SARS-CoV-2 infects blood monocytes to activate NLRP3 and AIM2 inflammasomes, pyroptosis and cytokine release

Short title: SARS-CoV-2 monocyte infection triggers inflammation

One sentence summary: Antibody-mediated SARS-CoV-2 infection of monocytes activates inflammation and cytokine release.

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SARS-CoV-2 causes acute respiratory distress that can progress to multiorgan failure and death in a minority of patients. Although severe COVID-19 disease is linked to exuberant inflammation, how SARS-CoV-2 triggers inflammation is not understood. Monocytes and macrophages are sentinel immune cells in the blood and tissue, respectively, that sense invasive infection to form inflammasomes that activate caspase-1 and gasdermin D (GSDMD) pores, leading to inflammatory death (pyroptosis) and processing and release of IL-1 family cytokines, potent inflammatory mediators. Here we show that expression quantitative trait loci (eQTLs) linked to higher expression of the inflammasome and pyroptosis-related genes *NLRP3, GSDMD,* and *IL1R2* increase the risk of severe COVID-19 disease. We find that about 6% of blood monocytes in COVID-19 patients are infected with SARS-CoV-2. Monocyte infection depends on viral antibody opsonization and uptake of opsonized virus by the Fc receptors CD16 and CD64. After uptake, SARS-CoV-2 begins to replicate in monocytes, as evidenced by detection of double-stranded RNA and subgenomic RNA and expression of a fluorescent reporter gene. However, infection is aborted, and infectious virus is not detected in infected monocyte supernatants or patient plasma. Instead, infected cells undergo inflammatory cell death (pyroptosis) mediated by activation of the NLRP3 and AIM2 inflammasomes, caspase-1 and GSDMD. Moreover, tissue-resident macrophages, but not infected epithelial and endothelial cells, from COVID-19 lung autopsy specimens showed evidence of inflammasome activation. These findings taken together suggest that antibody-mediated SARS-CoV-2 uptake of monocytes/macrophages triggers inflammatory cell death that aborts production of infectious virus but causes systemic inflammation that contributes to severe COVID-19 disease pathogenesis.

In a small subset of mostly elderly patients and patients with comorbidities, SARS-CoV-2 causes severe COVID-19 disease marked by acute respiratory distress that can progress to multiorgan failure and death[1]. Severe disease is linked to an overly exuberant inflammatory response, including elevated serum pro-inflammatory cytokines, C-reactive protein, and lactate dehydrogenase (LDH)[2-6]. Increased chronic inflammation is associated with aging ("inflammaging") and the comorbidities linked to severe COVID-19 disease[7]. Myeloid immune cells (monocytes, macrophages, dendritic cells) are sentinels that sound the innate immune alarm by sensing invasive infection and danger to activate inflammasomes[8]. They are often the most important source of inflammatory cytokines during inflammation, and their activation is required to process and release IL-1 family cytokines, arguably the most potent inflammatory mediators in the body[9]. However other pathways, including NF-κB activation by Toll-like receptors or the TNF receptor superfamily and T_H17 lymphocyte cytokines, can also cause severe inflammation. When inflammasomes sense danger or infection, they recruit the ASC adaptor and assemble into large supramolecular complexes that recruit and activate caspase-1, which in turn processes interleukin (IL)-1 family pro-cytokines and the pore-forming protein GSDMD that damages the cell membrane, leading to cell death and inflammatory cytokine release[8]. Cell membrane rupture during pyroptosis releases cytokines, chemokines and other alarmins that recruit and activate immune cells to sites of infection. Release of large proteins such as the tetramer LDH (144 kDa), is a pathognomonic feature of pyroptosis and other forms of necrotic cell death[8]. Elevated LDH is one of the best correlates of severe COVID-19 disease[6].

Circulating monocytes and plasma of COVID-19 patients show signs of pyroptosis

Because inflammasome activation in myeloid cells is a major mediator of inflammation[2,10,11], we examined blood of SARS-CoV-2-infected donors for signs of inflammasome activation and

pyroptosis. Freshly isolated mononuclear peripheral cells from 19 healthy donors (HD) and 22 COVID-19 patients seen in the emergency department (ED) of Massachusetts General Hospital were stained for hematopoietic cell markers, a small fixable dye (Zombie Yellow) that enters dying cells whose plasma membrane is damaged and annexin V, which identifies cells undergoing programmed cell death (Fig. 1a,b, Extended Data Fig. 1a, Table S1). Annexin $V^+Zombie^-$ apoptotic cells did not increase in any subpopulation in COVID-19 samples. However, ~6% of monocytes of COVID-19 patients on average took up Zombie dye, a sign of ongoing membrane damage consistent with pyroptosis. None of the lymphocyte subsets in COVID-19 samples showed any evidence of increased pyroptosis. Monocytes subset flow cytometry phenotyping indicated a reduced frequency of classical monocytes ($CD14^{hi}CD16^-$) in the monocyte gate of freshly isolated blood mononuclear cells from 15 COVID-19 patients compared to 13 HD, while intermediate monocytes ($CD14^{hi}CD16^+$) were significantly increased, but there was no change in the non-classical subset ($CD14^{lo}CD16^+$) (Fig. 1c, Extended Data Fig. 1b). A large proportion of the intermediate (~60%) and non-classical (~40%), but none of the more abundant classical, monocytes had taken up SARS-CoV-2 virus in vivo since they stained for nucleocapsid (N) (Fig. 1d). Since expression of FcγRIIIa (CD16), an important mediator of antibody-mediated phagocytosis by monocytes, correlated with virus uptake, titers of anti-Spike RBD IgG, in the plasma of 64 COVID-19 patients were measured by ELISA (in samples obtained at the same time the fresh blood cell samples were obtained on presentation to the emergency department (ED)) and compared to plasma from 20 HD and from 5 patients who presented to the ED with COVID-19-like symptoms but were PCR- for SARS-CoV-2 infection (non-COVID patients). Most of the COVID-19 patients, but not the HD or non-COVID controls, had elevated anti-Spike RBD IgG when they presented to the ED, suggesting that they had been

104 infected for at least a week[12]. Plasma from a large panel of COVID-19 patients who had diverse
105 disease outcomes and HD were assessed for specific inflammatory markers of pyroptosis
106 (GSDMD, IL-1β, IL-1RA, IL-18, LDH activity) (Fig. 1f) and for other markers of inflammation
107 that are not pyroptosis-specific (inflammatory cytokines IL-6, TNF, IL-17/17A; growth factors
108 IL-7, G-CSF; chemokines CCL7, CXCL9, CXCL10) and for interferons (IFNβ, IFNγ).
109 Consistent with published data[13-15] [16,17], all the markers that are elevated by all sorts of
110 inflammation, but are not specific for pyroptosis, were significantly elevated in COVID plasma,
111 except for IL-17/17A, and the IFNs were not detected above baseline (data not shown). All of the
112 pyroptosis markers were significantly elevated in COVID-19 patient plasma compared to HD.
113 The plasma concentration of IL-1β, although significantly higher in COVID-19 samples, was
114 low, which was not surprising since it is rapidly cleared from the blood and usually not detected
115 even in patients with severe disease caused by ongoing pyroptosis, such as those bearing
116 constitutively active *NLRP3* mutations[9] or with IL-1-mediated systemic juvenile idiopathic
117 arthritis[18]. However, its antagonist IL-1RA, which is sometimes used as a surrogate[9], was greatly
118 increased in COVID-19 samples. It is worth noting that IL-1 family cytokines and pyroptosis
119 potently activate the other elevated inflammation markers[19].

120 To determine if pyroptosis-related biomarkers correlate with COVID-19 disease severity, plasma
121 from 10 HD and 60 COVID-19 patients who presented to the ED was analyzed for GSDMD,
122 LDH, IL-1RA and IL-18 at presentation and on days 3 and 7 for patients who were hospitalized
123 (Fig. 1g, Table S2). Patients were grouped into mild, moderate or severe disease using the MGH
124 COVID Acuity scale [20,21]. Moderate disease patients required supplemental O_2 and severe
125 disease patients required mechanical ventilation or died. Plasma levels of GSDMD, LDH, IL-
126 1RA and IL-18, were all elevated in severe patient samples compared to those with mild or

moderate disease, but the increase in GSDMD did not reach significance. Taken together, these results provide evidence of ongoing pyroptosis in blood monocytes and plasma of COVID-19 patients that was more prominent in patients who developed more severe disease.

