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IgG Subclasses Shape Cytokine Responses by Human Myeloid Immune Cells through Differential Metabolic Reprogramming

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

3

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

5

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30 **Abstract**

31

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

44

45 **Keypoints:**

- 46 • Human IgG subclasses elicit distinct cytokine profiles by myeloid immune cells
- 47 • Subclass-specific effects are mediated by distinct metabolic reprogramming by Fc γ Rs
- 48 • IgG subclasses provide pathogen- and cell type-specific immunity

49 **Introduction**

50

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

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

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

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

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

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

109 **Material and Methods**

110

111 **Cells**

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

126

127 **Antibodies**

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

134

135 **Stimulation**

136 DCs and macrophages were harvested at day 6 by respectively using ice for 30 minutes or TrypLE
137 Select (Invitrogen). 2µg/ml IgG subclasses or IgG allotypes were coated on a 96-well high-affinity plate
138 (Nunc) or a 48-wells plate (Costar). High affinity plates were blocked with 10% FBS in PBS at 37°C,
139 1h prior to use. Cells were stimulated with 10µg/ml Pam3CSK4 (Invivogen) or 20µg/ml Poly I:C
140 (Sigma-Aldrich). To block glycolysis, 10mM 2-Deoxy-D-glucose (2DG, Sigma Aldrich) was added to
141 the cells simultaneously with the stimuli. For the bacteria experiment, *S.pneumoniae* were pre-
142 opsonized with 250 µg/ml IgG in PBS for 45 minutes. After washing, bacteria were diluted in X-vivo
143 media supplemented with gentamycin (Gibco) and stimulated with DCs afterwards (MOI 1:10) in a
144 round bottom plate (Costar) for 24h.

145

146 **ELISA**

147 To determine cytokine production, supernatants were harvested after 24h of stimulation and stored at
148 -20°C. Cytokines levels were measured using antibody pairs for TNF (eBioscience), IL-1β, IL-6, IL-23
149 (U-CyTech Biosciences), and IL-10 (BD Bioscience). IFN-β was detected using an IFN-β ELISA kit
150 (PBL Assay Science).

151

152 **Quantitative RT-PCR.**

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

161

162 **Caspase-1 activation.**

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

166

167 **Metabolism assays**

168 Glycolysis in DCs was determined by real-time analysis of the extracellular acidification rate
169 (ECAR)(XF-96e Extracellular Flux Analyzer; Seahorse Bioscience). A XF-96 culture plates was coated
170 with 4ug/ml IgG subclasses and 30,000 DCs were plated per well in unbuffered RPMI-1640 (Sigma),
171 which was filtered through a 0.22 μ filter system (Corning, Amsterdam, The Netherlands), the pH was
172 set to 7.4 using 37% HCl, and was supplement with 5% FCS. Cells were and rested at 37°C in 0% CO₂
173 for 1 hour. ECAR was monitored in response to glucose (10 mM; port A; Sigma) and 30 minutes later
174 to 10ug/ml Pam3CSK or 20 μ g/ml Poly I:C (Port B). ECAR values as shown in the graphs were
175 represent the increased in ECAR as determined 45 minutes after injection of port B over the baseline
176 ECAR values before port A injection.

177 **Results**

178

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

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

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

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

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

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

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

218

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

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

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

235

236 **Suppression of type I IFNs is dependent on IgG1 and IgG3**

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

247

248 **IgG subclass-specific cytokine production is not regulated by gene transcription and caspase-1 249 activation**

250 IgG-IC co-stimulation is known to change immune responses by modulating cytokine production by
251 human myeloid immune cells at three different levels. These are (1) altered cytokine gene transcription
252 (12); (2) activation of caspase-1, which promotes IL-1 β production by cleaving pro-IL-1 β into its
253 functional form (12, 14); and (3) increased cytokine gene translation which is dependent on glycolytic
254 reprogramming (29, 30). To determine at which level IgG subclasses regulate their distinct cytokine
255 profiles, we first assessed cytokine gene transcription upon co-stimulation with IgG1, IgG2, or IgG3.
256 Notably, all subclasses amplified gene transcription of pro-inflammatory cytokines *TNF* and *IL1B* in a
257 similar manner (Fig. 3A). Similarly, all subclasses also equally suppressed the transcription of *IFNB*
258 and *IFNL* (encoding IFN- λ 1) (Fig. 3B). Next, we set out to investigate whether IgG subclasses display
259 a different capacity to activate caspase-1. However, no differences in caspase-1 activation were

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

264

265 **IgG subclasses induce distinct metabolic reprogramming**

266 Since no differences between IgG subclasses were observed in cytokine gene transcription and caspase-
267 1 activation, we next studied IgG-IC-induced metabolic reprogramming. IgG-IC (from pooled IgG)
268 induces metabolic reprogramming by increasing the glycolytic rate, which is essential for post-
269 translational amplification of (particularly) pro-inflammatory cytokine production (30). To determine
270 whether the subclass-specific cytokine production is dependent on differences in glycolytic
271 reprogramming, we stimulated human DCs and analyzed them for changes in rates of extracellular
272 acidification (ECAR), as a measure of lactate production (a proxy for the glycolytic rate). Strikingly,
273 while co-stimulation of Pam3 with IgG1 and IgG2 amplified the glycolytic rate, co-stimulation with
274 IgG3 had very little effect on glycolysis (Fig. 4A).

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

287 Interestingly, the nature of modulation of cytokine production by IgG-IC is dependent on the co-
288 receptor involved. In general, IgG-IC co-stimulation with bacteria-sensing receptors such as TLR2, 4,
289 5, or NLRs leads to amplification of pro-inflammatory cytokines TNF, IL-1 β , and IL-23 (12, 14), while
290 co-stimulation with virus-sensing receptors such as TLR3, RLRs, and CDS particularly suppresses type
291 I IFNs, type III IFNs, and ISGs (16, 20). To determine whether IgG-IC also induce subclass-specific
292 glycolytic changes upon co-stimulation with virus-sensing receptors, we measured the ECAR upon co-
293 stimulation with TLR3 ligand Poly I:C. TLR3 stimulation, similar to TLR2 stimulation, increased the
294 glycolysis (Fig. 4C). Yet, surprisingly, co-stimulation with IgG1 and IgG2 lowered TLR3-induced
295 glycolysis, while IgG3 had little effect (Fig. 4C). These data indicate that the nature of metabolic
296 reprogramming by IgG-IC is strongly dependent on the co-receptor involved.

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

300 Discussion

301

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

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

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

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

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

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

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

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

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

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

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- 567

568 **Footnotes**

569

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573

574 **Author contributions**

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

578

579 **Conflicts of interest statement**

580 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 \pm SEM). **(c)** DCs
586 were incubated for 24h with *S.pneumoniae* (multiplicity of infection = 10) with were pre-opsionized 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 \pm 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 \pm 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 \pm 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 \pm 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 Fc γ RIIa-TLR2 co-stimulation (mimicking bacterial infections) strongly upregulates the production of
620 pro-inflammatory cytokines TNF, IL-1 β , and IL-23 via IRF5-mediated glycolytic changes. On the other
621 hand, Fc γ RIIa-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