine profile between DC/macrophages and monocytes. Since these cell types are located in different tissues (e.g. skin vs. blood), these cell type-specific responses by IgG subclasses may contribute to the generation of tissue/organ-specific immune responses, although more research would be required to confirm this. For future research it would be very interesting to determine the IgG subclass-specific cytokine profile on other cells, since IgG immune complexes elicit cytokine production by various different cell types including pDCs (18), intestinal DCs (29), synovial macrophages (14), and nasal and lung epithelial cells (30).

IgG immune complexes can modulate cytokine production at three levels, i.e. gene transcription, caspase-1 activation, and gene translation (13, 30). Interestingly, we identified that IgG subclass-specific cytokine production is regulated post-transcriptionally. Yet, our data also show that all IgG

subclasses are capable of modulating cytokine gene transcription and caspase-1 activation, which they do in a comparable manner. This suggests that there is a common pathway induced by all subclasses (modulating gene transcription and caspase-1 activation), as well as subclass-specific pathways (modulating only gene translation). When focusing on the subclass-specific pathways, IgG2 induces signaling by the kinase Syk and the transcription factor IRF5. This leads to an increased glycolytic rate, which in myeloid immune cells is associated with increased inflammatory responses by promoting *de novo* synthesis of fatty acids for the expansion of endoplasmic reticulum required for cytokine gene translation (29, 30, 37). How FcγRIIa (co-)stimulation increases the glycolytic rate and subsequent translation of selected cytokines is still unknown, but recent findings provide several potential mechanisms. One possibility is that IgG subclasses differentially activate TBK1/IKKε, which in DCs is essential for hexokinase activity and glycolysis (37), and is also required for FcγR-induced cytokine production (30). In addition, TBK1 could induce metabolic reprogramming through STAT3 activation, which promotes pro-inflammatory cytokine production by inducing glycolytic and mitochondrial reprogramming (38). Another option could be differential activation of Akt2 by IRF5, which enhances the transcription of glycolytic genes (31). How metabolic reprogramming specifically amplifies particular cytokines is still unclear, but similar effects have been observed upon stimulation of FcαRI(29) and other receptors, which show that show that loading of mRNA with ribosomes is gene-specific, with particular upregulation of genes such as *TNF* and *IL23A (39)*.

In contrast to IgG2, IgG3-induced signaling for the suppression of type I IFNs is not dependent on Syk and IRF5 (16), and did not affect the glycolytic rate in these myeloid immune cells. Still little is known about the molecular mechanisms that underlie this type I IFN suppression by IgG, although it could be related to decreased IRF1 expression (16) and/or differences in fatty acid oxidation (40). Intriguingly, the two distinct signaling/metabolic pathways that are activated by IgG2 or IgG3 are induced through the same receptor, i.e. FcγRIIa, since in human DCs both the amplification of pro-inflammatory cytokines and the suppression of type I IFNs is fully dependent on FcγRIIa (12, 15, 16). This is reminiscent of other receptors such as the C-type lectin DC-SIGN, which induces pathogen-specific immunity through distinct signaling upon recognition of either mannosylated or fucosylated structures (41). What is driving this IgG subclass-specific signaling by FcγRIIa is still unclear, but it

could be related to differences in affinity for the receptor (IgG3>IgG1>IgG2) or differences in glycosylation pattern, similar to what has recently been described for IgA subclasses (42). Interestingly, we identified even more diversity in IgG-induced signaling by showing that the activation of FcγRIIa by the same subclass can lead to the activation of different intracellular pathways, since IgG2 enhanced the glycolytic rate upon co-stimulation with TLR2, but lowered the glycolysis upon co-stimulation with TLR3. Yet, although blocking of FcγRIIa completely abrogated IgG-induced cytokine responses, a role for non-classical FcRs such as C-type lectin receptors cannot be ruled out, since these receptors simultaneously recognize the antibodies and are also capable of inducing signaling and metabolic pathways (43). Particularly Dectin-1 is an interesting candidate in this regard, since it is expressed by myeloid immune cells, is known to synergize with TLR activation (44), and can also signal through Syk and IRF5 (45). This would add another layer of complexity to the mechanism by which IgG antibodies are able to shape immune responses to the pathogen involved.

Taken together, these results indicate that human IgG subclasses shape cytokine responses through distinct metabolic reprogramming of human myeloid immune cells. While the physiological function of this phenomenon most likely is to provide pathogen- and cell type-specific immunity, aberrancies in this inflammatory mechanism may also lead to pathology. For example, in myeloid immune cells of lupus nephritis patients, a shift in balance is observed between the two FcγRIIa-induced signaling pathways, leading to over action of the Syk-dependent pathway (that promotes pro-inflammatory cytokines) and inactivation of the Syk-independent pathway (that suppresses type I IFN production) (20), thereby promoting local kidney inflammation. In addition, these findings may be relevant for future design of antibody-related therapies. Currently, the overwhelming majority of therapeutic antibodies is of the IgG1 subclass (46), while the use of other subclasses may help to skew immune responses depending on the infection of disease in question, e.g. as previously observed in the context of cancer therapy (47).