GSDMD **eQTLs increase the risk of severe COVID-19 infection**

To probe further whether pyroptosis might be associated with severe COVID-19 infection, we used Mendelian randomization analyses to examine whether expression quantitative trait loci (eQTLs) linked in eQTLGen[22] to increased blood expression of 18 immune genes were associated with severe COVID-19 disease. The genes analyzed mostly play roles in inflammation and/or programmed necrosis - 11 inflammasome and pyroptosis-related genes: *AIM2, NLRC4, NLRP1, NLRP3, NAIP, CASP1, CASP4, GSDMA, GSDMD, GSDME, GZMA;* 3 genes related to inflammatory cytokine/chemokine signaling: *IL1R1, IL1R2, CXCL10;* 2 necroptosis (another inflammatory programmed necrosis pathway) genes: *RIPK3, ZBP1;* and 2 death receptor or apoptosis signaling genes: *CASP8, APIP*. Data were from case-control study cohorts of the COVID-19 Host Genetics Initiative release #5 (January, 2021)[23] (Fig. 1h, Table S3a-b). Comparing 5582 severe COVID-19 cases with 709,010 population controls, eQTLs associated with higher expression of 3 inflammasome/pyroptosis related genes but none of the other genes were significantly enriched in patients with COVID-19 severe respiratory infection - *NLRP3*, the gene encoding for the most important inflammasome (Odds ratio (OR, 1.12; 95% confidence interval (CI), 1.02-1.21; $p<0.02$); *GSDMD,* the gene encoding the pyroptotic pore-forming protein in immune cells (OR, 1.19; 95% CI, 1.03-1.38; $p<0.02$); and *IL1R2*, the gene encoding for the receptor of IL-1 family cytokines processed and released during pyroptosis (OR, 1.13; 95% CI, 1.02- 1.25; $p<0.03$). Increased expression of *RIPK3*, whose gene product is critical for another inflammatory pathway (necroptosis), was associated with a significantly

lower risk (OR, 0.72; 95% CI, 0.54-0.96; $p < 0.03$). Since pyroptosis and necroptosis do not occur in the same cell and antagonize each other, the negative link of *RIPK3* to COVID-19 severity may be viewed as additional support for pyroptosis as opposed to necroptosis, the other known programmed necrotic cell death pathway. All of the significant findings obtained for severe COVID-19 respiratory disease had evidence of genetic colocalization according to the HEIDI test statistic (p-values of 0.32, 0.36, 0.93, and 0.49 for *NLRP3, GSDMD*, *IL1R2*, and *RIPK3*, respectively, Table S3c) and were supported by visual examination of each locus (Fig. S2a-f). The genetic link between increased *NLRP3, GSDMD,* and *IL1R2* eQTLs and severe respiratory COVID-19 infection further supports a role for pyroptosis in clinical deterioration that may be especially important in the immunopathogenesis that accompanies the transition from initial pneumonitis to respiratory failure and systemic disease.

A parallel analysis of eQTL links to COVID-19 hospitalization (12,888 hospitalized cases versus 1,295,966 population controls, Table S3a) identified only 1 significant association with *NLRC4*, another inflammasome gene. However, *NLRC4* eQTLs associated with increased expression led to a lower risk of COVID-19 hospitalization (OR, 0.91, 95% CI 0.84-0.99; $p<0.03$) and this association was not supported by the HEIDI test for colocalization ($p<0.04$). eQTLs for none of the 18 immune genes were significantly different in a smaller dataset that compared 5,773 cases of severe COVID-19 disease with 15,497 non-hospitalized COVID-19 patients (Table S3a).

Severe COVID-19 infection samples enriched for gene expression of monocyte functional and regulatory elements

Using data from the NIH Roadmap Epigenomics Project[24] and COVID-19 Host Genetics Initiative (phase 5 release)[23] we found evidence of significant enrichment of

functional/regulatory elements in monocytes for severe COVID-19 compared to the general population in a partitioned heritability LD Score regression analysis (h^2 = 0.44, enrichment = 6.15 (95% CI, 1.85-10.46); p<0.03). This finding was replicated for the genome wide association with COVID-19 hospitalization compared to the general population (h^2 = 0.43, enrichment = 6.07 (95% CI, 2.24- 9.9); p<0.03). However, it did not reach statistical significance when comparing hospitalized versus not hospitalized COVID-19 cases (h^2 = 0.51, enrichment = 7.14 [95% CI, -3.66-+17.94]; p>0.08) possibly because of the small sample size.

Circulating monocytes have activated NLRP3 and AIM2 inflammasomes

These data suggested that circulating monocytes in COVID-19 patients might die of pyroptosis and release inflammatory cytokines to cause cytokine storm and contribute to poor outcome. Not much is known about how viruses interact with the 27 potential human canonical inflammasome sensors (22 NOD-like receptors (NLR), 4 AIM2-like receptors (ALR) and pyrin)[8]. The NLRP3 inflammasome, which detects K^+ efflux generated by a variety of stimuli including extracellular ATP, bacterial toxins or disruption of the cell membrane, could be activated by lytic SARS-CoV-2 infection itself or by specific viral proteins[25-27]. Three SARS-CoV-2 proteins, Orf3a, Orf8 and the E envelope, are thought to be "viroporins" (ion channels) that potentially activate NLRP3 by K^+ efflux when ectopically expressed, as previously described for SARS-CoV[28-31]. Orf3 and Orf8 are encoded by the pathogenic, but not the avirulent, human CoVs. Interestingly, bats, the natural zoonotic hosts of SARS-CoV and SARS-CoV-2, have a dampened NLRP3 response to infection with multiple viruses, including MERS-CoV, which might explain their ability to tolerate these infections despite high viral loads[32,33]. To probe whether circulating monocytes from COVID-19 patients are undergoing pyroptosis, freshly isolated, enriched monocytes from 8-10 HD or COVID-19 patients with mixed disease severity (Table S1), and 4-5 non-COVID-19

patients were analyzed by imaging flow cytometry for expression and intracellular distribution of the common inflammasome adapter ASC, activated caspase-1 (by fluorochrome-labeled inhibitor of caspases assay (FLICA)) and GSDMD. Canonical inflammasome activation forms a large micron-sized inflammasome-ASC-caspase-1 speck[8 34]. About 4% of fresh monocytes from COVID-19 patients, 1% of non-COVID-19 patients, but no HD samples, had activated caspase-1 (FLICA speck) and ASC specks (Fig. 2a-c; Extended Data Fig. 3a,b). These results suggest that other respiratory infections or causes of respiratory distress activate inflammasomes in blood monocytes, but monocyte inflammasome activation is more extensive in SARS-CoV-2 infection. Most of the cells with ASC specks (~80%) from COVID-19 patients also had co-localized activated caspase-1 specks (Fig. 2d).

Many of the fresh monocytes with ASC specks from COVID-19 samples showed ballooning plasma membranes and GSDMD redistribution from the cytoplasm to form prominent cell membrane puncta, consistent with GSDMD pore formation and pyroptosis, but cells without ASC specks did not (Fig. 2e,f, Extended Data Fig. 3b). Immunoblots of lysates of freshly isolated HD and COVID-19 patient monocytes and of LPS plus nigericin-treated HD monocytes were probed for full-length GSDMD (GSDMD-FL) and its cleaved C-terminal fragment (GSDMD-CT) and the housekeeping proteins, β-actin and COX-IV (Fig. 2g). During pyroptosis, cleaved GSDMD and actin are released, the actin cytoskeleton disintegrates and pyroptotic cells no longer stain for actin, while membrane-bound proteins, like COX-IV, are mostly retained[35,36]. Although GSDMD-FL was observed in all HD samples, it was only detected in 1 of 3 COVID-19 samples. A prominent GSDMD-CT fragment was detected in COVID-19 monocytes and in the positive control (LPS + nigericin-treated HD monocytes). Although mitochondrial inner membrane-anchored COX-IV was detected in all the samples, FL β-actin was not detected in one

of the COVID-19 samples, but immunoreactive β-actin fragments were detected in all the COVID-19 samples and in nigericin-activated HD monocytes. Thus, circulating monocytes from COVID-19 patients show signs of GSDMD cleavage and pyroptosis.

To identify the activated inflammasome sensor, fresh HD and COVID-19 monocytes were co-stained for ASC and 3 canonical inflammasomes (NLRP3, AIM2 (activated by cytoplasmic DNA) or pyrin (activated by bacterial toxins) (Fig. 2d, h-j)[37,38]. In COVID-19 patient monocytes, ASC specks co-localized with NLRP3 and AIM2, but there were no pyrin specks. The activation of AIM2 was unexpected, although AIM2 has been shown, in rare cases, to be activated by RNA viruses by an unclear mechanism[39-41]. AIM2 might sense host genomic or mitochondrial DNA since mitochondrial membranes are damaged during pyroptosis[42]. Almost all of the cells with ASC specks had co-localized NLRP3 and AIM2 specks (Fig. 2d) and ASC, NLRP3 and AIM2 co-localized (Fig. 2j). We did not expect to find more than one inflammasome sensor stimulated in the same cell, although co-localization of 2 distinct inflammasome sensors to the same speck has been previously observed[43]. Confocal microscopy confirmed ASC, activated caspase-1, NLRP3 and AIM2 colocalization in inflammasomes selectively in COVID-19 monocytes (Extended Data Fig. 3c). These data showing NLRP3- and AIM2-ASC-caspase-1 inflammasomes and GSDMD membrane localization and cleavage, together with our detection of dying Annexin $V^-Zombie^+$ circulating monocytes and plasma GSDMD and IL-1 family cytokines (Fig. 1), indicate that some COVID-19 blood monocytes are dying of pyroptosis.

