High titers and low fucosylation of early human anti–SARS-CoV-2 IgG promote inflammation by alveolar macrophages

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Patients diagnosed with coronavirus disease 2019 (COVID-19) become critically ill primarily around the time of activation of the adaptive immune response. Here, we provide evidence that antibodies play a role in the worsening of disease at the time of seroconversion. We show that early-phase severe acute respiratory distress syndrome coronavirus 2 (SARS-CoV-2) spike protein–specific immunoglobulin G (IgG) in serum of critically ill COVID-19 patients induces excessive inflammatory responses by human alveolar macrophages. We identified that this excessive inflammatory response is dependent on two antibody features that are specific for patients with severe COVID-19. First, inflammation is driven by high titers of anti-spike IgG, a hallmark of severe disease. Second, we found that anti-spike IgG from patients with severe COVID-19 is intrinsically more proinflammatory because of different glycosylation, particularly low fucosylation, of the antibody Fc tail. Low fucosylation of anti-spike IgG was normalized in a few weeks after initial infection with SARS-CoV-2, indicating that the increased antibody-dependent inflammation mainly occurs at the time of seroconversion. We identified Fcγ receptor (FcγRI) IIa and FcγRIII as the two primary IgG receptors that are responsible for the induction of key COVID-19–associated cytokines such as interleukin-6 and tumor necrosis factor. In addition, we show that anti-spike IgG–activated human macrophages can subsequently break pulmonary endothelial barrier integrity and induce microvascular thrombosis in vitro. Last, we demonstrate that the inflammatory response induced by anti-spike IgG can be specifically counteracted by fostamatinib, an FDA- and EMA-approved therapeutic small-molecule inhibitor of Syk kinase.

INTRODUCTION
Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is characterized by mild flu-like symptoms in most patients (1, 2). However, about 20% of the cases have more severe disease outcomes, with bilateral pneumonia that may rapidly deteriorate into acute respiratory distress syndrome and even death by respiratory failure. With high numbers of infected people worldwide and limited treatments available, safe and effective therapies for the most severe cases of COVID-19 are urgently needed.

Many of the COVID-19 patients with severe disease show a marked worsening of the disease around 1 to 2 weeks after onset of symptoms (2, 3). This is suggested not to be a direct effect of viral infection but instead to be caused by the overactivation of the immune system in response to infection because worsening of disease coincides with the activation of adaptive immunity (2). This excessive

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immune response is frequently described as a “cytokine storm,” characterized by high concentrations of proinflammatory cytokines (3, 4). A detailed assessment of the cytokine profile in severe cases of COVID-19 indicates that some cytokines and chemokines are strongly elevated, such as interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF) (5–7). In contrast, type I and III interferon (IFN) responses, which are critical for early antiviral immunity, appear to be suppressed (8, 9). Together, high proinflammatory cytokines, known to induce collateral damage to tissues, and muted antiviral responses suggest that an unfavorable immune response may be driving disease patients with severe cases of COVID-19.

Antibodies pose a potential candidate of the adaptive immune system that could explain the observed worsening of disease during SARS-CoV-2 infection. Previous studies on dengue virus identified that immunoglobulin G (IgG) antibodies can increase the infection of cells by a process known as antibody-dependent enhancement (ADE) (10). However, thus far, there is little evidence for antibody-enhanced infection in COVID-19 (11). In addition to ADE (which increases viral infection of cells), human IgG antibodies can also worsen pathology by increasing the release of proinflammatory cytokines. Initial studies identified this phenomenon in autoimmune disorders such as rheumatoid arthritis, where IgG autoantibodies promote synovial inflammation (12, 13). More recently, antibody-dependent inflammation (ADI) has also been observed upon infection with SARS-CoV-1, and this was induced by anti-spike IgG (14). In both rheumatoid arthritis and SARS-CoV-1 infection, IgG antibodies convert wound-healing “M2” macrophages to a proinflammatory phenotype (12, 14, 15). Combined, these data hint toward a pathogenic role for IgG in severe cases of COVID-19. In this study, we explored the hypothesis that anti–SARS-CoV-2 antibodies drive excessive inflammation in severe cases of COVID-19 and define therapeutic approaches to suppress these responses.

RESULTS

High titers of anti-spike IgG promote inflammation by alveolar macrophages

We assessed the effect of anti-spike antibodies from serum of patients who were critically ill with COVID-19 on human M2-polarized macrophages. Our previous transcriptional analysis revealed that macrophage colony-stimulating factor (M-CSF) and IL-10 induce M2 monocyte differentiation that generates macrophages that most closely resemble primary human lung macrophages (16). Because activation of immune cells by IgG antibodies requires immune complex formation by binding of IgG to its cognate antigen (17, 18), we generated spike-IgG immune complexes by incubating SARS-CoV-2 spike–coated wells with diluted serum from patients with severe COVID-19 treated in the intensive care unit (ICU) at the Amsterdam University Medical Centers (UMC) who tested positive for anti–SARS-CoV-2 IgG (fig. S1A). Stimulation with spike protein alone did not induce cytokine production, whereas spike-IgG immune complexes elicited small amounts of IL-1β, IL-6, and TNF but very high IL-8 production by human macrophages (Fig. 1A). However, because in the later phase of infection (1 to 2 weeks after initial exposure) lung macrophages are simultaneously exposed to virus-associated stimuli and anti-spike IgG immune complexes, we also assessed the effect of the combination of these two stimuli. Combined stimulation of anti-spike IgG immune complexes and the Toll-like receptor 3 agonist, polyinosinic-polycytidylic acid [poly(I:C)], increased the production of COVID-19–associated proinflammatory cytokines IL-1β, IL-6, and TNF compared to IgG or poly(I:C) alone (Fig. 1A). Similar effects were observed with other viral and bacterial co-stimuli (fig. S1B). Induction of the anti-inflammatory cytokine IL-10 was also increased (Fig. 1A), similar to what is observed in patients with COVID-19 (19). We confirmed these findings in primary human alveolar macrophages that were obtained by bronchoalveolar lavage (BAL), which showed similar responses (Fig. 1B). Phenotypical analysis of these human alveolar macrophages showed significantly decreased expression of M2 markers upon costimulation with anti-spike IgG immune complexes, indicating the polarization toward a more inflammatory phenotype [P < 0.0001 (CD163) and P = 0.001 (CD209); fig. S1C].