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Footnotes

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Author contributions

Conceptualization: WH, JdD; Methodology: SWdT, LdB, SAJZ, MvW, RH; Investigation: WH, SA, HH, AJvdH; Resources: SWdT, LdB, GV; Writing the original draft: WH, JdD; Reviewing and editing the manuscript: DB, GV, BE, JdD; Supervision: JdD

Conflicts of interest statement

D.L.P.B is employee of Union Chimique Belge, the other authors have no financial conflicts of interest.

581 **Figure 1.** IgG-induced pro-inflammatory cytokine production is dependent on IgG1 and IgG2**. (a-b)**
582 Human monocyte-derived dendritic cells (DCs) were stimulated for 24h with 10µg/ml Pam3CSK4
583 (Pam3) and IgG-IC. **(a)** 7-9 independent experiments performed in triplicate, with different donors
584 (one way ANOVA with Bonferroni's multiple comparisons test; *P<0.05). **(b)** Representative example
585 of three independent experiment with different donors performed in triplicate (Mean ± SEM). **(c)** DCs
586 were incubated for 24h with *S.pneumoniae* (multiplicity of infection = 10) with were pre-opsonized or
587 not with 250ng/ml of the different IgG subclasses (IgG1-3) for 45 min and washed. Representative
588 example of three independent experiments performed in triplicate (Mean ± SEM). **(a-c)** Cytokine
589 production in supernatant was determined by ELISA.
590
591 **Figure 2**. IgG subclasses induce cell type-specific cytokine responses by human myeloid immune cells.
592 **(a)** Human monocytes or **(b)** human monocyte-derived macrophages were stimulated for 24h with
593 10µg/ml Pam3CSK4 (Pam3) and IgG-IC. **(a-b)** Cytokine production in supernatant was determined by
594 ELISA. Experiments were performed in triplicate with 6 **(a)** and 4 **(b)** independent donors. (one way
595 ANOVA with Bonferroni's multiple comparisons test; *P<0.05, **P<0.01).
596
597 **Figure 3.** IgG subclass-specific cytokine production is not regulated by gene transcription and caspase-
598 1 activation. **(a,b)** DCs were stimulated for 3h with 10µg/ml Pam3CSK4 (Pam3), 20µg/ml Poly I:C, or in
599 combination with IgG-IC from different subclasses, and *TNF*, *IL1B*, *IFNB*, and *IFNL* mRNA transcription
600 was measured by quantitative RT-PCR (qPCR) (normalized to housekeeping gene expression). Data of
601 **(a)** 5 and **(b)** 7 independent experiments is shown as fold increase for each donor after co-stimulation,
602 normalized to mRNA expression when stimulated with Pam3 or Poly I:C alone (set to 1) (Mean ± SEM).
603 **(c)** Caspase-1 activity was determined by stimulating DCs at indicated time points with 10µg/ml
604 Pam3CSK4 (pam3), or in combination with IgG-IC from different subclasses. After stimulation, cells
605 were incubated for 1h with the caspase-1 binding compound FAM-YVAD-FMK and fluorescence was
606 measured by flow cytometry. Representative example of 3 independent experiments.

607

608 **Figure 4.** IgG subclasses induce distinct metabolic reprogramming. **(a,c)** DCs were stimulated with
609 Pam3CSK4 (Pam3) or Poly I:C, in combination with the different IgG subclasses and extracellular
610 acidification rate (ECAR) was determined. Values are showed as fold increase after Pam3 or Poly I:C
611 stimulation alone (set to 1). Mean ± SEM of 6 independent donors. **(b)** IRF5 in DCs was silenced (70%
612 silencing efficiency) using control or IRF5 si-RNA, and the cells were subsequently stimulated with
613 Pam3, IgG2 or the combination, during which the ECAR was determined. Experiments were performed
614 in triplicate (Mean ± SEM).

615

616 **Figure 5.** Proposed model of division of labor by IgG subclasses. (Top panel) IgG2 is the main subclass
617 that controls pro-inflammatory cytokine production, while IgG3 antibodies are able to strongly induce
618 phagocytosis, ADCC, and complement activation. IgG1 appears to be in the middle. (Bottom panel)
619 FcγRIIa-TLR2 co-stimulation (mimicking bacterial infections) strongly upregulates the production of
620 pro-inflammatory cytokines TNF, IL-1β, and IL-23 via IRF5-mediated glycolytic changes. On the other
621 hand, FcγRIIa-TLR3 co-stimulation (mimicking viral infections) induces suppression of type I IFN
622 responses. IgG1 can mediate both effects. TLR: Toll-like receptor, IFN: Interferon