Circulating monocytes are infected with SARS-CoV-2 and infected cells are undergoing pyroptosis

But what activates inflammasomes in COVID-19 patient monocytes? Since inflammasomes sense invasive infection, the monocytes might be infected. A few recent reports suggest that monocytes[15,44] and tissue macrophages[45] can be infected with SARS-CoV-2 and we detected nucleocapsid staining within patient monocytes (Fig. 1d). However, monocytes are generally thought not to express ACE2, the viral receptor for entry[46,47]. Indeed, ACE2 was not detected or barely detected by flow cytometry and qRT-PCR on both healthy donor (HD) monocytes, even when they were activated, and COVID-19 monocytes (Extended Data Fig. 4a,b). Both HD and COVID-19 patient monocytes also expressed similar levels of CD147 (basigin or EMMPRIN), an immunoglobulin superfamily receptor implicated in bacterial, parasite and viral entry, which has been reported to bind to SARS-CoV-2 spike protein and facilitate viral uptake and infection, although this finding is controversial[48,49,50] (Extended Data Fig. 4c,d). Monocytes express 3 Fcγ receptors – CD64 (FcγRI) and CD32 (FcγRII), expressed on most blood monocytes, and CD16 (FcγRIIIa), expressed on a small minority of blood monocytes (~10% in healthy donors)[51,52] that are activated and increased in number in COVID-19 patients[14]. These receptors could recognize antibody-opsonized viral particles and mediate their entry via antibody-dependent phagocytosis (ADP)[53]. Anti-SARS-CoV-2 spike protein antibodies are detected early in SARS-CoV-2 infection, about the same time as patients start developing inflammatory symptoms[12,45,54,55], as observed in our patient cohort (Fig. 1e). To examine whether COVID-19 patient blood monocytes are infected, we co-stained isolated HD and COVID-19 patient monocytes for SARS-CoV-2 nucleocapsid (N) (Fig. 3a-d) or dsRNA (J2 antibody) (Fig. 3e-h) and ASC. N staining indicates virus internalization, but J2 staining indicates active infection[56]. HD monocytes did not

stain for N, dsRNA or ASC. About 10% of COVID-19 patient blood monocytes stained for N or dsRNA (Fig. 3b,f), indicating that circulating monocytes in COVID-19 patients are infected. Moreover, virtually all the infected cells showed ASC specks (Fig. 3c,g) and virtually all the ASC speck$^+$ cells were infected (Fig. 3d,h). Thus SARS-CoV-2 infection of monocytes activates inflammasomes and pyroptosis.

Lung macrophages are infected in COVID-19 autopsies and have activated inflammasomes

Since the lung and airways are the main site of infection, we next assessed whether cells in lung autopsy specimens that stain for CD14, a marker of tissue macrophages and dendritic cells, were infected with SARS-CoV-2 and had active inflammasomes. Fixed slides from five human autopsy specimens and three uninfected trauma victims without lung pathology were co-stained for CD14, ASC, SARS-CoV-2 N and DAPI (Fig 3i-k). In the COVID-19 lungs, 15.1±2.9% of CD14$^-$ cells and 8.3±4.2% of CD14$^+$ cells stained for N, but no N staining was detected in the control trauma victim lungs (Fig 3i-k). As expected, N staining was detected in both E-cadherin$^+$ epithelial and CD31$^+$ endothelial CD14$^-$ cells selectively in the infected lungs (Fig 3k). However, ASC specks were detected only in CD14$^+$, but not in CD14$^-$, COVID-19 lung cells, indicating that tissue-resident macrophages have activated ASC-containing inflammasomes, but infected lung epithelial and endothelial cells do not. Most CD14$^+$N$^+$ cells had ASC specks (Fig. 4j). We also did not detect prominent ASC specks in the uninfected control autopsy specimens. About a quarter of the CD14$^+$ lung cells had ASC specks, although only ~8% of them were N$^+$. This discrepancy suggests that inflammasomes in uninfected macrophages of infected lungs may be activated by danger-associated molecular patterns (DAMPs), such as cellular alarmins like HMGB1 or ATP, released from infected and/or other damaged cells in the tissue. Identifying the

284 noninfectious stimulators of inflammasome activation of macrophages in the lung will require
285 further study.

286 **CD16 uptake of antibody-opsonized virus infects healthy donor monocytes**

287 To confirm that monocytes can be infected by SARS-CoV-2, purified HD monocytes were
288 infected with an engineered infectious clone (icSARS-CoV-2-mNG) derived from the 2019-
289 nCoV/USA_WA1/2020 strain encoding a Neon Green (NG) fluorescent protein gene as a
290 reporter of viral replication[57]. Monocytes, primed or not with LPS, were infected (MOI 1) with
291 reporter virus that was preincubated with IgG1 isotype control antibody (mAb114), anti-Spike
292 mAbs (non-neutralizing C1A-H12, neutralizing C1A-B12)[58] or pooled HD or COVID-19 patient
293 plasma (heat inactivated or not). Antibodies and plasma were also present during culture. After
294 48 h, monocytes were co-stained for SARS-CoV-2 N or dsRNA (J2) and ASC and analyzed by
295 imaging flow cytometry (Fig. 4a-g, Extended Data Fig. 5). Without LPS or anti-Spike antibody
296 or COVID-19 pooled plasma, few HD monocytes took up or replicated the virus, but infection
297 increased significantly in the presence of anti-Spike mAb or COVID-19 plasma. Nonetheless, N,
298 J2 and NG positive monocytes were reproducibly detected at low levels above background after
299 HD monocyte infection with virus preincubated with isotype control mAb or with HD plasma,
300 suggesting that there is inefficient anti-SARS-Cov-2 antibody-independent uptake and infection
301 of monocytes in addition to more efficient infection with spike-antibody opsonized virus. The
302 highest in vitro infection rate was ~3% in HD monocytes pretreated with LPS and incubated with
303 patient plasma. N and J2 staining were comparable across the different conditions with a low
304 background of ~0.1% in uninfected samples; fewer cells were NG fluorescent (about half as
305 many) and there was no background NG fluorescence in uninfected samples. More of the J2 or N
306 staining cells in the samples with the highest infection rates (treated with LPS and patient plasma

or anti-Spike antibodies) were also NG fluorescent (Extended Data Fig. 5e). NG may be less often detected than N or dsRNA because it is highly expressed later in the viral lifecycle and/or is more difficult to detect than N or double-stranded RNA. Nonetheless detection of NG is another indication of active viral replication in monocytes. ASC specks were barely detected in uninfected control HD monocytes but increased with SARS-CoV-2 infection (Fig. 4c, Extended Data Fig. 5d). More cells were ASC speck^{+} when SARS-CoV-2 was preincubated with neutralizing or non-neutralizing anti-Spike than with isotype control antibody and still more when virus was preincubated with pooled patient plasma. To verify that fluorescent molecular clone infection used for in vitro infection was similar to infection with clinical isolates, icSARS-CoV-2-mNG infection of ACE2-overexpressing A549 (A549-ACE2) and HD monocytes was compared to infection by the parent Washington (WA) strain (from which the clone was engineered) and the more infectious Delta variant (Extended Data Fig. 5f,g). As expected, the molecular clone less efficiently infected A549-ACE2 than the WA strain and infection by the Delta variant virus was significantly higher than both of the other viruses, consistent with its increased infectivity. However, infection of HD monocytes was similar for all three viruses, suggesting the possibility of ACE2-independent viral entry into monocytes.

The neutralizing activity of the antibody did not consistently affect infection. Heat inactivation of COVID-19 plasma did not reduce infection (Extended Data Fig. 5), suggesting that complement did not facilitate infection. HD plasma or isotype control antibody only weakly increased infection (Extended Data Fig. 5e-h), suggesting that opsonization of virus with anti-viral antibodies might be required for efficient infection. Indeed, IgG-depletion of COVID-19 plasma nearly abrogated viral infection assessed by NG fluorescence, but IgA depletion had no effect on infection (Fig. 4e,k).

To assess whether disease severity or antibodies raised by vaccination increased monocyte virus uptake, LPS-activated monocytes were infected in the presence of pooled plasma from uninfected donors, mRNA vaccine recipients or infected patients with mild or severe disease severity. Importantly, uninfected HD and post-vaccination plasma did not facilitate virus uptake or replication (Fig. 4f,g). However, non-COVID-19 patient pooled plasma slightly increased infection, but the increase was not significant, suggesting possible inefficient COVID-independent viral uptake by some plasma component. Disease severity did not seem to affect the infection enhancing property of COVID-19 patient plasma since plasma pooled from mild and/or severe COVID-19 patients induced similar amounts of infection.

