SARS-CoV-2 host-shutoff impacts innate NK cell functions, but antibody-dependent NK activity is strongly activated through non-spike antibodies

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Abstract

The outcome of infection is dependent on the ability of viruses to manipulate the infected cell to evade immunity, and the ability of the immune response to overcome this evasion. Understanding this process is key to understanding pathogenesis, genetic risk factors, and both natural and vaccine-induced immunity. SARS-CoV-2 antagonises the innate interferon response, but whether it manipulates innate cellular immunity is unclear. An unbiased proteomic analysis determined how cell surface protein expression is altered on SARS-CoV-2-infected lung epithelial cells, showing downregulation of activating NK ligands B7-H6, MICA, ULBP2, and Nectin1, with minimal effects on MHC-I. This occurred at the level of protein synthesis, could be mediated by Nsp1 and Nsp14, and correlated with a reduction in NK cell activation. This identifies a novel mechanism by which SARS-CoV-2 host-shutoff antagonises innate immunity. Later in the disease process, strong antibody-dependent NK cell activation (ADNKA) developed. These responses were sustained for at least 6 months in most patients, and led to high levels of pro-inflammatory cytokine production. Depletion of spike-specific antibodies confirmed their dominant role in neutralisation, but these antibodies played only a minor role in ADNKA compared to antibodies to other proteins, including ORF3a, Membrane, and Nucleocapsid. In contrast, ADNKA induced following vaccination was focussed solely on spike, was weaker than ADNKA following natural infection, and was not boosted by the second dose. These insights have important implications for understanding disease progression, vaccine efficacy, and vaccine design.
Introduction

COVID-19 studies have focused on interferon responses during the early innate phase of infection, and neutralising antibodies and virus-specific T-cells during the adaptive phase. In contrast, and despite their importance in antiviral protection, considerably less is known about the role of Natural Killer (NK) cells in infection. NK cells bridge the innate and adaptive responses, and individuals with NK cell deficiencies suffer severe viral infections. NK cells respond rapidly to viruses, directly killing infected cells by releasing cytotoxic granules. They promote inflammation through the release of TNFα and IFNγ, and influence the induction of both B- and T-cell responses. Once virus-specific antibodies are induced, antibody-dependent NK activation (ADNKA) is generated through antibody linking cell-surface viral antigens with NK cell Fc receptors and subsequent antibody-dependent cellular cytotoxicity (ADCC).

The majority of studies on NK cells have focussed on their adaptive roles in established COVID-19 infection, with highly activated NK cells seen in both the periphery and the lung. The genetic loss of NKG2C (a hallmark of adaptive NK cells, capable of enhanced ADCC) is also a risk factor for severe disease, implying a protective role in COVID-19 infection. In support of this, in animal models, Fc-mediated functions correlate with protection in vaccination, and monoclonal antibody-mediated control of SARS-CoV-2 infection is enhanced by Fc-dependent mechanisms, which enable control of disease even in the absence of neutralising activity. Furthermore, although neutralising monoclonal antibodies are effective when administered prophylactically, Fc-activity is required for efficacy when administered to animals therapeutically.

These animal studies demonstrate potentially important roles for adaptive NK cell responses in COVID-19. Despite this, our understanding of ADCC following infection and vaccination in humans remains limited. Studies to date have assumed spike protein is the dominant ADCC target, and have tested plate-bound protein or transfected cells. Using these systems, they have shown that ADNKA-inducing antibodies are generated following SARS-CoV-2 infection, are induced by vaccination in humans, and can be mediated by a subset of neutralising monoclonal antibodies targeting the spike protein. However, these responses have not been tested against SARS-CoV-2 infected cells; the presence,
abundance, accessibility, and conformation of viral glycoproteins can be very different
during productive infection. Furthermore, although viral entry glycoproteins such as spike
can be found on the infected cell surface, and can mediate ADCC, other viral proteins can be
the dominant mediators of ADCC during infection; indeed, we have shown that in other
viruses, non-structural proteins are often the dominant targets.

In addition, there is little information on the NK cell response to SARS-CoV-2 infected cells
during the innate phase of infection, despite this process being important to virus
pathogenesis; successful viruses must counteract robust NK activation to establish infection,
and their ability to do this is critical to their ability to cause disease. Whether SARS-CoV-
2’s ability to infect humans is dependent on viral evasion of innate cellular immunity, is
unknown. NK cell activation is complex and depends on the balance of ligands for a wide
range of inhibitory or activating receptors. These ligands can be induced on the surface of
target cells in response to stress, infection, or transformation. For example, the stress
ligands MICA, MICB, and ULBP1-6, all bind to the ubiquitously expressed activating receptor
NKG2D. To limit NK cell activation, many viruses manipulate these NK activating ligands,
reducing NK-cell mediated control. Whether similar manipulations underlie the ability of
SARS-CoV-2 to cause disease in humans remains unclear.