To assess whether the inflammatory response is dependent on anti-spike antibodies, we compared the effect of sera from 33 intensive care lung disease patients that either (i) did not have COVID-19, (ii) had COVID-19 but were still negative for anti-spike IgG, or (iii) had COVID-19 and were positive for anti-spike IgG (table S1). Whereas serum of non–COVID-19 patients and anti-spike IgG–negative COVID-19 patients showed no up-regulation of proinflammatory cytokines compared to individual poly(I:C) stimulation, IL-1β, IL-6, IL-8, and TNF production was amplified by serum of COVID-19 patients with anti-spike IgG (P < 0.0001; Fig. 1C). To further confirm that the observed inflammation is induced by anti-spike IgG and not by other inflammatory components in serum, we purified IgG from serum of critically ill COVID-19 patients that were seropositive and healthy controls that were seronegative for anti–SARS-CoV-2. Whereas proinflammatory cytokine production was strongly amplified by purified IgG from severely ill COVID-19 patients, no amplification was observed by purified IgG from controls (fig. S1D).

To determine whether the inflammatory responses are specific for severely ill COVID-19 patients or are also induced by patients who have mild symptoms, we directly compared cytokine amplification by serum obtained from patients with mild COVID-19 or patients in the ICU (table S2). Amplification of proinflammatory cytokine production was specific for severely ill patients (P < 0.0001; Fig. 1D), which was in line with the substantially lower anti-spike titers in mild patients (fig. S1E), whereas the fucosylation was comparable (fig. S1F).

RNA sequencing analysis of macrophages stimulated with sera from anti-spike IgG–positive COVID-19 patients showed induction of a proinflammatory gene program, as highlighted by induction of TNF, ILs, chemokines, and macrophage differentiation factors (Fig. 2A). IFN-β and IFN-γ were induced also by anti-spike–positive serum, whereas the classical downstream IFN response gene, CXCL10, was reduced (P < 0.0001; fig. S1, G to I), which is in line with recent findings by others (20).

In patients with COVID-19, high anti-spike IgG titers are strongly associated with disease severity (21, 22). To determine whether anti-spike titers correlate with higher cytokine responses by human macrophages, we performed a principal component analysis of the combined cytokine production data for all samples that, upon overlaying with anti-spike IgG titers, suggested that the inflammatory response of macrophages was associated with IgG titers (Fig. 2B). Subsequent analysis similarly demonstrated that anti–receptor binding domain (RBD) IgG titers and cytokine production correlate for the cytokines IL-1β (P < 0.0001), IL-6 (P < 0.0001), IL-8 (P < 0.0001), IL-10 (P < 0.0001), and TNF (P < 0.0001; Fig. 2C and fig. S1J).
Similar correlations were observed for IL-6 and total anti-spike IgG ($P < 0.0001$; fig. S1K). IFN-β ($P = 0.0004$) and IFN-γ ($P < 0.0001$) also showed a positive correlation, whereas CXCL10 showed a negative correlation ($P < 0.0001$; fig. S1I), which may be related to reduced expression of IFN receptors (fig. S1L). Stimulation with immune complexes made from three serum samples with different titers using serial-step dilutions showed a dose-dependent induction of proinflammatory cytokines (Fig. 2D), thereby confirming that high anti-spike titers drive proinflammatory cytokine production by human macrophages.

To assess whether inflammatory responses are induced directly upon virus opsonization or whether this requires spike expression by infected cells, we stimulated macrophages with anti-spike IgG-opsonized pseudo-typed virus. Virus opsonization had no detectable effect on cytokine production (fig. S1M), which is in line with previous findings that small IgG immune complexes are unable to
trigger cytokine production (23). In contrast, IgG-opsonized spike-expressing 293F cells, which mimic SARS-CoV-2–infected cells and induce the formation of larger immune complexes, did amplify IL-6 production by macrophages (fig. S1N). These results indicate that anti-spike–induced inflammation requires large IgG immune complexes, as occurs upon host cell infection. Combined, these data demonstrate that high titers of anti-spike IgG from serum of severely ill COVID-19 patients induce a strong proinflammatory response by otherwise immunosuppressive human M2 macrophages, which is characterized by the production of classical cytokine storm mediators such as IL-1β, IL-6, IL-8, and TNF.

Aberrant glycosylation of anti-spike IgG contributes to inflammation

In addition to the anti-spike antibodies from serum, we tested the effect of the recombinant anti-spike IgG COVA1-18, which we generated previously from B cells isolated from a patient with COVID-19 (24). We stimulated macrophages with anti-spike immune complexes made with a high concentration of recombinant anti-spike antibody COVA1-18 (mimicking a serum concentration of 100 μg/ml in our assay). This concentration is higher than the average anti–SARS-CoV-2 IgG concentration in patients with severe COVID-19, which, according to previous studies, on average peaks at 16.5 μg/ml...
One of the critical characteristics that determine IgG pathogenicity is the glycosylation of the IgG Fc tail at position 297 (26, 27). Recently, we and others (28, 29) have shown that anti-spike IgG of patients with severe COVID-19 have aberrant fucosylation and galactosylation, both compared to the total IgG within these individual patients, as well as compared to anti-spike IgG from mild or asymptomatic patients. We determined the glycosylation pattern of

(25). The high concentration of COVA1-18 immune complexes elicited substantially less IL-1β, IL-6, and TNF than anti-spike immune complexes made from COVID-19 serum (Fig. 3A). We did not observe this difference for the induction of anti-inflammatory cytokine IL-10 (Fig. 3A). These data suggest that the anti-spike IgG in severe cases of patients with COVID-19 is intrinsically more proinflammatory than a recombinant IgG against the same target.
a subset of COVID-19 serum samples in the present study, which showed significantly decreased fucosylation ($P = 0.0003$) and increased galactosylation of anti-spike IgG compared to total IgG within the tested patients ($P = 0.0096$; Fig. 3B), similar to the study of Larsen et al. (28). Fucosylation of anti-spike IgG correlated negatively with macrophage production of proinflammatory cytokines IL-6 ($P = 0.0358$) and IL-8 ($P = 0.0234$; Fig. 3C). No correlation was observed for TNF, IL-1β, or IL-10 (Fig. 3C and Fig. S2A). CXCL10 showed a positive correlation ($P = 0.0443$; Fig. S2A). RNA sequencing data from patients with relatively low fucosylation (sera 07, 09, and 14) and from patients with relatively normal fucosylation (sera 04 and 05) showed a very pronounced induction of inflammatory mediators and proinflammatory pathways specifically in low-fucosylation patients (Fig. 3, D and E).