Patients with severe acute COVID-19 disease have increased antiviral IgGs that are afucosylated in their Fc region that bind more strongly to CD16[59-61]. To test whether afucosylation affects HD monocyte infection, which occurs selectively in CD16+ monocytes (Fig. 1d), the effect of IgG purified from pooled HD or COVID-19 plasma or purified from COVID-19 patients with relatively low (~10%) or high (~30%) afucosylation (2 patients of each) on in vitro infection of HD monocytes was compared (Fig. 4h,i). As expected, purified HD plasma did not lead to infection, measured by N staining and NG fluorescence, while IgG purified from pooled COVID-19 plasma did. IgG with low afucosylation did not significantly increase infection compared to HD IgG, but the COVID-19 IgGs that had more afucosylation significantly increased the number of N+ cells that took up the virus. However, NG fluorescence did not increase significantly after adding either low or high afucosylated COVID patient IgG, compared to HD IgG, perhaps because this assay is less sensitive than N staining and because the proportion of afucosylated anti-spike antibodies in these samples was low even in the more highly afucosylated samples. It is worth noting that purified IgG is less active at enhancing

353 infection of HD monocytes than patient plasma (i.e., compare Fig. 4l,m with Fig. 4f,g) and that
354 non-COVID-19 plasma and sometimes HD plasma supports low level infection in LPS-primed
355 HD monocytes (i.e., Fig. 4c,g,i). These findings suggest that there might be another Ig-
356 independent plasma component that weakly promotes infection that remains to be uncovered.

357 To identify the monocyte receptor responsible for viral uptake, purified HD monocytes were
358 infected with the reporter virus in the presence of COVID-19 patient plasma that was depleted or
359 not of IgG or in the presence of blocking antibodies to potential monocyte receptors - ACE2,
360 CD147 and the three FcγRs, CD16, CD32 and CD64, expressed on monocytes (Fig. 4j,k,
361 Extended Data Fig. 6a,b). Blocking CD16 or CD64 or Ig depletion strongly inhibited infection,
362 while blocking the other receptors had no significant effect. The combination of anti-CD16 and
363 anti-CD64 blocking antibody did not increase inhibition of virus uptake more than either
364 blocking antibody on its own. It is possible that these 2 FcγRs, which use the same γ-chain to
365 signal, might colocalize after IgG binding so that blocking one blocks binding to the other.
366 Thus, SARS-CoV-2 infection of monocytes is mostly mediated by CD16 and/or CD64 uptake of
367 opsonized virus.

368 CD16 is also highly expressed on neutrophils and NK, which could be infected by a similar
369 antibody-dependent mechanism. We did not observe cell death in patient NK, making CD16-
370 dependent pyroptosis unlikely and therefore didn't study them further (Fig. 1a). However,
371 neutrophils are thought to play an important role in the immunopathology and inflammatory
372 syndrome elicited by SARS-CoV-2[62]. To determine whether neutrophils are also infected by
373 SARS-CoV-2 by a similar mechanism, HD neutrophils and monocytes were infected side by side
374 in vitro in the presence of COVID-19 plasma (Extended Data Fig. 6b,c). Even in the absence of

blocking antibodies, SARS-CoV-2 infection of HD neutrophils was low compared to monocyte infection (~0.2% vs almost 3% in monocytes) and not significantly increased above background N staining. To assess whether neutrophils are infected with SARS-CoV-2 in vivo, the frequency of in vivo neutrophil infection in COVID-19 samples of mixed disease severity and HD was assessed by N staining of negatively selected, freshly isolated blood neutrophils (Extended Data Fig. 6d). Infection was not detected in either HD or COVID-19 patient neutrophils. These data indicate that neutrophil infection may not occur and, if it does, is unlikely to be a significant contributor to neutrophil pathogenesis in COVID-19 complications, which may result from infection-independent neutrophil activation.

SARS-CoV-2 infection of monocytes is aborted

Detection of dsRNA and the fluorescent NG reporter strongly suggested that SARS-CoV-2 is not just taken up by monocytes, but also begins to replicate. To confirm viral replication in monocytes and verify that viral uptake is not mediated by the canonical ACE2 receptor, HD monocytes were infected in the presence of COVID-19 plasma and the antiviral drugs, Remdesivir, an inhibitor of the viral RNA-dependent RNA polymerase, and Camostat mesylate, an inhibitor of the TMPRSS2 serine protease, which primes the Spike protein for ACE2 mediated entry [63] (Fig. 4l,m, Extended Data Fig. 6e,f). Infection, assessed by counting N or NG positive cells, was not affected by Camostat, but was significantly and comparably inhibited by Ig depletion or Remdesivir, confirming antibody-dependent, ACE2-independent uptake and viral replication. Early in viral replication, a series of plus strand subgenomic RNAs are transcribed with a common leader sequence that are highly specific indicators of viral replication[33]. To further confirm viral replication, qRT-PCR was used to detect genomic and subgenomic SARS-CoV-2 RNAs. Genomic and subgenomic RNA were assessed after qRT-PCR amplification in

SARS-CoV-2-infected HD monocytes using primers to the N1 region of the N gene and to the shared leader sequence and 3'UTR sequences of the subgenomic RNAs, respectively. Genomic and subgenomic RNAs were detected in SARS-CoV-2-infected HD monocytes, but not in uninfected monocytes (Fig. 4n,o). The most abundant amplified sgRNA fragment migrated on agarose gels at the expected size of the N gene subgenomic RNA (1560 nt), and its identity was confirmed by sequencing the excised band.

Although multiple assays indicated that monocytes initiate viral replication, we next wanted to know if infected monocytes release infectious virus. Previous studies have not cultured SARS-CoV-2 from COVID-19 plasma, which we confirmed in 9 COVID-19 plasma samples, suggesting that monocyte infection may not produce infectious virus. Indeed, culture supernatants of infected HD monocytes contained infectious virus that formed plaques in Vero E6 cells when the culture supernatant was harvested immediately after infection but not 48 hours later (Fig. 4p). By contrast plaques were easily detected in culture supernatants from infected Vero E6 harvested 48 hours post-infection (hpi). Thus, monocyte infection does not produce infectious virus.

Discussion

Here we show that antibody-opsonized SARS-CoV-2 can infect and replicate in blood monocytes and lung macrophages. As many as 10% of circulating monocytes and 8% of lung macrophages in COVID-19 patients in our study were infected with SARS-CoV-2 and a comparable number of circulating monocytes had activated inflammasomes and took up a small membrane-impermeable dye, indicating that they were undergoing pyroptosis. This is a very large number, considering that dying cells are usually difficult to detect in vivo since they are

rapidly eliminated from the body. It may be surprising that monocyte infection and cell death has not been widely recognized. However, we think this is due to three reasons – (1) many studies of COVID-19 blood cells use thawed, frozen cells, and dying or activated cells do not survive freeze-thawing, (2) published studies have not specifically looked at whether circulating mononuclear cells are dying and (3) few researchers have looked at whether monocytes might be infected because they do not express ACE2. In support of our findings, a few studies have shown evidence of increased IL-1 family cytokines in COVID-19 patient plasma, in vitro SARS-CoV-2 entry in myeloid cells or NLRP3 inflammasome-caspase-1 activation in COVID-19 patient blood cells[13-15,44,64]. However, none of these studies has shown that SARS-CoV-2 infection of monocytes is antibody-mediated, identified the receptor responsible for viral uptake or shown evidence of viral replication without production of infectious virions. However two previous studies have suggested that monocyte-derived macrophages (MDM) can also be abortively infected[64,65]. In contrast to our findings in monocytes which don't express ACE2, MDM weakly express ACE2 and their infection may be partly mediated by ACE2, since in vitro infection in the absence of a source of anti-Spike antibody is blocked by anti-ACE2[64,65]. No previous study identified SARS-CoV-2 infection of monocytes as the cause of inflammasome activation or showed evidence of ongoing pyroptosis.

We found a one-to-one correspondence between monocyte infection and inflammasome-caspase-1 activation. Inflammasome activation and pyroptosis likely abort viral infection before infectious viruses are fully assembled because pyroptosis occurs quickly after infection is sensed and a viable host cell is needed to complete replication. Given the high frequency of infected monocytes, induction of pyroptosis in most patients is a protective response that reduces viral burden. The activation of pyroptosis in infected myeloid cells also sounds a potent immune

443 alarm that recruits and activates innate and adaptive immune cells to sites of infection, including
444 the lung, and contributes to immune defense. However, pyroptotic myeloid cells are likely to be
445 a major cause of the serious inflammatory sequelae that lead to acute lung injury, multiorgan
446 damage, vascular leak, and respiratory distress in the minority of patients with severe disease. In
447 particular, COVID-19 patients who developed severe disease had increased plasma biomarkers
448 of pyroptosis than patients with mild or moderate disease. However, we did not find any
449 correlation of severe disease with antibody titers at time of presentation to the ED. Nor did we
450 find disease severity correlated with the proportion of monocytes that were infected or had ASC
451 specks indicative of inflammasome activation. However, the number of patient samples in which
452 we measured monocyte infection and inflammasome activation was low.