To address these critical gaps in our understanding of the SARS-CoV-2-specific NK cell
response, we used quantitative proteomics to determine how SARS-CoV-2 infection affected
cell surface protein expression. We found that SARS-CoV-2-infected cells downregulate
multiple activating NK ligands, resulting in a reduced NK cell response to infected cells. This
suggests a novel mechanism by which SARS-CoV-2 counteracts innate immunity early in
infection, to establish disease. The development of humoral immunity following natural
infection resulted in robust ADNKA against infected cells, and high levels of proinflammatory
cytokines; these responses persisted for at least 6 months in most patients. Proteomics
identified four cell-surface viral proteins capable of driving this response, leading to the
surprising discovery that ADNKA is not primarily driven by spike antibody, but was
dominated by antibody to other viral proteins such as nucleocapsid, membrane and ORF3a;
this defines a novel role for antibodies targeting the immunodominant nucleocapsid
protein. In agreement with these findings, and in contrast to plate-bound protein or
transfected cells, spike was a poor activator of ADNKA following vaccination when tested
using infected cells. This implies that the breadth and magnitude of ADNKA responses is limited in current vaccination strategies. The inclusion of antigens such as nucleocapsid would recruit additional effector mechanisms, and may help maintain vaccine efficacy following mutation of spike in naturally circulating virus variants.
Results

SARS-CoV-2 remodels the Plasma Membrane Proteome

Immune cells recognise and interact with infected cells through cell surface ligands. We therefore wanted to gain a comprehensive and unbiased overview of how SARS-CoV-2 infection changes the plasma membrane protein landscape. We performed plasma membrane enrichment through selective aminooxy-biotinylation (Plasma Membrane Profiling; PMP), a methodology that we previously developed\(^{28,29}\) and have validated extensively against human immunodeficiency virus (HIV)\(^{30}\), human cytomegalovirus (HCMV)\(^{23,29,31-33}\), and herpes simplexvirus (HSV)\(^{34}\) infection.

High infection rates are critical to minimise confounding effects from bystander cells. We therefore applied PMP to SARS-CoV-2 infected A549-ACE2-TMPRSS2 (AAT) cells, which are highly permissive to SARS-CoV-2\(^{27}\) and infection at a high multiplicity of infection (MOI) results in over 90% infection by 24h (Fig. 1A). We utilised TMT based quantification to compare plasma membrane (PM) protein abundance in uninfected cells, and cells infected for 12, 24, and 48 h (Fig 1B). In total, 4953 proteins were quantitated, including 2227 proteins with gene ontology annotation associated with the cell surface and plasma membrane (Fig 1-supplement 1A). Importantly however, the plasma membrane annotated proteins made up nearly 80% of the total protein abundance, indicating a high degree of enrichment for plasma membrane proteins, comparable with our previous PMP datasets\(^{23,28-34}\). The detection of low abundance proteins that are not plasma-membrane annotated reflects a combination of incorrect annotations, and contaminating intracellular proteins that were incompletely removed following PMP.

Five SARS-CoV-2 viral proteins were detected (see later), including spike, ORF3A, and membrane, which have previously been annotated as having a cell surface localisation (Fig. 1C). Consistent with prior reports, the viral receptor ACE2 was strongly downregulated after infection\(^{35}\), while TMPRSS2 expression was unaffected (Fig. 1C). Overall, of the 2227 PM proteins quantified with more than one peptide, 914 showed changes which were both significant and with a greater than 1.5-fold alteration over the course of the experiment. Clustering of proteins by their temporal pattern of change has previously shown utility in defining classes of regulated proteins and predicting underlying mechanisms\(^{30,36,37}\). Here, we...
used K-means clustering to define 5 groups of significantly altered proteins by their temporal profile.

To assess the innate immune response triggered by SARS-CoV-2 infection, the clusters were interrogated for the presence of a predefined list of interferon alpha-inducible genes. Thirteen of the 23 detected PM genes associated with the interferon response fell into cluster 5 (Fig 1-Supplement 1B, Supplementary File 2), which describes proteins with an increase in expression at 48 h, examples shown in Fig S1C. As the SARS-CoV-2 viral replication cycle is completed in less than 24 h\(^{38}\), SARS-CoV-2 is able to evade innate immune recognition and/or suppress IFN signalling until long after virus egress has been achieved.

To identify potential innate and adaptive immune evasion strategies employed by SARS-CoV-2, we focused on cluster 1, which describes proteins downregulated from early in the time course. This cluster was subject to gene ontology and pathway analysis to define proteins related by functional and structural similarities (Fig 2). Several classifications enriched in this cluster are particularly relevant, including the downregulation of several cytokine receptors, including IFNAR1. Much of the SARS-CoV-2 genome is dedicated to antagonising a type I interferon response within an infected cell\(^{38}\) and the plasma membrane downregulation of IFNAR1 presents a previously undescribed strategy to prevent signalling from exogenous IFN.

Also of note is the downregulation of heparan sulfate proteoglycans, the syndecan (SDC), glypican (GPC) and neurophilin (NRP) proteins, which together encompass the families of heparan sulfate modified proteins expressed on epithelial cells\(^{39}\). NRP1 is a cell entry factor for SARS-CoV-2\(^ {40,41}\), and SARS-CoV-2 spike is also reported to bind heparan sulphated proteins\(^ {42}\). The decrease in cell surface expression of heparan sulfated proteins may therefore enable the egress of viral particles and prevent super-infection, and is reminiscent of the capacity of other viruses to downregulate both primary viral receptors and co-receptors\(^ {43}\).
SARS-CoV-2 inhibits the synthesis of multiple NK cell ligands and reduces NK activation

Our experience with PMP has emphasised the modulation of NK ligands as an important immune evasion mechanism\textsuperscript{31,37}. As these ligands are poorly annotated in public databases, we investigated the PMP dataset for previously described ligands\textsuperscript{26}, of which 18 were detected (Fig 2-supplement 1). MHC-I proteins HLA-B to HLA-E were upregulated in cluster 5, likely reflecting a late IFN-mediated upregulation, while HLA-A was unchanged. By contrast, the activating NK-cell ligands MICA, ULBP2, B7-H6 (NCR3LG1), and Nectin1 (which is inhibitory in mice, but recently reported as activating in humans\textsuperscript{44}) were significantly downregulated in cluster 1. This was confirmed by flow cytometry in multiple cell types (Fig 3A, Fig 3-supplement 1). These responses were dependent on virus replication, because no alterations in ligand abundance were observed when virus was heat-inactivated (Fig 3-Supplement 2A).