To determine whether anti-spike glycosylation directly modulates cytokine induction, we stimulated macrophages with regular monoclonal COVA1-18 or modified COVA1-18 that had low fucosylation or high galactosylation (table S3). COVA1-18 with low fucosylation showed an increased capacity for amplification of proinflammatory cytokines ($P < 0.0001$; Fig. 4A). High galactosylation alone or in combination with low IgG fucosylation did not lead to elevated cytokine production (Fig. 4A). COVA1-18 with low fucose and high galactose showed a similar amplification of IL6, IL8, and TNF mRNA expression over time (Fig. 4B), whereas CXCL10

![Image](http://stm.sciencemag.org/)

**Fig. 4. Low fucosylation of IgG promotes inflammatory cytokine production.** (A) Macrophages stimulated with spike protein were costimulated with combinations of poly(I:C), COVA1-18 [wild-type (WT), recombinant anti-spike IgG1], or COVA1-18 that had been modified to express low fucose or high galactose. IL-6 production was measured after 24 hours. Each line represents one macrophage donor, performed in triplicate. Statistics were calculated with two-way ANOVA. **$P < 0.01$; ****$P < 0.0001$. mAb, monoclonal antibody. (B) Time-dependent fold changes (to 0-hour unstimulated M2 macrophages) in gene expression are depicted in line chart for IL6, IL8, and TNF. Macrophages stimulated with poly(I:C) were costimulated with immune complexes of wild-type antibody (Ab) or an antibody that had been modified to express low fucose or high galactose. Representative example of six independent macrophages donors is shown. Cytokine production was measured after 0.5, 1.5, 3, 6, and 24 hours. (C) Enriched motifs for significantly up-regulated genes when comparing macrophages stimulated for 6 hours with spike and poly(I:C), with or without anti-spike IgG. (D) Enriched motifs for significantly up-regulated genes when comparing macrophages stimulated for 6 hours with spike, poly(I:C), and serum with low-fucosylated IgG to the same stimulation with high-fucosylated IgG. (E) Heatmap showing scaled log2 expression (z score) of IFN-stimulated genes assessed by RNA sequencing after a 6-hour stimulation of human macrophages with poly(I:C) with or without spike protein and serum from five seropositive patients with COVID-19. (F) IgG fucosylation and galactosylation of anti-spike specific antibodies were determined in serum samples over time for patients in the ICU with COVID-19. Each line represents one donor.
mRNA expression was again inhibited (fig. S2B). To gain more insight into the molecular mechanisms that underlie the enhanced inflammatory response induced by anti-spike IgG with aberrant glycosylation, we focused on the transcriptional responses induced by the anti-spike IgGs. Motif analyses of genes differentially induced by anti–SARS-CoV-2 monoclonal IgG COVA1-18 showed clear enrichment for classical inflammatory transcription factors like EGR, p65 (RELA), and Maf (Fig. 4C). Upon comparison of genes affected by the differential glycosylation in patient samples, we identified IFN-stimulated response elements as a key enriched motif (Fig. 4D), suggesting amplification of macrophage activation via IFN pathways. This was further indicated by increased IFN-β and IFN-γ secretion (fig. S2C) by afucosylated IgG compared to IgG with normal fucosylation and by increased expression of a series of classical IFN response genes (Fig. 4E) (30). These data suggest that afucosylated anti–SARS-CoV-2 IgG promotes inflammation by engagement of IFN pathways, which are classical cofactors to promote macrophage activation (31).

Last, we determined whether the aberrant glycosylation pattern of anti-spike IgG is stable over time, by analyzing fucosylation and galactosylation over time of the patients in our study. Both fucosylation and galactosylation normalized within days to weeks after ICU admission (Fig. 4F). Similar results were observed for the other types of IgG glycosylation (fig. S2D). These data indicate that, in patients critically ill with COVID-19, the first anti-spike IgG antibodies that are produced after infection are intrinsically more inflammatory by bearing different glycosylation patterns.

**Anti-spike IgG induces activation of endothelium and platelets in vitro**

The excessive lung inflammation in severely ill COVID-19 patients often leads to pulmonary edema, after disruption of the microvascular endothelium (32), and coagulopathy, which in many patients is characterized by pulmonary thrombosis (33). To test whether the excessive macrophage activation by anti-spike IgG may contribute to pulmonary edema and thrombosis, we applied in vitro models for endothelial barrier integrity (34) and in situ thrombosis (35) using primary human pulmonary artery endothelial cells (HPAECs), where thrombocytes are added under flow conditions. For this, we stimulated macrophages and used the supernatant to assess endothelium and platelet activation. Although conditioned medium of poly(I:C)-stimulated macrophages induced only a transient drop in endothelial barrier integrity, costimulation of macrophages with spike protein and serum isolated from patients with severe COVID-19 induced long-lasting endothelial barrier disruption (Fig. 5A). In addition, during platelet perfusion, we observed significantly increased platelet adhesion to endothelium exposed to conditioned medium of macrophages that had been costimulated with spike protein and serum (P < 0.0001; Fig. 5B). This effect was paralleled by an increase in von Willebrand factor release from the endothelial cells (Fig. 5C), indicative of an active procoagulant state of the endothelium. These data suggest that anti-spike IgG–induced inflammation by macrophages may contribute to permeabilization of pulmonary endothelium, microvascular thrombosis, and subsequent severe pulmonary problems.