453 Four times as many lung-resident macrophages appeared to have inflammasome activation as
454 were infected (as assessed by staining for viral nucleocapsid). Further studies will be needed to
455 identify what stimulates inflammation in uninfected macrophages, but alarmins released by lung
456 tissue damage are likely culprits. An intriguing finding of this study was the absence of
457 inflammasome activation in lung epithelial cells compared to our finding of inflammasome
458 activation in virtually every infected monocyte. Why lung epithelial cells resist inflammasome
459 activation will also require further study. Are the genes needed for inflammasome activation, the
460 inflammasome sensors or ASC not adequately expressed? In fact, the lung epithelial cells in
461 autopsy specimens did not stain appreciably for ASC. Another possibility is that an
462 uncharacterized viral ORF might suppress the inflammasome activation pathway selectively in
463 lung epithelial cells.

464 SARS-CoV-2-infected monocytes had detectable NLRP3 and AIM-2 inflammasomes that
465 recognize cell membrane damage and cytosolic DNA, respectively. Moreover, eQTLs that

indicate higher expression of critical genes involved in inflammasome activation and pyroptosis, *NLRP3*, *GSDMD* and *IL1R2,* were significantly increased in patients with severe respiratory COVID-19 infection. Further work is needed to understand how these inflammasomes get activated by SARS-CoV-2 and whether inflammasome activation is restricted to virulent coronaviruses. It will also be worth studying whether other inflammasomes are activated, such as NLRP1 and NLRP6, which were recently shown to sense dsRNA[66,67] or whether other viral infections activate multiple inflammasomes.

At the time of diagnosis, plasma biomarkers of pyroptosis, including IL-1RA, IL-18, LDH and GSDMD, were increased in patients who developed severe disease. This finding suggests that they might be incorporated into a diagnostic panel to help predict who might be susceptible to overexuberant inflammatory complications and benefit from immune modulating therapy. Repurposing FDA-approved drugs that inhibit inflammatory cytokines or GSDMD, the final mediator of both cytokine release and inflammatory death, is worth assessing in controlled clinical trials. Although anti-IL-1β (canakinumab) did not meet its endpoint for efficacy in hospitalized hypoxic COVID-19 patients (Novartis press release, 11/06/2020), an unpublished manuscript of a randomized control trial of anakinra (IL-1RA) in patients with SARS-CoV-2 pneumonia showed a highly significant reduction in the development of severe respiratory failure and overall clinical severity[68]. The possible efficacy of antagonizing IL-1 implicates GSDMD activation in severe COVID-19 disease since IL-1 secretion depends on GSDMD pore formation. Antagonists of IL-6 signaling, however, have had weak, at best, effects on COVID-19 infection[69,70]. The disappointing results of inhibiting IL-6 may be due to suboptimal timing (it is hard to stop a fulminant inflammatory cascade once it has started) or because IL-6 is only one of many inflammatory mediators that are released and increased during severe disease. Two FDA-

approved inhibitors of GSDMD, the critical mediator of pyroptosis and IL-1 family cytokine release - disulfiram (Antabuse)[71] and dimethyl fumarate (Tecfidera)[72] - are also worth evaluating and are currently being evaluated in clinical studies (NCT04485130, NCT04594343, NCT04381936). Administering disulfiram or dimethyl fumarate in mouse models of sepsis, which has many overlapping features with severe COVID-19 disease, strongly improved not only survival, but also plasma levels of IL-6 and TNF.

In human studies like this, it is difficult to assess how much monocyte and macrophage infection and inflammasome activation contribute to COVID-19 inflammation, cytokine release syndrome and severe disease. However, given the large percentage of infected cells, the large number of monocytes in the blood (~1-3 $x10^9$), the fact that a quarter of lung macrophages appear to have activated inflammasomes and that myeloid cells are the major source of IL-1 and other inflammatory cytokines, it is likely that monocyte/macrophage infection and inflammasome activation are important contributors to the pathogenesis of severe COVID-19 disease. The relative importance of blood monocytes versus tissue macrophages in inflammation and its serious consequences is also not clear and will require further study. Although neutrophils express CD16 and could potentially be infected, we did not detect infection in freshly isolated COVID-19 patient neutrophils and infection was at or near baseline in in vitro-infected HD neutrophils. Thus, neutrophil infection is not likely to be a major contributor to SARS-CoV-2 pathogenesis, although neutrophil activation of GSDMD-dependent netosis or other features of neutrophil activation may well be important drivers of pathogenesis. However, it will also be worthwhile to study other infected cells that express GSDMD as sources of inflammation, and to understand what aspects of monocyte/macrophage activation enhance SARS-CoV-2 infection.

Our findings implicate opsonizing antibodies in monocyte SARS-CoV-2 infection and inflammasome activation and raise the possibility that antibodies contribute to deleterious immune reactions associated with severe disease[59,73]. Infusion of anti-Spike neutralizing monoclonal antibodies or antibody-containing convalescent sera has not yet been shown to improve clinical outcome in SARS-CoV-2 patients in clinical trials[74], suggesting that it is worth considering whether some antibodies might have both protective and deleterious effects. Antibodies are clearly beneficial for blocking infection of ACE2-expressing lung and airway epithelia, where the virus completes replication to produce infectious progeny. Antibody properties that rely on the FcR, which mediates cellular uptake, phagocytosis, cytotoxicity and complement activation, can affect disease pathogenesis[53]. Here we show that CD16 and CD64 FcRγ-mediated uptake of antibody-coated virus triggers viral entry, inflammasome activation and pyroptosis, which is a double-edged sword. In vitro infected monocytes did not produce detectable infectious virus. For this reason, we do not consider antibody-mediated viral uptake in monocytes an example of antibody-dependent enhancement. Uptake into monocytes/macrophages is a dead end for the virus - it removes virions from the extracellular milieu, blocks them from producing infectious progeny and prevents them from disseminating to cells it can productively infect. On the other hand, the inflammatory mediators spewed out from pyroptotic monocytes and macrophages can cause severe inflammatory side effects and cytokine storm. It may not be a coincidence that clinical deterioration coincides temporally with the detection of SARS-CoV-2 antibody responses[12,45,54,55,75]. In fact, some recent studies suggest that higher antibody titers correlate with disease severity[54,75].

Patients with severe COVID-19 disease have a strong increase in antiviral IgGs that are afucosylated in their Fc region and bind more strongly to CD16[59-61,76], a receptor responsible for

uptake of antibody-opsonized SARS-CoV-2. Here we showed that IgG isolated from COVID-19 patients who had a higher proportion of afucosylated antibodies significantly increased in vitro monocyte infection but IgG from patients with fewer afucosylated antibodies did not, adding additional support for the role of CD16 in antibody-dependent viral uptake. Afucosylated antibodies may be increased during acute infection with enveloped viruses like SARS-CoV-2 but are not abundant after vaccination or other types of antigen exposure[77]. Indeed, pooled plasma from HD who received SARS-CoV-2 mRNA vaccines did not enhance monocyte in vitro infection, suggesting, fortunately, that antibody-dependent viral uptake is not a concern with respect to vaccination. Characterizing how antibody features, such as glycosylation and choice of constant region, change the ratio of protective vs deleterious functions of anti-spike antibodies will be important not only for understanding SARS-CoV-2 pathogenesis, but also for choosing the best preparations of convalescent patient plasma and monoclonal antibodies for therapy and/or prevention of severe disease and for comparing whether different vaccines generate antibodies that enhance monocyte infection and inflammation.

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Supplementary Information is available for this paper.

Supplemental Table S1: Demographic and clinical information of the fresh PBMCs and plasma cohort. Age, race and ethnicity, body mass index, co-morbidities, symptoms, MGH

Acuity score, hospitalization details and clinical information of the patients in the fresh PBMCs and plasma cohort.

Supplemental Table S2: Demographic and clinical information of the frozen plasma cohort. Age, body mass index, co-morbidities, symptoms, MGH Acuity score, hospitalization details and clinical information of the patients in the frozen plasma cohort.