Many of the best characterised mechanisms by which viruses inhibit the function of NK ligands involve inhibition of cell-surface transport and/or proteasomal or lysosomal degradation of the mature protein\textsuperscript{24,25}. To determine whether SARS-CoV-2 targeted NK ligands in the same way we measured their abundance, and their sensitivity to digestion with EndoH, which cleaves N-linked glycans from proteins that have not yet transited (or have been retained in) the endoplasmic reticulum (ER). Digestion with PNGaseF (which cleaves N-linked glycans irrespective of subcellular trafficking) served as a control to show the effect of complete deglycosylation. We were unable to detect endogenous NK ligands by western blot (data not shown), and therefore overexpressed MICA, ULBP2, and B7-H6 using replication deficient adenovirus vectors (RAd). Following infection with SARS-CoV-2, levels of all three NK ligands were substantially reduced, however there was no alteration in the pattern of EndoH sensitivity (Fig 3B). Thus SARS-CoV-2 infection does not result in intracellular retention of NK ligands, but instead reduces the overall abundance of protein.

To determine whether this occurred by inhibition of synthesis or degradation of mature protein, we treated cells with proteasomal (MG132) or lysosomal (Leupeptin) inhibitors after infection (Fig 3C). When tested with overexpressed MICA or B7-H6 (ULBP2 was not tested), MG132 did not result in recovery of NK ligand levels. Leupeptin resulted in a small recovery of B7-H6, however this was seen in both Mock and infected cells, implying that it
reflects constitutive turnover of the ligand rather than virus-specific targeting, and recovery in infected cells remained reduced compared to untreated mock infected cells. Overall, these results are consistent with the virus targeting NK ligands at the level of synthesis, rather than post-translationally. We therefore investigated whether NK ligands were particularly sensitive to inhibition of synthesis, by treating cells expressing MICA, B7-H6, or a control protein (GFP) with the translation inhibitor cycloheximide (ULBP2 was not tested). This resulted in rapid loss of MICA, ULBP2, and B7-H6, but not GFP (Fig 4A). Finally, to determine which viral proteins were responsible for these effects, we transfected cells with a library of previously validated plasmids expressing all 29 SARS-CoV-2 ORFs individually, and assessed them for levels of NK ligands on the cell surface. Only two SARS-CoV-2 ORFs resulted in a reduction of staining; Nsp1 and Nsp14 (Fig 4B). For MICA and ULBP2, Nsp14 consistently resulted in the strongest reduction, with Nsp1 being weaker. For Nectin1 and B7-H6, effects were weaker, but both Nsp1 and Nsp14 resulted in similar reductions.

Both Nsp1 and Nsp14 are involved in the ability of SARS-CoV-2 to degrade host RNA (Nsp1), and inhibit host protein translation (Nsp1 and Nsp14), and these activities have been implicated in their ability to counteract the interferon response within the infected cell. Together with our biochemical analysis, this data suggests that NK ligands are highly susceptible to inhibition of synthesis, and that SARS-CoV-2 manipulation of host protein production could impact the NK response in addition to the interferon response. To determine whether this was the case, we incubated SARS-CoV-2 infected cells with PBMC, and measured (i) degranulation of CD3\(^+\)CD56\(^+\) NK cells (Fig 4C; Figure 3-supplement 2B), (ii) production of IFN\(\gamma\) (Fig 4D) and (iii) TNF\(\alpha\) (Fig 4E, Fig 3-Supplement 2B). Consistent with data using other viruses, CD107a was recirculated on a higher proportion of NK cells than IFN\(\gamma\) and TNF\(\alpha\). Nevertheless, all three markers of NK activation were significantly reduced in SARS-CoV-2 infected versus mock infected cells, suggesting that the virus has evolved to inhibit NK cell responses. Comparable results were seen at both 24h and 48h post-infection (data not shown).

**NK-cell evasion is overcome by antibody-dependent activation**

In addition to their role in innate immunity through interactions with activating and inhibitory NK receptors early in infection, NK cells play additional roles following the
development of adaptive immunity via ADNKA/ADCC, in which CD16 interacts with the Fc portion of antibodies bound to targets on the surface of infected cells. To analyse this aspect of NK function, PBMC were incubated with infected cells in the presence of sera from individuals that were seronegative or seropositive for SARS-CoV-2. Certain NK cell subsets degranulate more effectively in response to antibody bound targets. These NK cells can be differentiated by the presence of NKG2C and CD57 on the cell surface. However, NKG2C is only found in a subset of donors who are HCMV seropositive, and a proportion of these individuals are NKG2Cnull due to a deletion in the KLRC2 gene. As a result, NKG2C cannot be used as a marker in all donors. We have previously shown that CD57 is sufficient to identify higher responding NK cells in all donors, and activation of this population correlates with the ability of antibodies to mediate ADCC. We therefore focussed on this population here; identical patterns of activation were observed in the CD57 negative population, albeit of slightly smaller magnitude (not shown). Non-specific activation was controlled for by testing against mock-infected cells throughout.