**Fostamatinib counteracts inflammation induced by anti-spike IgG**

Anti-spike IgG from severely ill COVID-19 patients promoted inflammatory cytokines, endothelial barrier disruption, and microvascular thrombosis in vitro, which are key phenomena underlying pathology in patients with severe COVID-19. Hence, counteracting this antibody-induced aberrant immune response could be of potential therapeutic interest. To determine how to counteract this ADI, we first set out to investigate which receptors on human macrophages are activated by the anti–SARS-CoV-2 IgG immune complexes. IgG immune complexes can be recognized by Fcγ receptors (FcγRs), which include FcγRI, FcγRIIa, and FcγRIII (18), which are all expressed on our human M2 macrophages (Fig. 6A). To determine whether FcγRs are involved in activation by anti-spike immune complexes, we blocked the different FcγRs with specific antibodies during stimulation and analyzed cytokine production after exposure to
Fig. 6. Anti-spike IgG–induced inflammation is Fcγ/R dependent and can be counteracted by fostamatinib. (A) Membrane expression of FcγRI, FcγRII, and FcγRIII by human macrophages was determined by flow cytometry. FMO, fluorescence minus one control. (B) FcγRI, FcγRII, FcγRIII, and FcγRI were blocked by specific antibodies, after which macrophages were stimulated with spike, COVID-19 serum, poly(I:C), or a combination. IL-6 production was measured after 24 hours. Triplicate values from a representative experiment with serum from three different patients with COVID-19 and two different macrophage donors (means ± SD). (C) FcγRI, FcγRII, and FcγRIII were blocked by specific antibodies, after which macrophages stimulated with poly(I:C) and immune complexes of wild-type antibody or an antibody that had been modified to express low fucose and high galactose. Each dot represents cytokine production after 24 hours by a different macrophage donor (means ± SEM). (D) and (E) Macrophages were preincubated with Syk inhibitor R406, after which cells were stimulated as in (B). Cytokine production was measured after 24 hours. A representative donor is shown (D), and data are presented as means ± SD. The response for multiple donors with or without preincubation with R406 is shown (E). Every pair of dots represents cytokine production after 24 hours by a different serum donor. Statistics were calculated with a paired t test. ***P < 0.001; ****P < 0.0001. (F) Volcano plot depicting up- and downregulated genes when comparing macrophages stimulated for 6 hours with spike, poly(I:C), and serum to the same stimulation in the presence of R406. (G) Gene set enrichment analysis (GSEA) of curated gene sets suppressed by R406: IL-1–mediated signaling pathway (GO:0070498), TNF production (GO:0032640), response to TNF (GO:0034612). NES, normalized enrichment score; adj. P, Benjamini-Hochberg (BH)–adjusted P value.

anti-spike immune complexes. All FcγRs contributed to anti-spike–induced cytokine induction, but the most pronounced inhibition was observed upon blockade of FcγRIIa (Fig. 6B). No inhibition was observed upon blocking of FcαRI, suggesting that IgA does not play a substantial role in the observed cytokine induction (Fig. 6B).

Because changes in glycosylation of the Fc tail can differentially affect the interaction of IgG with the different FcγRs, we also blocked the three different FcγRs upon costimulation with monoclonal IgG that either had conventional Fc glycosylation or low fucose and high galactose. Cytokine induction by both IgG with conventional
glycosylation and low fucose and high galactose was mostly dependent on FcγRIIA (Fig. 6C). However, FcγRIII appeared to be the primary receptor responsible for the enhanced cytokine production by aberrant IgG glycosylation, because blocking FcγRIII specifically counteracted IL-6 and TNF production induced by IgGs with low fucose and high galactose (Fig. 6C). We did not observe this for IL-1β (fig. S3A), which may be related to the activation of caspase-1 that is known to be mainly dependent on FcγRIIA (36).

FcγRs are known to induce signaling that critically depends on the kinase Syk (12, 36). To determine whether we could counteract anti-spike–induced immune activation, we blocked Syk using R406, the active component of the small-molecule inhibitor fostamatinib, a U.S. Food and Drug Administration (FDA)– and European Medicines Agency (EMA)–approved drug for the treatment of immune thrombocytopenia (ITP) (37). R406 significantly reduced proinflammatory cytokine production induced by anti-spike IgG from patients with severe COVID-19 (P < 0.0001; Fig. 6, D and E). Inhibition by R406 appeared to be specific, because it selectively blocked anti-spike–induced amplification of cytokines but did not substantially affect cytokine production induced by poly(I:C) alone (Fig. 6D). Similar effects were observed with primary human macrophages obtained from BAL fluid (fig. S3B).

To assess the consequences of inhibition by fostamatinib in greater detail, we analyzed the effects of R406 on macrophages stimulated with spike, serum from patients with COVID-19, and poly(I:C) by RNA sequencing. In total, 4386 genes were suppressed by R406 treatment, whereas 3976 genes were induced [false discovery rate (FDR) < 0.05; Fig. 6F]. Many of the classical proinflammatory mediators were present in the list of genes down-regulated by R406 treatment, including TNF, IL1B, IL6, and CCL2. Pathway analyses showed no clear pathways in the up-regulated genes, although suppressed genes were linked to inflammatory pathways (fig. S3C). Last, gene set enrichment analysis (GSEA) showed that genes associated with several proinflammatory pathways, including IL-1 signaling and TNF production and response, were significantly down-regulated by R406 (P = 0.013; Fig. 6G). Response to type I IFN, FcγR signaling, glycosylation, and platelet activation gene sets were suppressed (fig. S3D). These data demonstrate that the excessive inflammatory response by anti-spike IgG from severely ill COVID-19 patients can be counteracted by the Syk inhibitor fostamatinib.

**DISCUSSION**

It is still not well understood why many patients with COVID-19 become critically ill around the time of activation of adaptive immune responses. Here, we identified the induction of pathogenic IgG antibody responses against the spike protein as a potential cause, which not only amplifies proinflammatory responses by human macrophages but also induces subsequent endothelial barrier disruption and thrombosis (fig. S4). The induction of inflammation by anti-SARS-CoV-2 IgG is both dependent on anti-spike IgG titers and on low fucosylation of these antibodies, which increases their inflammatory potential, most likely by overactivation through FcγRIII. During the course of infection, both these inflammatory parameters change. Anti-spike IgG titers rapidly increase after seroconversion followed by a gradual decline (38). In contrast, only the first wave of anti-spike IgG displays aberrant Fc glycosylation (characterized by low fucose and high galactose), which rapidly normalizes in the following days to weeks. On the basis of these two parameters, the induction of excessive inflammation by anti-spike IgG is particularly likely to occur in the days right after seroconversion, when titers are high and glycosylation is most aberrant. This correlates with the observed pathology in severely ill COVID-19 patients, which show a peak in inflammation, edema, and thrombosis around the time of seroconversion (2, 3). In addition, this also correlates with the common absence of excessive inflammation in people that become reinfected with SARS-CoV-2 (39, 40), because the anti-spike IgG in these reinfected individuals will have lower titers and most likely will have normalized Fc glycosylation.