Supplemental Table S3: eQTL data. Tables show the (**a**) primary findings, (**b**) underlying data for the primary findings, (**c**) summary data-based Mendelian randomization (SMR) and heterogeneity in dependent instruments (HEIDI) analysis, and (**d**) list of genes considered in the analysis. Column headings: (**a**) MR, Mendelian randomization; SNP, single nucleotide polymorphisms; OR, odds ratio; 95% CI, 95% confidence interval; MR Method, method used to perform Mendelian randomization analysis; n SNP, number of SNPs used as instrumental variants in the analysis; OR (95% CI), odds ratio and 95% confidence interval for association of increased gene expression and risk of COVID-19 outcome from Mendelian randomization analysis; (**b**) BP_37, the base position of the genetic variant on GRCh37/hg19; EA, effect allele [beta represents association for this allele]; NEA, non-effect allele; eaf.exposure, the effect allele frequency in the eQTL dataset; eaf.outcome, the effect allele frequency in the COVID-19 outcome GWAS; palindromic, an indicator of whether the genetic variant is a palindrome (e.g. C/G on the forward strand, G/C on the reverse strand); ambiguous, an indicator as to whether it was not possible to infer the effect allele using allele frequency information; beta.exposure, the beta coefficient from the eQTL GWAS; beta.outcome, the natural-log odds ratio from the COVID-19 outcome GWAS; se.exposure, the standard error of the beta as reported in the eQTL GWAS; se.outcome, the standard error of the natural-log odds ratio as reported in the COVID-19 outcome GWAS; pval.exposure, the p-value of the variant association in the eQTL GWAS; pval.outcome, the p-value of the variant association in the COVID-19 outcome GWAS; samplesize.exposure, the sample size reported for the variant in the eQTL GWAS; samplesize.outcome, the sample size reported for the variant in the COVID-19 GWAS; (**c**) BP_37, the centre base position of the gene on GRCh37/hg19; topSNP, the genetic variant with the lowest p-value in the eQTL dataset; topSNP_BP, the base position of the genetic variant with the lowest p-value in the eQTL dataset; EA, effect allele [beta represents association for this allele]; NEA, non-effect allele; the effect allele frequency in the eQTL dataset; b_GWAS, the natural-log odds ratio from the COVID-19 outcome GWAS for the genetic variant with the lowest p-value in the eQTL dataset; se_GWAS, the standard error of the natural-log odds ratio from the COVID-19 outcome GWAS for the genetic variant with the lowest p-value in the eQTL dataset; p_GWAS, the p-value from the COVID-19 outcome GWAS for the genetic variant with the lowest p-value in the eQTL dataset; b_eQTL, the beta coefficient from the eQTL GWAS; se_eQTL, the standard error of the beta coefficient from the eQTL GWAS; p_eQTL, the p-value from the eQTL GWAS; b_SMR, the beta coefficient from the SMR analysis of the association of gene expression and COVID-19 outcome; se_SMR; the standard error of the beta coefficient from the SMR analysis of the association of gene expression and COVID-19 outcome; p_SMR, the p-value from the SMR analysis of the association of gene expression and COVID-19 outcome; p_HEIDI, the p-value from the HEIDI test for colocalisation; nsnp_HEIDI, number of genetic variants used to perform the HEIDI test for colocalisation

Supplemental Table S4: Reagents and materials used for this manuscript. Antibodies, chemicals and commercial kits (with sources and catalog numbers) described in Methods.

Methods

Human subjects

Fresh PBMCs and plasma cohort The study was approved by the Investigation Review Boards of Boston Children's Hospital and Massachusetts General Hospital (MGH), and all enrolled patients signed an informed consent. 73 patients 18 years or older with clinical symptoms suggestive of COVID-19 infection were enrolled at the time of presentation to the MGH emergency department (ED) from 7/9/20 to 10/15/21. A 10-ml EDTA blood sample was transported to Boston Children's Hospital and processed within 2 h of collection. COVID-19 samples were all qRT-PCR verified for SARS-CoV-2 infection. Patients who presented to the ED but were PCR- were used as non-COVID-19 samples. Patients who had received SARS-CoV-2 vaccination prior to presentation were excluded from the study. Demographic and clinical data are summarized in Table S1. Healthy donor (HD) samples were processed and analyzed in parallel with patient samples. Subjects were enrolled from 7/9/20 to 01/10/21 at Boston Children's Hospital (BCH) with IRB-approved waiver of informed consent. Vaccinated HD (n=6), who received 2 doses of the Pfizer-BioNtech mRNA vaccine, were enrolled 3 weeks after the second dose and their plasma was pooled to evaluate whether it promoted monocyte infection.

Frozen plasma cohort 60 patients 18 yr or older with clinical symptoms suggestive of COVID-19 infection were enrolled in the MGH ED from 3/15/20 to 4/15/20 with an IRB-approved waiver of informed consent. Enrolled patients had at least one of the following: (i) tachypnea ≥22 breaths per minute, (ii) oxygen saturation ≤92% on room air, (iii) requirement for supplemental oxygen, or (iv) positive-pressure ventilation. A 10-ml EDTA tube was obtained with the initial clinical blood draw in the ED (n=60). Blood was also obtained on days 3 (n=42) and 7 (n=35) if the patient was hospitalized on those dates. Clinical course was followed for 28 d post-enrollment or until hospital discharge if after 28 d. SARS-CoV-2-confirmed patients (by qRT-PCR) were assigned a maximum acuity score (A1-A5) (A1 – died, A2 – required mechanical ventilation, A3 – hospitalized requiring supplemental oxygen, A4 – hospitalized but not requiring supplemental oxygen, A5 – discharged and not requiring hospitalization)[20,21]. Patients were grouped based on their worst acuity score over 28 d and divided into three groups for comparison (A1 and A2, severe disease; A3, moderate disease; A4 and A5, mild disease). Only 1 patient was in A4; therefore, most mild patients represent those that were discharged immediately from the ED and thus have only a day 0 sample. Demographic and clinical data are summarized for each outcome group (Table S2).

Lung tissue specimens Lung samples from 5 individuals who died from COVID-19 and 3 individuals who died from trauma and without lung disease were obtained from MGH. The study was approved by the institutional review board of MGH IRB # 2020P001147. Informed consent was obtained from relatives of study participants. Lung tissue specimens were obtained within 24 h of autopsy and immediately formalin fixed and embedded in paraffin.

Reagents and Antibodies A listing of reagents and antibodies and their sources is provided in Table S4.

Plasma, PBMC, neutrophils and monocyte isolation Samples were processed using recommended safety precautions in a BSL-2+ facility. Blood tubes were centrifuged at 2000 rpm for 10 min to separate plasma from blood cells. Plasma was collected to a new tube and incubated or not with 1% Triton X-100 for 1 h on ice before aliquoting and freezing at -80ºC. Blood cells were resuspended in PBS and layered over Ficoll for density centrifugation. PBMC were collected from the interface and subjected to red blood cell lysis (if necessary) with Red Blood Cell Lysing Buffer Hybri-Max for 5 min on ice, followed by quenching with RPMI medium supplemented with 10% FBS and 1% Penicillin/Streptomycin. PBMC were washed once more with RPMI and one fraction was stained for flow cytometry, while the remaining cells were used for monocyte purification by negative selection using RosetteSep Human Monocyte Enrichment Cocktail. COVID-19 patient neutrophils were isolated from the whole blood by immunomagnetic negative selection using the EasySep Direct Human Neutrophil Isolation Cocktail, according to the manufacturer's instructions. HD monocytes for in vitro infection were purified from PBMC by positive selection with $CD14^+$ magnetic beads. The red blood cell pellet from the Ficoll density centrifugation was used to isolate neutrophils from the same HD samples. Neutrophils were separated from the RBC pellet by hypotonic lysis.

Multiplex Luminex, Immunoassay and LDH activity assay IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, IL-18, IL-21, IL-23, CCL3, CCL7, CCL9, CXCL10, G-CSF, TNF, IFN-β and IFN-γ were measured in plasma samples using a custom Luminex assay (R&D Systems), following the manufacturer's instructions. Plates were analyzed using a Luminex MAGPIX Analyzer at the Analytical Instrumentation Core Lab of Boston University. Plasma levels of IL-1β were measured using Simple Plex cartridge Ella (ProteinSimple), following the manufacturers' instructions at Boston Children's Hospital (BCH). All samples were diluted 1:3 with the dilution buffer and the analytical performance were conducted on the ProteinSimple Ella automated immunoassay platform (Bio-Techne). GSDMD was measured in the same samples using the Human GSDMD ELISA kit (MyBiosource) following the manufacturer's instructions and LDH activity was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Results from the latter assays were analyzed using a Biotek Synergy 2 analyzer; GSDMD absorbance was measured at 450 nm and LDH absorbance was measured at 490 nm. Absorbance levels were quantified by linear regression based on the standard curve

Anti-Spike RBD ELISA Enzyme-linked Immunosorbent Assay (ELISA) kit anti-Spike RBD (BioLegend) was used to quantify antigen-specific IgG in plasma from HD, non-covid-19 and COVID-19 patients. ELISA was performed as per manufacturer's instructions. Anti-Spike RBD absorbance was measured at 450 nm and 570 nm and quantified by linear regression based on the standard curve.

Intracellular staining for imaging flow cytometry and confocal microscopy Fixed monocytes were permeabilized with 0.1% Triton X-100 for 10 min and washed twice with PBS + 3% FBS. Monocytes were then blocked for 30 min with PBS + 5% FBS, washed twice and then stained with unconjugated primary antibodies for ASC (1:200, mouse or rabbit), NLRP3 (1:200, goat), AIM2 (1:200, mouse), GSDMD (1:200, mouse), pyrin (1:200, rabbit), dsRNA (J2, mouse) (1:500) or SARS-CoV-2 nucleocapsid protein (1:500, rabbit) for 2 h, followed by 3 washes with PBS + 3% FBS. Cells were then stained with secondary antibodies (donkey anti-mouse, rabbit or

goat conjugated with AlexaFluor 488, 546 or 647, at 1:1000) for 1 h in PBS + 3% FBS, followed by 3 washes.