Degranulation (Fig 5A), as well as production of IFNγ (Fig 5B) and TNFα (Fig 5C), were all significantly increased in the presence of serum containing anti-SARS-CoV-2 antibodies, across a range of different NK donors (Fig 5D-F). ADNKA was dependent on virus replication, since heat inactivated virus did not lead to serum-dependent NK degranulation (Fig 5-Supplement 1A). NK cells were activated equivalently whether purified NK cells or PBMC were used (Fig 5-Supplement 1B), thus these responses are due to direct stimulation of NK cells, rather than other cells responding to antibodies and releasing NK stimulating cytokines. Viral immune evasion can therefore be overcome through antibody dependent mechanisms, suggesting that in addition to neutralising virus, SARS-CoV-2 antibodies may aid virus clearance through ADCC.

The magnitude of the neutralising antibody response to SARS-CoV-2 correlates with severity of disease; individuals with more severe disease generate higher levels of neutralising antibody. Although memory B-cell responses persist, levels of circulating antibody can decrease over time following resolution of disease, such that in some mildly infected people they become undetectable after a few months. To determine whether the same was true of ADCC responses, we assessed ADNKA in a cohort of donors with known disease
status, for whom longitudinal sera were available (Fig 5G). ADNKA responses were generally of greater magnitude in individuals with more severe disease, with responses remaining detectable during the 150-200 day follow-up. Amongst those with milder disease, strong initial responses were maintained over time, while weaker responses decreased, becoming virtually undetectable by around 200 days. There was a moderate correlation between the ability of antibodies to neutralise SARS-CoV-2, and to activate ADNKA in response to SARS-CoV-2 (Fig 5H), with a $R^2$ value of 0.7. Thus, although the antibodies mediating these activities may be induced in a similar manner, the antibodies mediating the two responses may not be identical.

**Multiple SARS-CoV-2 ORFs mediate ADCC**

There has been a major focus on spike as an activator of ADCC\textsuperscript{13-22}. It is certainly a potential target during infection as, despite the fact that virion particles bud internally rather than from the cell membrane, both our PMP and flow cytometric analysis detected substantial levels of spike protein on the infected cell surface (Fig 6A, 6C, 5-Supplement 1C, 5-Supplement 1D). However, our studies with other viruses have demonstrated that although viral proteins involved in virion binding and entry are found on the cell surface, and can be bound by antibody, they are not necessarily the strongest mediators of ADCC activity\textsuperscript{23}. PMP also detected membrane (M), nucleocapsid (N), ORF3a, and ORF1ab on the cell surface. ORF1ab is expressed as a polyprotein, which is proteolytically cleaved into multiple non-structural proteins (NSPs). Analysis of individual peptides representative of the proteolytically processed NSPs did not reveal any specific NSP enrichment. Furthermore, comparing the relative abundance of viral proteins in PMP, ORF1ab was only present at very low levels (1% of all viral proteins, significantly lower than any other SARS-CoV-2 protein). Taken together, it is unlikely that any NSPs are surface-exposed. Nevertheless, we performed bioinformatic analysis of all viral proteins for the presence of potential transmembrane domains or signal peptides, revealing five additional candidate cell-surface proteins (Nsp4, Nsp6, ORF6, ORF7a, ORF7b). We transfected cells with previously validated expression constructs for each of these genes\textsuperscript{45}, and screened them for their ability to activate NK cells in an antibody dependent manner. We used sera from patients with severe COVID-19 disease, that had previously generated the strongest ADNKA signals against infected cells (Fig 6B). As expected, spike primed a substantial ADNKA response. However, in
support of our PMP data, expression of ORF3a, membrane, and nucleocapsid, were also capable of priming ADNKA in the presence of serum containing SARS-CoV-2 antibodies, while the Nsps did not.

Interestingly, nucleocapsid readily activated ADNKA despite the fact that this protein has previously been assumed to be intracellular. We have previously observed the exquisite sensitivity of ADNKA, which can be activated in response to levels of virus protein that are difficult to detect by flow cytometry. We have also shown that antigen levels can be very different in the context of virus infection, compared with transfection. In support of this, nucleocapsid levels were much higher on the surface of infected cells than transfected cells (Fig 6C); detection of nucleocapsid was dependent on virus replication (Fig 5-Supplement 1C), and occurred following infection of multiple different cell types (Fig 5-Supplement 1D).

The presence of nucleocapsid on the cell surface was also supported by immunofluorescence staining of non-permeabilised infected cells (Figure 6D), where distinct patches of nucleocapsid were observed that sometimes, but not always, coincided with cell-cell junctions. Given how well even very low levels of surface nucleocapsid activated ADNKA, this suggested that multiple proteins could be major contributors to ADCC during SARS-CoV-2 infection.

Monoclonal anti-spike antibodies only weakly mediate ADNKA despite binding strongly to infected cells

To determine the relative contribution of spike, and of different antigenic sites on spike, to ADNKA during SARS-CoV-2 infection, we tested a panel of 26 human anti-spike monoclonal antibodies, which had been isolated from naturally infected donors, and cloned as IgG1 constructs. The panel included antibodies targeting the RBD, S1, NTD, and S2 domains, neutralising and non-neutralising, and those targeting multiple distinct epitopes (Supplementary File 4).

All 26 antibodies bound efficiently to infected cells, at levels comparable to polyclonal serum (Fig 7-supplement 1). However, only five of these antibodies were capable of triggering degranulation (Fig 7A), of which two triggered TNFα (Fig 7-Supplement 2A), and one IFNγ (Fig 7-Supplement 2B). Importantly, these NK responses were at dramatically lower
levels than seen with polyclonal serum from a naturally infected donor, even when combined (Fig 7B).