In general, antibodies are beneficial for host defense by providing various mechanisms to counteract infections, including pathogen neutralization, phagocytosis, complement activation, antibody-dependent cellular cytotoxicity (ADCC), and cytokine production (41). These different effector functions of antibodies are induced to a greater or lesser extent depending on antibody-intrinsic characteristics, such as isotype, subclass, allotype, and glycosylation (26). In patients who are severely ill with COVID-19, the glycosylation of anti-spike IgG is changed, which can lead to pathology by overactivation of IgG effector functions, as we show here by particularly amplifying the production of COVID-19–associated cytokines such as IL-6 and TNF (5, 42). Decreased IgG fucosylation, as observed in severe cases of COVID-19, has previously been observed in patients infected with HIV or dengue virus (43, 44) and may actually be a general phenomenon in a response to enveloped viruses (28). For dengue virus, decreased IgG fucosylation has been described to contribute to the worsening of the course of disease after reinfection (10). However, it is important to realize that the underlying mechanism by which low-fucose IgG contributes to disease exacerbation is very different between dengue virus and SARS-CoV-2. In dengue virus infections, decreased IgG fucosylation worsens the pathology by binding to the virus and increasing the infection of host cells through enhanced uptake by FcγRs, a process known as ADE (10). For SARS-CoV-2, there is very little evidence for ADE. Instead, increased pathology by afucosylated IgG in patients with COVID-19 likely results from excessive immune activation. To make this difference clear, we propose to not use the term ADE but instead to use ADI to denote antibody-induced pathology as observed in patients with COVID-19.

The combination of decreased fucosylation and increased galactosylation of IgG is known to increase the affinity for FcγRIII (26). Whereas FcγRIII was the primary receptor responsible for the inflammatory responses that were specifically induced by IgG with low fucose and high galactose, FcγRIIA contributed most to anti-spike–induced inflammation overall. These findings indicate that collaboration between multiple FcγRs is required for ADI by anti-spike IgG. The observed FcγR-dependent overactivation of human alveolar macrophages, which generally have a wound-healing M2 phenotype, is in line with the general concept that the effect of ADI is most pronounced in immune cells that have a tolerogenic or anti-inflammatory phenotype, such as synovial M2 macrophages (12) or intestinal CD103+ dendritic cells (45). Although we focused on alveolar macrophages in this study, FcγRII and FcγRIII are also expressed by various other myeloid immune cells that are found in the inflamed lungs of patients with severe COVID-19, such as monocytes and neutrophils (3, 46). Overactivation of neutrophils by COVID-19 patient plasma can also be inhibited by fostamatinib (47). In addition, the high degree of aberrantly glycosylated anti-spike IgG could also contribute to pathology by activating nonimmune cells. For example, airway epithelial cells express FcγRIII, are one of the main target cells of...
infection by SARS-CoV-2, closely interact with activated macrophages (48), and are a major source of IL-6 (49). In addition, anti-spike IgG may activate platelets through FcγRIIA (50, 51), which would provide a direct way of platelet activation in addition to the indirect activation by macrophages and pulmonary endothelium that we observed in this study, thereby further promoting microvascular thrombosis.

It is still unclear how severe SARS-CoV-2 infections lead to the generation of IgG antibodies with aberrant glycosylation. Regarding the total amount of IgG in circulation, changes in glycosylation are associated with age and sex, which results in slightly decreased IgG fucosylation with age (52, 53). However, in severely ill patients with COVID-19, it is specifically the anti-spike IgG that shows lower fucosylation. Although production of afucosylated IgG seems to be a general mechanism in response to enveloped viruses (28), it is unclear why afucosylation is more pronounced in patients with COVID-19 who develop severe disease as compared to mild disease. The quick normalization of glycosylation of anti-spike IgG after seroconversion hints toward the aberrant activation of B cells that are responsible for the first wave of anti-SARS-CoV-2 antibodies, mostly likely the short-lived plasmablasts. Critically ill COVID-19 patients are characterized by extrafollicular B cell activation, which coincides with early production and high concentrations of SARS-CoV-2–specific neutralizing antibodies (54). The molecular processes that underlie the production of IgG with aberrant glycosylation in these cells are still unclear but could be related to increased endoplasmic reticulum stress or different expression of proteins such as Jagunal homolog 1 (55). For future studies, it would be very interesting to study how risk factors of severe COVID-19 (such as age, obesity, and comorbidities) affect these glycosylation processes in B cells. In addition to IgG, the extrafollicular B cells also produce IgM and IgA (54). Whether these isotypes are also aberrantly glycosylated in patients with severe COVID-19 is still unknown. However, in particular, antibodies of the IgA isotype can promote inflammation depending on the glycosylation profile (56). On top of this, IgG subclasses (IgG1 to IgG4) could also play a role in both the amplitude and the kinetics of anti-spike–induced inflammatory responses (57). For example, IgG3 is typically the first IgG subclass to be produced in response to viruses and generally shows a glycosylation pattern similar to IgG1 (26, 58). Last, cell-intrinsic differences in macrophages may also contribute to increased or decreased susceptibility of particular individuals to IgG glycosylation differences. This could be related to genetic polymorphisms, such as FCGR2A and FCGR3A single-nucleotide polymorphisms or downstream signaling molecules, but could also be related to epigenetic differences in macrophages or their precursors.