For microscopy, cells were fixed and stained cells were stained with DAPI (1:1000) for 10 min, washed 3 times and cytospun onto glass slides (VWR); sealed using polyvinyl alcohol and 1.5 mm coverslips (VWR). Confocal images were acquired using a Zeiss LSM 800 with 405, 488, 561 and 633 nm lasers (emission filters, 465, 509, 561 and 668 nm, respectively) and a 40x or 63x 1.4 oil immersion objective. Images were processed using Zen Blue 3.2.

For imaging flow cytometry, cells were resuspended in PBS + 3% FBS for analysis. Data were acquired using an ImageStream X MKII (Amnis) with 60x magnification and analyzed using Ideas software (Amnis). Monocytes were gated based on area/aspect ratio. ASC, NLRP3, AIM2 and pyrin specks were gated and quantified based on fluorophore intensity/max pixels.

Flow cytometry PBMC were washed and stained for viability with Zombie Yellow in PBS (1:200) for 15 min on ice. Cells were washed with PBS, centrifuged, and then stained with Annexin V PE (1:200) in 1x Annexin Buffer for 15 min on ice. After washing with 1x Annexin V buffer, cells were blocked for 10 min with anti-CD32 (1:100) in PBS + 3% FBS, and then stained for 15 min on ice with a cocktail of antibodies to identify lymphocyte and myeloid cell subsets (all 1:200 except CD19 BV650, CD123 PerCP-Cy5.5 and CD56 APC-Cy7, 1:100). Purified monocytes and an A549 cell line overexpressing ACE2 were blocked with anti-CD32, then stained with primary antibodies for ACE2 (1:100) for 15 min on ice. The secondary anti-goat AF488 was co-incubated with CD14 PE-Cy7 (1:200) and CD147 APC (1:100). After the last wash, cells were resuspended in 2% PFA and kept at 4ºC until flow cytometry analysis. In vitro-infected monocytes were fixed and permeabilized with 0.1% Triton X-100, then blocked with PBS + 5% FBS. Cells were stained with primary antibodies for dsRNA (J2, mouse) (1:500), then stained with secondary antibody (donkey anti-mouse conjugated with AlexaFluor 647, at 1:500) and anti-CD14 PE-Cy7. Cells were acquired using a FACS Canto II or LSR II and data were analyzed using FlowJo Version 10.

FLICA assay Freshly isolated monocytes were washed and resuspended in RPMI 10% FBS with FLICA substrate (BioRad FAM-FLICA Caspase-1 kit) and cultured for 1 h at 37º C. Cells were then washed twice with 1X Apoptosis Buffer (from the kit) and fixed with 1x Fixative (from the kit). Cells were kept at 4ºC until further staining and analysis.

Immunoblot Lysates of enriched monocytes from HD and COVID-19 patients, the former treated or not for 16 h at 37^{o}C with 100 ng/ml LPS and 20 μM nigericin, were resolved on 12% SDS PAGE gels, transferred to nitrocellulose membranes and blotted to detect GSDMD using (Abcam ab210070) primary rabbit mAb and secondary anti-rabbit IgG. Membranes were also blotted for β-actin and COX-IV.

eQTL analysis To assess whether a causal association exists between *GSDMD* and other immune gene eQTLs and severe COVID-19, *in silico* analyses were performed using two sample Mendelian randomization[78]. Mendelian randomization is a form of instrumental variable analysis that exploits the random allocation of alleles at meiosis to draw causal inferences using observational data by attempting to emulate randomization procedures that would be adopted in a clinical trial (although Mendelian randomization analyses represent the causal effects of lifelong perturbation in an exposure, not necessarily acute changes). To be considered an

appropriate instrument for an exposure, a genetic variant must satisfy the following assumptions: i. be associated with the exposure of interest (relevance assumption); ii. not associate with a potential confounder of the exposure and outcome (i.e., does not display evidence of "horizontal" pleiotropic effects) (independence assumption); and iii. affect the outcome only through its effect on the exposure of interest (exclusion restriction).

Uncorrelated single-nucleotide polymorphisms (SNPs) ($r^2 < 0.01$ in European ancestry individuals in the 1000 Genomes Project, Phase 3 release) associated with whole-blood RNA expression of *GSDMD* and other immune genes at genome-wide significance ($P<5\times10^{-8}$) from the eQTLGen consortium were used in the analysis[22]. These SNPs were cross-referenced against a large phenotypic database of publicly available genetic associations to ensure that they were not associated with potential confounding factors[79,80].

Summary statistics from a genome-wide association study[23,81] of severe respiratory confirmed COVID-19 were used for outcome data. Analysis was performed with data from release #5 (January 2021) in which there were 5582 severe COVID-19 cases versus 709,010 population control subjects, 12,888 hospitalized COVID-19 patients versus 1,295,966 control subjects, and 5,773 cases of severe COVID-19 requiring hospitalization versus 15,497 non-hospitalized COVID-19 patients. These analyses were based on trans-ancestry meta-analysis of different sample sets depending on whether the original investigators collected the relevant information either when planning the study or were able to obtain it retrospectively. In particular 35 studies contributed data to "COVID-19 hospitalized patients vs control population" and 18 studies contributed to "severe COVID-19 patients vs control population". The control populations were a mix of subjects who were not COVID-19 infected (i.e., negative test result(s)) or were assumed to be not COVID-19 infected (i.e., there was no record of COVID-19 in their linked data).

All datasets were converted to VCF format[82] aligned to GRCh37 (hg19) and mapped to dbSNP build 153 using FASTA files provided by the Integrative Epidemiology Unit, University of Bristol (which also provided 1000 Genome, Phase 3 PLINK files used to calculate linkage disequilibrium (LD)). Variants were aligned so that the effect alleles were consistent across studies in R v4.0.4 using the TwoSampleMR package[83]. For all analyses, palindromic variants with ambiguous allele frequencies were discarded as were genetic variants with potential stand issues that could not be resolved.

Our primary analysis was based on the inverse variance weighted method of performing Mendelian randomization (this method combines the causal effect estimates from each individual genetic variant, computed as the ratio of the variant-expression association to the variant-COVID-19 association, into a single causal effect using an inverse-weighted meta-analysis of the Wald ratios for each genetic instrument). A range of sensitivity analyses was performed relaxing some of the stricter assumptions underlying this method including the weighted median, modal and MR-Egger methods[78]. The MR-Egger method calculates a causal effect as the slope from the weighted regression of the variant-outcome associations on the variant-exposure associations, and the average pleiotropic effect as the intercept. This allows all genetic variants to have pleiotropic effects under the Instrument Strength Independent of Direct Effect (InSIDE) assumption (i.e. it requires that the pleiotropic effects are independent of the variant–exposure associations). Median- and mode-based methods are typically more robust to outlying genetic

instruments and make weaker assumptions about invalid instruments (e.g., at least half, and more than not, of variants for the median- and mode-based methods, respectively, estimate the true causal effect). These three methods are recommended in practice for sensitivity analyses as they require different assumptions to be satisfied, and therefore if estimates from such methods are similar, then any causal claim inferred is more credible.

Effect estimates are presented as odds ratios (OR) per standard deviation increase in *GSDMD* expression. A p-value < 0.05 was considered significant. All summary data used in this work are publicly available, together with a description of relevant participant consent and ethical approval secured in the original investigation.

Summary data-based Mendelian randomization (SMR) was used to calculate the HEIDI (heterogeneity in dependent instruments) p-value, using multiple SNPs in a *cis*-eQTL region to distinguish pleiotropy (i.e., gene expression and phenotype association owing to a single shared genetic variant) from linkage (i.e., two or more distant genetic variants in LD affect gene expression and phenotype independently)[84]. Values of > 0.05 are considered to likely represent colocalization. In the analyses, we excluded variants in high LD ($r^2 > 0.9$) with the sentinel *cis*-eQTL as they are not informative for the HEIDI test and SNPs in the *cis*-eQTL regions with p $>1.6\times10^{-3}$ to avoid weak instrumental variables[84]. These analyses were supplemented by graphical comparison of -log10 p-values for expression of the gene of interest and the COVID-19 outcome prioritised via Mendelian randomization using LocusComparer[85].