A number of previous publications have used cells transfected with spike expression constructs to suggest that monoclonal anti-spike antibodies can induce potent ADNKA, which would appear to contradict our data$^{6-11}$. We therefore tested a subset of our monoclonal antibodies against transfected, as opposed to infected, cells (Fig 7C). mAbs that induced ADNKA against infected cells also induced ADNKA against transfected cells (P008-014 and P054-044). However, of 12 antibodies that failed to activate ADNKA against infected cells, 5 induced potent ADNKA against transfected cells. Furthermore, in contrast to infected cells, the level of ADNKA induced by mAbs was extremely high against transfected cells, with many exceeding that of our control seropositive serum. Thus, despite a substantial ability to activate ADCC against cells transfected with spike protein, and high levels of antibody binding, spike was a relatively poor ADCC target in the context of natural infection when using mAbs.

**Following natural infection, ADNKA responses are dominated by non-spike antibodies**

Although our data using monoclonal antibodies indicated that spike was a comparatively weak activator of ADNKA, the antibodies that mediate ADNKA against infected cells may represent a minor proportion of the overall anti-spike response; we simply may not have captured those antibodies in our panel. To directly address this concern, we specifically depleted anti-spike antibodies from the serum of naturally infected individuals, and assessed changes in activity. ELISA analysis confirmed the effective depletion of spike-specific antibodies (Fig 8A), which was accompanied by a significant loss of neutralisation activity (Fig 8B), across multiple donors (Fig 8C). However, there was no loss of ADNKA activity from any of these samples following spike-antibody depletion (Fig 8D, E), consistent with the results of our monoclonal antibody data. We conclude that spike-specific antibodies dominate the neutralising antibody response, but play a minor roles in the ADCC response following natural infection.

Since nucleocapsid, ORF3a, and membrane had activated ADNKA when expressed in isolation, we therefore investigated whether they also mediated ADNKA against virus infected cells. A nucleocapsid-specific human monoclonal antibody was capable of
activating ADNK A against infected cells (Fig 8F) and, although not to the extent of polyclonal serum, was superior to our best anti-spike antibody. Furthermore, when we used the serums from which Spike antibodies had been depleted (Fig 8D-E), and also depleted anti-nucleocapsid antibodies, we saw a significant reduction in ADNK A activity (Fig 8G-H), although considerable activity remained. Reagents are not available to deplete antibodies targeting ORF3a or membrane, so we instead deleted ORF3a from the virus genome. In line with existing data, this resulted in a small reduction (~1log) in virus titres (not shown). We therefore assessed levels of viral antigens on the cell surface following infection, which revealed that deletion of ORF3a resulted in a small reduction in levels of spike and nucleocapsid, but not membrane (Fig 8I). To ensure that these alterations did not affect readouts, we used serums that had been depleted for antibodies targeting both Spike and Nucleocapsid, and compared ADNK A responses against wildtype or \( \Delta \)ORF3a virus (Fig 8J-K). Deletion of ORF3a resulted in a dramatic reduction in ADNK A activity, although this was not reduced completely to the level of seronegative serum. We therefore conclude that nucleocapsid and ORF3a are targets for ADNK A activity against SARS-CoV-2 infected cells, with membrane potentially also contributing.

**Spike-specific antibodies following vaccination are weak mediators of ADNK A**

Since non-spike antigens are required to efficiently prime ADCC following natural infection, we investigated individuals who have been vaccinated against spike, but not exposed to the virus - since they will only have spike-specific antibodies. As with serum from naturally infected individuals, ELISA demonstrated effective depletion of spike antibodies (Fig 9A), which was accompanied by a marked loss of virus neutralisation activity (Fig 9B, C). In functional NK assays, these sera only mediated weak ADNK A (Fig 9D, E) and, unlike sera from naturally infected individuals, ADNK A activity was abrogated following depletion of spike antibodies. When we compared responses following natural infection or vaccination, vaccine mediated ADNK A was significantly weaker than responses seen following natural infection, and was not boosted following the second dose of the vaccine (Fig 9F). This was in stark contrast with the neutralisation activity of those same serum samples, which were dramatically increased following the second dose, with activity comparable to patients with severe COVID-19 disease (Fig 9G).
Thus, anti-spike antibodies can mediate ADNKA, but this activity is significantly weaker than responses seen during natural infection, which targets a broader repertoire of viral antigens.
Discussion

The host innate immune system plays a critical role in both early SARS-CoV-2 infection, and later COVID-19 disease. This is emphasised by the identification of multiple interferon antagonists encoded by SARS-CoV-2, and the increased disease severity in patients with genetic and acquired interferon deficiencies. To determine whether the virus evades other components of the host immune system we took a proteomic approach to systematically analyse how SARS-CoV-2 infection manipulates the cell surface during the time course of SARS-CoV-2 infection. We find that SARS-CoV-2 remodelling of the plasma membrane leads to the downregulation of multiple cell surface immune ligands involved with the interferon response, cytokine function, and NK activation. This may enable SARS-CoV-2 to evade numerous effector arms of the host immune system. Of particular interest, NK activating ligands appear particularly susceptible to viral shutoff of host gene expression, and this results in inhibition of NK cell activation. This provides an explanation for how small RNA viruses may be able to target the NK response without encoding the large repertoire of specific NK-evasins seen in the large DNA viruses, and indicates that shutdown of host transcription and translation is likely to play a broader role in the ability of the virus to establish initial infection than previously appreciated. Later in disease, following the production of antibody, ADNKA leads to significant degranulation, and production of pro-inflammatory cytokines. Somewhat surprisingly, spike antibody is not responsible for the robust ADNKA which develops after natural infection, and only weak ADNKA is generated following spike vaccination.