A limitation of our current study is that we have not been able to perfectly match mild and severe COVID-19 patients in terms of age and several other parameters because of practical limitations. This study required serum from mild patients quickly after seroconversion, which is generally difficult to obtain because mildly ill patients with COVID-19 are not hospitalized (or even diagnosed) and therefore difficult to follow over time. Although, here, we were able to match for the most important parameter (day of onset), it will be relevant to additionally match for age, sex, body mass index, and comorbidities in future studies. In addition, it is not yet clear whether ADI is specific for severe SARS-CoV-2 infection or whether it may also occur upon infection with other viruses. Although induction of afucosylated IgG may be a common trait of enveloped viruses (28), excessive inflammation right after seroconversion appears to be a rare event for most viral infections in humans, with the exception of SARS-CoV-1 (14). However, theoretically, ADI may still occur during other viral infections but in a less pronounced manner that does not lead to pathology. Last, it is important to realize that, although we specifically focused on ADI in this study, antibodies have additional effector functions that will be activated simultaneously in patients with COVID-19. Whereas overactivation of ADI leads to pathology, increased activation of ADCC or phagocytosis of infected cells by afucosylated IgG could simultaneously have beneficial effects such as increasing viral clearance. Therefore, in future work, it will be interesting to determine how afucosylation affects other antiviral IgG effector functions in patients with COVID-19.

We here showed that the observed inflammatory response induced by anti-spike IgG from severe patients could be specifically counteracted by the Syk inhibitor R406, the active component of fostamatinib. Fostamatinib is an FDA- and EMA-approved drug that is currently used for the treatment of ITP (37), which may facilitate repurposing for the treatment of patients with severe COVID-19. A study indicates that fostamatinib may also counteract acute lung injury by inhibiting Mucin-1 expression on epithelial cells, suggesting that fostamatinib may target multiple pathways simultaneously (59). In addition to fostamatinib, other drugs that also interfere with FcγR activation could be efficacious to counteract anti-spike IgG–induced inflammation in patients with COVID-19. Previous studies already showed the beneficial effects of treatment with intravenous immunoglobulin, which can interfere with FcγR activation (60). Alternatively, it could be interesting to target critical molecules in FcγR downstream signaling. For example, the Syk-dependent FcγR signaling pathway critically depends on the transcription factor IFN regulatory factor 5 (IRFS) (17, 36), which can be targeted using cell-penetrating peptides (61). Furthermore, FcγR stimulation is known to induce metabolic reprogramming of human macrophages (36), which is also observed in patients with COVID-19 (62), and therefore may provide additional targets for therapy. These findings may not only be valuable to find new ways to treat the most severely ill COVID-19 patients but may also have implications for the therapeutic use of convalescent serum, for which it may be wise to omit the afucosylated IgGs that are present in severely ill patients. Similarly, for recombinant neutralizing antibodies, the composition of the Fc tail needs to be carefully considered, because extreme activation of Fc effector functions by afucosylation needs to be prevented, while at the same time, Fc effector functions should remain partially intact to provide optimal therapeutic protection (63). Because ADI appears to lead to excessive inflammation upon infection with both SARS-CoV-1 (14) and SARS-CoV-2 viruses, these findings may also be relevant in case of an emergence of a future outbreak with related coronaviruses. In conclusion, our data indicate a pathogenic role for anti–SARS-CoV-2 antibodies in patients who are severely ill with COVID-19 caused by high titers and low fucosylation of anti-spike IgG. Moreover, we define therapeutically relevant approaches to suppress the induced cytokine release. These data thus warrant future investigations into the therapeutic potential of targeting this inflammatory mechanism in patients with COVID-19.

**MATERIALS AND METHODS**

**Study design**

The study was designed to investigate the effect of SARS-CoV-2 immune complexes on macrophage activation and clinically relevant

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in vitro parameters. We applied a human monocyte-derived macrophage model of IL-10–polarized macrophages, resembling human alveolar macrophages (16). We verified these data in primary human macrophages obtained via BAL. In the studies, we analyzed sera from patients hospitalized at Amsterdam UMC ICUs (n = 27) and compared these to sera from ICU patients negative for SARS-CoV-2 (n = 2), sera from patients positive for SARS-CoV-2 but negative for IgG against spike protein (n = 4), and to the response induced by recombinant anti-spike IgG, COVA1-18 (24). The patients with COVID-19 were included on the basis of serology (positive for anti-spike), except for the control COVID-19 patients in Fig. 1D, who not only needed to have a positive quantitative polymerase chain reaction (qPCR) result but also had to be seronegative. No other selection criteria were used, and there were no outliers. For the comparison with mild patients, we worked with sera from patients who tested positive for SARS-CoV-2 but were not hospitalized (n = 10). Mild patients’ sera were selected by matching gender and serum collection date as comparable as possible with the ICU sera. Smaller subsets of sera were used for selected experiments as described in the respective methods. Cytokine production assays were repeated at least in two donors. Investigators were not blinded for the patient status of the serum used. Samples were randomly assigned to positions in culture plates.

Cells

Buffy coats from healthy anonymous donors were acquired from the Sanquin blood supply in Amsterdam, The Netherlands. All the individuals provided written informed consent before donation to Sanquin. Monocytes were isolated from the buffy coats through density centrifugation using Lymphoprep (Axis-Shield) followed by human CD14 magnetic bead purification with the MACS cell separation columns (Miltenyi Biotec) as previously described (16). The resulting monocytes were seeded on tissue culture plates and subsequently differentiated to macrophages for 6 days in the presence of human M-CSF (50 ng/ml; Miltenyi Biotec) with Iscove’s modified Dulbecco’s medium (Lonza) containing 5% fetal bovine serum (FBS; Biowest) and gentamicin (86 μg/ml; Gibco). The medium was renewed on the third day. After a 6-day differentiation period, the medium was replaced by culture medium without M-CSF and supplemented with IL-10 (50 ng/ml; R&D Systems) for 24 hours to generate alveolar macrophage-like monocyte-derived macrophages. These macrophages were then detached with TrypLE Select (Gibco) for further treatment and stimulation.

PAECs were obtained from resected pulmonary artery tissue, obtained from lobectomy surgery performed at Amsterdam UMC, and isolated according to the previously published protocol (35). Briefly, the endothelial cell layer was carefully scraped onto fibronectin-coated (5 μg/ml) culture dishes (Corning, #3295) and maintained in culture in endothelial cell medium (ECM; ScienCell, #1001) supplemented with 1% penicillin/streptomycin, 1% endothelial cell growth supplement (ECS), 5% FBS, and 1% nonessential amino acids (NEAA; Biowest, #X055-100). Cells were grown until passages 4 to 6 for experiments.