Functional/regulatory enrichment for monocytes Enrichment of monocyte-related functional and regulatory elements in the COVID-19 Host Genetic Initiatives GWAS of COVID-19 outcomes[23] were derived using GenoSkyline-plus annotations[86] from data from the NIH Epigenomics Roadmap Project's consolidated reference epigenomes database (using epigenetic marks H3k4me1, H3k4me3, H3k36me3, H3k27me3, H3k9me3, H3k27ac, H3k9ac, DNase I Hypersensitivity, plus DNA methylation/CpG islands and RNA-seq data)[24]. We assessed heritability explained by variants in the monocyte category using LD score regression[87,88]. We used pre-calculated LD weights for European ancestry populations and restricted our analysis to HapMap 3 SNPs as recommended by the developers. Given that LD score weights were derived from European data, in contrast to the eQTL-based Mendelian randomization analyses, we used summary data based on only European subjects from the COVID-19 Host Genetic Initiatives for these analyses. This represented 4606 cases of severe respiratory confirmed COVID-19 versus 792,801 controls; 9373 hospitalised COVID-19 cases versus 1,197,256 controls; and 4839 severe COVID-19 cases requiring hospitalisation versus 11,816 non-hospitalised COVID-19 cases. Enrichment was calculated as the ratio of the percentage of heritability explained to the overall percentage of variants covered by the monocyte category[86].

Immunofluorescence (IF) of lung specimens Formalin fixed and paraffin embedded lung parenchymal samples were stained for SARS-CoV-2 nucleocapsid (N), ASC, and CD14 and IF was analyzed on the Leica Bond RX automated staining platform using the Leica Biosystems Refine Detection Kit (Leica). The antibody for SARS Nucleocapsid (Novus) was run with citrate antigen retrieval and tagged with Alexa Fluor 488 Tyramide (Life). Following citrate stripping, the antibody for CD14 (Cell Signaling) was incubated and tagged with Alexa Fluor 594 Tyramide (Life). Following EDTA stripping, staining for ASC (Santa Cruz) was analyzed using antibody tagged with Alexa Fluor 647 Tyramide (Life). EDTA stripping was performed prior to anti-CD31 or anti-E-cadherin staining tagged to Alexa Fluor 555 Tyramide (Life). Samples were

counterstained with DAPI. Slides were scanned using an Aperio Versa Digital Pathology Scanner (Leica) and analyzed with Aperio ImageScope v12.4.3 software (Leica). Slides were also analyzed by confocal microscopy as described above.

In vitro SARS-CoV-2 infection icSARS-CoV-2-mNG (a molecular clone of SARS-CoV-2 expressing Neon Green (NG) fluorescent protein) was a gift to AEG from Shi Pei Yong and the World Reference Center for Emerging Viruses and Arboviruses, Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX)[57]. The NG fusion protein is only expressed during viral replication. The SARS CoV-2 US-WA1/2020 ancestral (WA) variant was obtained from BEI Resources. The B.1.617.1 (Delta) variant isolate was obtained from the MassCPR variant repository. In brief, the variant was isolated at the Ragon BSL3 by rescue on Vero-E6 cells from primary clinical specimens. The whole genome of subsequent viral stocks was sequenced to confirm that no additional mutation arose during virus expansion. HD monocytes/neutrophils were purified from apheresis leukoreduction collars collected at Brigham and Women's Hospital. Monocytes were incubated overnight with medium or 100 ng/ml LPS, and then infected with icSARS-CoV-2-mNG, SARS-CoV-2 (WA), and SARS CoV-2 B.1.617.1 (Delta) (MOI =1) in a BSL-3 facility. Infection of $ACE2^{+}$A549 cells with MOI 0.01 was used as a control. The viral inoculum was treated with 10 μg/ml of antibody (isotype control mAb114, anti-Spike C1A-H12, or anti-Spike C1A-B12), or 5% HD (n=3), COVID-19 patients of mixed disease severity (n=12, 4 mild, 4 moderate, 4 severe) or vaccinated HD (n=6) pooled plasma (heat inactivated or not; Ig-depleted or not, as indicated) before infection with SARS-CoV-2 for 30 min at room temperature. 100 μl of treated virus was added to monocytes ($2x10^6$ cells/well) in 48 well plates. Infected cells were incubated at 37°C, 5% CO_2 with gentle shaking every 10 min for 1 h, after which the culture volume was increased to 500 μl with RPMI supplemented with 5% heat inactivated normal AB human serum and 10 μg/ml of the aforementioned antibodies or 5% pooled HD or COVID-19 patient plasma. Cultures were then incubated at 37°C, 5% CO_2 for 48 h at which time cells were harvested and fixed for 20 min with 4% PFA and then stained.

Immunoglobulin G (IgG) from COVID-19 patient pooled plasma were depleted by protein A/G agarose resin and IgA depleted by Peptide M agarose. Control samples were incubated with agarose resin without coupled protein. C1A-B12 and C1A-H12, two SARS-CoV-2 Spike-targeting human monoclonal antibodies, were produced as previously described[58]. For blocking experiments, cells were incubated with 10 μg/ml monoclonal antibodies, α-CD16, α-CD32 (Clone IV.3 - Fig. 4j and ED Fig. 6a; Clone 6C4 - Fig. 4k and ED Fig. 6b,c), α-CD64, α-ACE2, and α-CD147 for 30 min, before virus infection. For antiviral drug treatment, monocytes were incubated at 37°C, 5% CO_2 for 1 h with 10 μM Remdesivir (GS-5734) or Camostat mesylate prior to infection. To find an appropriate Remdesivir concentration, serial dilutions between 10 and 80 μM were analyzed. To compare plasma obtained from patients with different disease severity, plasma was pooled based on the MGH acuity score (A1-A5), as described above.

To test the role of IgG afucosylation, IgG purified from COVID-19 patient serum samples, was analyzed by mass spectrometry to define the percentage of afucosylation as described[60]. Low afucosylated samples, kindly provided by Prof. Taia Wang (Stanford University), contained 8.41±0.67 % afucosylated IgG and high afucosylated samples, 30.1±1.5% afucosylated IgG. IgG was also purified from HD and COVID-19 patient pooled plasma using the Melon gel IgG spin purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Virus was preincubated with 10 μg/ml of purified IgG and the infection was performed as described above.

qRT-PCR RNA was extracted using Trizol reagent (Invitrogen) from COVID patient monocytes or from uninfected or infected HD monocytes (stimulated or not with LPS (100 ng/ml for 16 h)), then reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Random primers were used to generate cDNA for detection of cellular RNAs (*ACE2*, *BSG*, *ACTB*) and SARS-CoV-2 specific primers were used to generate cDNA to detect viral genomic RNAs (N1 region of *N* gene)[89]. cDNA was analyzed by qRT-PCR using the Sso Fast EvaGreen Supermix (BioRad) (30 sec at 95ºC, 40 cycles (3 sec at 95ºC; 3 sec at 54 ºC) using a CFX96 Touch Real-Time PCR Detection System (BioRad). To detect SARS-CoV-2 subgenomic RNA, qRT-PCR was carried out using a primer pair with the forward primer annealing to the 5' leader region of the viral genome and the reverse primer annealing to the 3' UTR. With the cycling conditions used (30 sec at 95 °C, 40 cycles (30 sec at 95°C, 30 sec at 60 °C, 90 sec at 72 °C)), full-length genomic RNA was not amplified, but small subgenomic RNA segments (<3 kB) could be amplified[33,90,91]. For each sample, Ct values were normalized to the *ACTB* Ct value. Primer sequences are given in Table S4. Subgenomic RNA qPCR products were also analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized on a Chemidoc imager (BioRad). The ~1600 nt band was excised and sequenced to confirm its origin as the SARS-CoV-2 subgenomic RNA encoding for N.

Plaque assays Vero E6 cells were seeded as monolayers in 24-well plates 1 d prior to infection. Virus-infected sample culture supernatants were serially diluted in DMEM. The plates were washed once with DPBS and then infected with 100 μl of diluted sample and incubated at 37 °C, 5% CO_2 for 1 h with rocking every 15 min. After 1 h, the inoculum was removed and an overlay of 1% methylcellulose (Sigma) in complete MEM (Gibco) was applied to each well. The plates were incubated at 37 °C until plaques were observable in positive control wells. To visualize plaques, the overlay was removed, and the cell monolayer was fixed with 4% PFA and stained with crystal violet. Plaques were then counted to quantify the virus titer in PFU/ml.

Statistical Analysis Statistical analysis was performed using GraphPad Prism V7.0. Normal distribution of the data was evaluated by the D'Agostino and Pearson normality test prior to applying statistical methods. Distributions were considered normal if $P \leq 0.05$. Parametric or non-parametric (Mann-Whitney test) two-tailed unpaired *t*-tests were used to compare two unpaired groups. Multiple group comparisons were analyzed by one-way ANOVA with Sidak's or Tukey's multiple comparisons tests, or non-parametric Kruskal-Wallis with Dunn's post-test. Multiple groups were compared by two-way ANOVA with additional Sidak's or Tukey's multiple comparisons test. Mean plasma values from hospitalized COVID-19 patients on each day were compared between severity groups by multiple unpaired *t*-tests. Correlations of plasma levels were determined by simple linear regression and Pearson correlation coefficient.

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