Genetic variation in both viruses and their hosts affects the ability of viruses to modulate NK responses, and influence pathogenesis. SARS-CoV-2 appears to have evolved to antagonise NK activation by targeting the expression levels of multiple activating NK ligands. Nectin-1 is a ligand for CD96/TACTILE, which activates human NK cells, B7-H6 is a ligand for Nkp30, while ULBP2 and MICA are ligands for NKG2D. NKG2D is ubiquitously expressed on γδ cells and CD8+, as well as some CD4+, T-cells in addition to NK cells. Targeting NKG2D ligands may therefore enable the virus to impact additional effector mechanisms beyond just NK cells. Amongst NKG2D ligands, MICA is particularly polymorphic (100+ alleles), A549 cells express MICA*001/004, while the truncated MICA*008 is the most common allele worldwide. This diversity is largely driven by co-evolution of humans with
virus infections, and reflects the broad repertoire of viral immune-evasins. MICA variation therefore has the potential to play a role in the extremely variable outcome of SARS-CoV-2 infection.

A recent study demonstrated that NK cells stimulated with IL-12 and IL-15 were capable of controlling SARS-CoV-2 following infection of VeroE6 or Calu3 cells. NK ligands are highly diverse between species, thus VeroE6 (i.e. African green monkey) cells cannot inform on the ability of human NK cells to control infection, while expression levels of NK ligands were not assessed on Calu3 cells, making it difficult to draw comparisons between the studies. Nevertheless, NK cell activity is heavily dependent on stimulation, and it is highly likely that strongly activating cytokines such as IL-12/IL-15 are capable of stimulating NK cells sufficiently to contribute to viral control despite viral evasion mechanisms.

In contrast to the effect on NK ligands, we observed minimal downregulation of MHC-I, implying that SARS-CoV-2 cannot prevent peptide presentation to antagonise adaptive cytotoxic cellular immunity. Nor do infected cells bind human IgG from seronegative donors, indicating that it does not encode Fc receptors as ADNKA decoys. These observations are consistent with SARS-CoV-2 acting as an acute ‘hit and run’ virus, which has replicated and transmitted before host adaptive immunity develops. A previous study has demonstrated that SARS-CoV-2 downregulates HLA-A2 via ORF8, which contrasts somewhat with our results. This may indicate a HLA- or cell-type specific effect, it will be interesting to determine whether coronaviruses evade innate and adaptive immunity in their original bat host, and whether this contributes to their persistence in that species.

In animal models of vaccination and monoclonal antibody administration against SARS-CoV-2, antibody-mediated activation of cellular immunity is a critical component of the protective immune response. However, inflammation dominates severe COVID-19 disease. The high levels of TNFα and IFNγ observed following ADNKA are likely to exacerbate disease under these circumstances. In support of this, TNFα and IFNγ correlate with severe disease, and critically ill patients have a higher proportion of afucosylated antibodies that may promote a stronger ADCC response. Given that ADCC can potentially contribute to both protection and immunopathology, it is important to understand what determines the magnitude and quality of the ADCC response. Viral entry glycoproteins are a
common focus as mediators of ADCC. E.g. ADCC mediated by Env antibodies have been a major focus in HIV\textsuperscript{68}, and gB-specific antibodies have been studied in HCMV\textsuperscript{69}. Although antibodies targeting these antigens can clearly bind infected cells, using similar approaches to the present study we recently showed that non-structural accessory proteins were more potent ADCC targets during HCMV infection\textsuperscript{23}. This conclusion is reinforced here, where the neutralising antibody response is dominated by spike, but spike antibodies are poor activators of ADNKA, with other viral antigens driving more efficient ADNKA following natural infection. In support of this data, monoclonal anti-spike antibodies were only effective at activating Fc-mediated control of infection in animal models following engineering to enhance activity\textsuperscript{12}. Numerous spike mAbs were capable of potently activating ADNKA against transfected cells, but failed to stimulate NK cells in the presence of infected cells; this may result from differential post-translational modifications of spike between these contexts, the fact that the reduction in activating NK ligands as a result of infection alters the threshold for activation through ADNKA, or the fact that viral CPE affects the formation of an immunological synapse\textsuperscript{70}. Nevertheless, this underscores the importance of testing immune responses against live virus infected cells in order to assess efficacy. Importantly, the ability of monoclonal spike antibodies to bind to infected cells did not correlate with their ability to mediate ADNKA, implying that the epitope, position, or angle, at which the antibody binds to the cell surface is critical; it’s notable that 4/5 anti-spike antibodies that activated ADNKA bound the NTD, suggesting that this site is structurally advantageous for Fc-mediated responses.