Primary macrophages were prepared from BAL fluid that was obtained as spare material from the ongoing DIVA study (Netherlands Trial Register: NL6318; AMC Medical Ethical Committee approval number: 2014_294). The DIVA study includes healthy male volunteers aged 18 to 35. In this study, the individuals are given lipopolysaccharide (LPS) intravenously and, 2 hours later, a dose of either
elsewhere (28). Samples were eluted into neutralization buffer (214 mM tris and 22 mM sodium phosphate buffer) to obtain neutral pH. Concentrations of purified IgG were determined by absorbance at 280 nm (NanoDrop, Thermo Fisher Scientific).

### Titer determination
Total IgG to RBD was measured as described previously (66), using RBD proteins as described by Vogelzang et al. (67). In short, MaxiSorp plates were coated with RBD (1.0 μg/ml) in PBS overnight. After washing, samples were diluted 10,800-fold in PBS supplemented with 0.02% polysorbate 20 and 0.3% gelatin (PTG) and incubated for 1 hour at room temperature. After washing, horseradish peroxidase (HRP)–conjugated monoclonal mouse anti-human IgG (0.5 μg/ml; MH16, Sanquin) was added for 1 hour at room temperature, diluted in PTG. Afterward, enzymatic conversion of the tetramethylbenzidine substrate was used to evaluate antibody binding by measuring the difference in absorbance at 450 and 540 nm. Antibody binding was quantified using a serially diluted calibrator consisting of pooled convalescent plasma that was included on each plate. This calibrator was arbitrarily assigned a value of 100 arbitrary units (AU)/ml. Results are expressed as arbitrary units per milliliter and represent a semiquantitative measure of the concentrations of IgG antibodies.

### Stimulation
Macrophages (50,000 per well) were stimulated in precoated plates as described above in combination with poly(I:C) (20 μg/ml; Sigma-Aldrich), LPS (100 ng/ml; from Escherichia coli o111:B4, Sigma-Aldrich), CL097 (5 μg/ml; InvivoGen), R848 (100 ng/ml; Sigma-Aldrich), or Pam3CSK4 (10 μg/ml; InvivoGen). To block Syk, cells were preincubated with 0.5 μM R406 (Selleckchem) or dimethyl sulfoxide (Sigma-Aldrich) as a control, for 30 min at 37°C. To block the different FcRs, cells were preincubated with 20 μg/ml of the antibodies anti-FcγRI (CD64; 10.1; BD Biosciences), anti-FcγRIIA (CD32a; IV.3; STEMCELL Technologies), anti-FcγRIII (CD16; 3G8; BD Biosciences), and anti-FcαRI (CD89; MIP8a; Abcam) for 30 min at 4°C. Then, media was added to a final antibody concentration of 5 μg/ml.

### Virus and HEK293F opsonization
To mimic opsonized SARS-CoV-2 or infected cells, SARS-CoV-2 pseudovirus or SARS-CoV-2 spike–expressing human embryonic kidney (HEK) 293F cells were generated. Transient transfection of HEK293F cells with SARS-2 spike was performed as previously described (24). To obtain spike surface expression, 62.5 ml of HEK293F cells (at a density of 1 x 10^6/ml) was transfected with 20 μg of SARS-CoV-2 full-length spike plasmid DNA and 60 μg of PEI MAX. After 60 to 72 hours, cells were harvested and preincubated with COVA1-18. Then, HEK293F cells were washed three times and added to the macrophages at a 1:1 ratio in combination with or without poly(I:C). After 24 hours, supernatant was harvested, and cytokine production was assessed with enzyme-linked immunosorbent assay (ELISA). To produce a SARS-CoV-2 S–pseudo–type HIV-1 virus, a SARS-CoV-2 spike expression plasmid was cotransfected in HEK293T cells (American Type Culture Collection, CRL-11268) with an HIV backbone expressing firefly luciferase (pNL4-3.Luc.R-E-) as previously described (24). After 3 days, culture supernatants were harvested and stored at −80°C. To quantify pseudovirus production and determine the viral input for the macrophage activation assay, a capsid p24 antigen ELISA was performed (68). Monocyte-derived macrophages were incubated in 96-well flat-bottom plates at 37°C with SARS-CoV-2 pseudo-typed particles (an equivalent of 0.2 ng of CA p24 antigen) in the presence or absence of poly(I:C) (20 μg/ml; Sigma-Aldrich) and COVA1-18 antibody (0.4 ng/ml). After 24 hours, supernatant was harvested, and cytokine production was assessed by ELISA.

### Endothelial barrier function
PAEC passage 4 to 6 cells were seeded 1:1 in 0.1% gelatin-coated 8-well (8W10E) or 96-well (96W10idf PET) ibidi culture slides for electrical cell-substrate impedance sensing, as previously described (34). Cells were maintained in culture in ECM (Sciencell, #1001) supplemented with 1% penicillin/streptomycin, 1% ECGS, 5% FBS, and 1% NEAA (Biowest, #X055-100), with medium change every other day. From seeding onward, electrical impedance was measured at 4000 Hz every 5 min. Cells were grown to confluence, and after 72 hours, ECM medium was removed and replaced by the supernatant of alveolar macrophage–like monocyte-derived macrophages stimulated for 6 hours as described above with poly(I:C) or in combination with patient serum. Within every experiment, triplicate measurements were performed for each condition. For every experiment, PAECs and macrophages obtained from different donors were used.

### Platelet adhesion on PAEC under flow
PAECs were seeded in 0.1% gelatin-coated μ-Slide VI 0.4 ibiTreat flow slides (ibidi, #80606) and cultured for 7 days. PAECs were preincubated for 24 hours with the supernatant of alveolar macrophage–like monocyte-derived macrophages stimulated for 6 hours as described above with poly(I:C) or in combination with patient serum before flow experiments were performed. On the day of perfusion, citrated blood was collected from healthy volunteers, and platelets were isolated as previously described (69). Platelets were perfused for 5 min, and phase-contrast and fluorescent images were taken with an Etaluma LS720 microscope using a 20× phase-contrast objective. Platelet adhesion was quantified in ImageJ by determining the area covered by platelets per field of view.