A similar phenomenon may underly the greater ability of non-spike antigens to generate a ADNKA response. Nucleocapsid, ORF3a, and membrane are all present on the infected cell surface, and can promote ADNKA if appropriate antibodies are present. In some studies, antibodies targeting ORF3a and membrane have been reported as present in only a subset of patients, while almost everyone developed antibodies targeting nucleocapsid\textsuperscript{71}. However other studies have shown that antibodies targeting ORF3a and membrane are much more common and found in most patients\textsuperscript{72}. Our functional work is consistent with this latter data, and suggests that antibodies targeting all three proteins may be major targets for ADCC during natural SARS-CoV-2 infection, identifying a potential functional role for antibodies binding these immunodominant targets for the first time. Although it is
surprising to observe nucleocapsid on the cell surface, Influenza nucleoprotein is also detected at the plasma membrane\textsuperscript{73-75}, and antibodies targeting nucleoprotein can mediate ADCC\textsuperscript{76-78}. Structural proteins involved in genome packaging may therefore represent a common target for ADCC across multiple virus families.

The identification of the antigens that drive ADCC is important to our understanding of the mechanisms that underlie pathogenesis and disease. A skewing of the spike:nucleocapsid antibody ratio is associated with severe disease\textsuperscript{79-81}, where ADCC-mediating nucleocapsid antibodies might be promoting inflammation. Both the breadth of the nucleocapsid antibody response, and the particular epitopes targeted, have also been correlated with disease severity\textsuperscript{71,82}. These correlations now need to be followed up functionally, to determine how the epitopes bound by anti-nucleocapsid antibodies affect ADCC. Critically, the same surface-bound antibodies that activate NK cells are likely to promote inflammation by activating myeloid cells and complement\textsuperscript{83-85}, both of which are hallmarks of severe COVID-19.

Although Fc-dependent mechanisms of cellular immune activation could contribute to immunopathology via multiple mechanisms in severe disease, all patients with mild/asymptomatic disease also developed strong and persistent ADNKA responses. Furthermore, lineage defining mutations in the viral variants of concern B.1.1.7, B.1.351, and P.1, include non-synonymous mutations in membrane, ORF3a, and nucleocapsid, and these can result in reduced antibody binding\textsuperscript{86,87}. Virus variants may therefore evolve to evade ADCC responses as well as neutralising responses, supporting an important role for ADCC in protection. It is therefore significant that spike-vaccine generated antibody responses were poor ADNKA inducers when tested against infected cells. The addition of other viral proteins such as nucleocapsid to vaccines would engage a wider range of immune effector pathways. This approach might improve efficacy against both viral transmission and disease, resulting in vaccines that are more resistant to viral variants containing mutations that diminish antibody neutralisation\textsuperscript{88}. 
Acknowledgements

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### Materials and methods

#### Key Resources Table

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**Cells and viruses**

All cell lines tested negative for mycoplasma, and A549 were authenticated by Short tandem Repeat analysis. A549 were transduced with lentiviruses expressing human ACE2, and TMPRSS2 (AAT cells), as previously described\(^27\). The England2 strain of SARS-CoV-2 was obtained from Public Health England (PHE), and grown on VeroE6 cells. This strain has a genome that is identical to the original Wuhan isolate. A virus lacking ORF3a, along with the parental virus, was a kind gift from Luis Martinez-Sobrido (Texas Biomedical Research Institute)\(^89\). Virions were concentrated by pelleting through a 30% sucrose cushion to remove contaminating soluble proteins, and titrated by plaque assay on both VeroE6, AAT, and Caco2, as previously described\(^27\). All cell lines were grown in DMEM containing 10% FCS (Gibco), at 37°C and in 5% CO\(_2\). Throughout the study, multiple batches of virus were used, and no overt differences were noted between them. For assays, cells were plated out the day before, then infected at a multiplicity of infection (MOI) of 5, for 1h on a rocking platform. The inoculum was removed, and fresh DMEM containing 2% FCS was added, then cells were incubated until the assay. To confirm that phenotypes were dependent on active virus replication, virus was inactivated by heating to 56°C for 30 minutes.

**Plasma membrane profiling**

Cell surface proteins were labelled essentially as described\(^29,90\). Briefly, cells were incubated in a solution containing sodium periodate, aniline and aminooxy biotin to label predominantly sialic acid containing glycans at the cell surface. Cell lysates were then enriched for labelled proteins using streptavidin-agarose beads. After extensive washing
trypsin was added to liberate peptides of the enriched proteins. The resulting peptide pools were dried prior to labelling with TMT reagents.

TMT Labelling and clean-up

Samples were resuspended in 21µL 100mM TEAB pH 8.5. After allowing to come to room temperature, 0.2mg TMT reagents (Thermo Fisher) were resuspended in 9µL anhydrous acetonitrile (ACN) which was added to the respective samples and incubated at room temperature for 1h. A 3µL aliquot of each sample was taken and pooled to check TMT labelling efficiency and equality of loading by LC-MS. After checking each sample was at least 98% TMT labelled total reporter ion intensities were used to normalise the pooling of the remaining samples such that the final pool was as close to a 1:1 ratio of total peptide content between samples as possible. This final pool was then dried in a vacuum centrifuge to evaporate the majority of ACN form labelling. The sample was acidified to a final 0.1% Trifluoracetic Acid (TFA) (~200µL volume) and fluoroacetic acid (FA) was added until the SDC visibly precipitated. 4 volumes of ethyl acetate were then added and the sample vortexed vigorously for 10s. Sample was then centrifuged at 15’000g for 5mins at RT to effect phase separation. A gel loading pipette tip was used to withdraw the lower (aqueous) phase to a fresh low adhesion microfuge tube. The sample was then partially dried in a vacuum centrifuge and brought up to a final volume of 1mL with 0.1% TFA. FA was added until the pH was <2, confirmed by spotting onto pH paper. The sample was then cleaned up by SPE using a 50mg tC18 SepPak cartridge (Waters). The cartridge was wetted with 1mL 100% Methanol followed by 1mL ACN, equilibrated with 1mL 0.1% TFA and the sample loaded slowly. The sample was passed twice over the cartridge. The cartridge was washed 3x with 1mL 0.1% TFA before eluting sequentially with 250µL 40% ACN, 70% ACN and 80% ACN and dried in a vacuum centrifuge.