### Enzyme-linked immunosorbent assay
To determine cytokine production, supernatants were harvested after 24 hours of stimulation, and cytokines were detected using the following antibody pairs: IL-1β and IL-6 (U-CyTech Biosciences), TNF (eBioscience), and IL-8 (Invitrogen). Concentration of (anti-spike) antibodies present in patient serum was determined as described before (28).

Flow supernatant was collected after perfusion, and von Willebrand factor concentrations were measured with ELISA. A 96-well high-affinity ELISA plate was coated with polyclonal anti–von Willebrand factor (1:1000; Dako, #A0082) and blocked with 2% bovine serum albumin. Samples were loaded, and bound von Willebrand factor was detected with HRP-conjugated rabbit polyclonal anti–von Willebrand factor (1:2500; Dako, #A0082). Normal plasma with a stock concentration of 50 nM von Willebrand factor (gifted from Sanquin) was used as a standard for determination of concentration, measured at 450 and 540 nm.

### Quantitative polymerase chain reaction
Total RNA was isolated with the RNeasy Mini Kit (Qiagen) and RNase–Free DNase Set (Qiagen) as per the manufacturer’s protocol. RNA was then converted to complementary DNA (cDNA) with
Mesoscale Discovery multiplex assay

V-PLEX Custom Human Cytokine 10-plex kits for Proinflammatory Panel 1 and Chemokine Panel 1 (K151A0H-2, for IL-1β, IL-6, IL-8, IL-10, TNF, CCL2, and CXCL10) and U-PLEX human Interferon Combo SECTOR (K15094K-2, for IFN-α2a, IFN-β, IFN-γ, and IFN-λ1) were purchased from Mesoscale Discovery (MSD). The lyophilized cocktail mix calibrators for Proinflammatory Panel 1 and Chemokine Panel 1 and four calibrators for U-PLEX Biomarker Group 1 (calibrators 1, 3, 6, and 9) were reconstituted in provided assay diluents, respectively. U-PLEX plates were coated with supplied linkers and biotinylated capture antibodies according to the manufacturer’s instructions. Proinflammatory cytokines and chemokines in supernatant collected at 24 hours after stimulation were detected with precoated V-PLEX, and IFNs in the 6-hour supernatant were measured by coated U-PLEX plates. The assays were performed according to the manufacturer’s protocol with overnight incubation of the diluted samples and standards at 4°C. The electrochemiluminescence signal was detected by a MESO QuickPlex SQ 120 plate reader (MSD) and analyzed with Discovery Workbench Software (v4.0, MSD). The concentration of each sample was calculated on the basis of the four-parameter logistic fitting model generated with the standards (concentration was determined according to the four-parameter logistic fitting model generated with default settings and the -noad or -rpkm options for raw counts and RPKM reporting [72] for further analyses).

Flow cytometry

After detachment, macrophages were stained with antibodies against FcyRs: FcyRI (CD64, catalog no. 305014, BioLegend), FcyRII (CD32, catalog no. 555448, BD Biosciences), and FcyRIII (CD16, catalog no. 562293, BD Biosciences). Fluorescence was measured with the CytoFLEX flow cytometer and analyzed with Flowjo software version 7.6.5 (FlowJo LLC). Fluorescence Minus One (FMO) controls were used for each staining as negative controls.

Functional analyses of transcriptomic data

All analyses were performed in the R statistical environment (v3.6.3). Differential expression was assessed using the Bioconductor package edgeR (v3.28.1) [73]. Lowly expressed genes were filtered with the filterByExpr function, and gene expression was called differentially regulated with an absolute log2 (fold change) higher than 1 using the Metascape (http://metascape.org/gp/index.html) [74] on 26 June 2020. For heatmaps, normalized expression values (counts per million) of each gene were calculated and plotted using pheatmap (v1.0.12) with values scaled by gene. GSEA was performed with Bioconductor package fgsea (v1.12.0) [75] with genes ranked by effect size (Cohen’s d) with respect to the “R406+serum+spike+poly(I:C) vs serum+spike+poly(I:C)” against the curated gene sets obtained from gene ontology (GO) by Bioconductor package biomaRt (v2.42.1) [76]. A total of 5000 permutations of gene sets were performed to estimate the empirical P values for the gene sets. Normalized enrichment scores and the Benjamini-Hochberg (BH)-adjusted P values are provided in the figures. De novo transcription factor motif analysis was performed by using Homer (v4.11) [77] using the following parameters: -start -200 -end 100 -len 8, 10, 12.

Statistical significance

Statistical significance of the data was performed in GraphPad Prism version 8 (GraphPad Software). For t tests or nonparametric analysis comparing two sets of measurements, data were first examined with D’Agostino-Pearson normality test with α = 0.05. For the data following normal distribution, paired or unpaired t tests were conducted on the basis of the experiment design. For unpaired data not following a normal distribution, Mann-Whitney test was applied. For multiple comparison tests, one-way or two-way analysis of variance (ANOVA) was applied on the basis of the addressed scientific question. Brown-Forsythe and Welch’s ANOVA test was applied when not assuming that the compared groups were sampled from populations with equal variances (examined by Brown-Forsythe test), otherwise an ordinary one-way ANOVA was performed. For differential analysis and GSEA of transcriptomic data, P values were adjusted by BH procedure to control the FDR. The analysis methods applied for each figure are stated in the legends.

SUPPLEMENTARY MATERIALS

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Figs. S1 to S4
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Activating antibodies

Excessive inflammation is a characteristic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, particularly in patients that are hospitalized with coronavirus disease 2019 (COVID-19). Here, Hoepel et al. investigated how human antibodies specific to SARS-CoV-2 spike protein may contribute to exacerbated inflammation. The authors found that spike protein–specific antibodies from patients with COVID-19 who were hospitalized had altered glycosylation, with an enrichment in low-fucosylated antibodies. These antibodies were able to activate human macrophages in vitro to secrete proinflammatory cytokines. Thus, altered antibody glycosylation may contribute to disease severity in COVID-19.