Basic pH reversed phase fractionation

TMT labelled samples were resuspended in 40µL 200mM Ammonium formate pH10 and transferred to a glass HPLC vial. BpH-RP fractionation was conducted on an Ultimate 3000 UHPLC system (Thermo Scientific) equipped with a 2.1 mm × 15 cm, 1.7µ Kinetex EVO column (Phenomenex). Solvent A was 3% ACN, Solvent B was 100% ACN, solvent C was 200 mM ammonium formate (pH 10). Throughout the analysis solvent C was kept at a constant
The flow rate was 500 µL/min and UV was monitored at 280 nm. Samples were loaded in 90% A for 10 min before a gradient elution of 0–10% B over 10 min (curve 3), 10-34% B over 21 min (curve 5), 34-50% B over 5 mins (curve 5) followed by a 10 min wash with 90% B. 15s (100µL) fractions were collected throughout the run. Fractions containing peptide (as determined by A280) were recombined across the gradient to preserve orthogonality with on-line low pH RP separation. For example, fractions 1, 25, 49, 73, 97 are combined and dried in a vacuum centrifuge and stored at -20°C until LC-MS analysis. 24 Fractions were generated in this manner.

**Mass Spectrometry**

Samples were analysed on an Orbitrap Fusion instrument on-line with an Ultimate 3000 RSLC nano UHPLC system (Thermo Fisher). Samples were resuspended in 10µL 5% DMSO/1% TFA and all sample was injected. Trapping solvent was 0.1% TFA, analytical solvent A was 0.1% FA, solvent B was ACN with 0.1% FA. Samples were loaded onto a trapping column (300µm x 5mm PepMap cartridge trap (Thermo Fisher)) at 10µL/min for 5 minutes at 60 degrees. Samples were then separated on a 75cm x 75µm i.d. 2µm particle size PepMap C18 column (Thermo Fisher) at 55 degrees. The gradient was 3-10% B over 10mins, 10-35% B over 155 minutes, 35-45% B over 9 minutes followed by a wash at 95% B for 5minutes and reequilibration at 3% B. Eluted peptides were introduced by electrospray to the MS by applying 2.1kV to a stainless-steel emitter (5cm x 30µm (PepSep)). During the gradient elution, mass spectra were acquired with the parameters detailed in Fig 1-Supplement 2 using Tune v3.3 and Xcalibur v4.3 (Thermo Fisher).

**Data Processing**

Data were processed with PeaksX+, v10.5 (Bioinformatic Solutions). Processing parameters are shown in detail in Supplementary Fig S9. Briefly, .raw files were searched iteratively in three rounds, with unmatched de novo spectra (at 1% PSM FDR) from the previous search used as the input for the next. The three iterations were as follows 1) Swissprot Human (27/03/2020) + common contaminants 2) The same databases as search 1 but permitting semi-specific cleavage 3) trEMBL Human (27/03/2020), with specific cleavage rules. Proteins were then quantified using the parameters outlined in Fig 1-supplement 3. Identified proteins and their abundances were output to .csv format and further subjected to
statistical analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository\(^91\) with the dataset identifier PXD025000 and 10.6019/PXD025000. Supplementary File 1 contains the analysed data.

**Statistical analysis and K-means clustering.**

Prior to statistical analysis, proteins were filtered for those quantified across all TMT reporter channels and with more than one unique peptide. Only proteins with a plasma membrane related GO:CC annotation as previously described\(^28\) were then carried forward for statistical analysis. All SARS-CoV-2 proteins quantified with more than one unique peptide were also included. Statistical tests were performed with the `aov` and `p.adjust` functions in the R base stats package (version 4.0.3) to calculate Benjamini-Hochberg corrected p-values for changes in protein abundance across the measured time-points.

Proteins with a p-value < 0.05 and a maximum fold-change across the time-course of > 1.5-fold were selected for k-means clustering. Mean intensities of each time-point were scaled using the `scale` function in base R and utilised to cluster proteins into 5 groups using the `kmeans` function in R base stats package.

**Functional Annotation Clustering**

Assessment of enriched gene annotation terms in temporal cluster one was carried out using the Functional Annotation Clustering tool at DAVID (david.ncifcrf.gov) v6.8\(^92\), using the default clustering settings for medium stringency and the following libraries: Uniprot UP_Keyword, GOTERM:MF_ALL, BIOCARA,, KEGG_PATHWAY and REACTOME_PATHWAY. Output clusters were curated with representative names based on the enriched terms within. The background for enrichment was a list of proteins detected in the PMP dataset with more than one peptides and a plasma membrane associated GO:CC term. Functional annotation clusters with an enrichment score of >1.5 are shown, and any clusters in which no individual annotation term had a Benjamini-Hochberg correct p-value of <0.05 were excluded.
Biochemical analysis of NK ligands

Replication deficient adenovirus vectors (RAd) expressing B7-H6, MICA, and ULBP2, have been described previously. To analyse glycosylation of these proteins, cells were infected with RAd-MICA and RAd-GFP at MOI=100 and RAd-B7-H6 and RAd-ULBP2 at MOI=500, then 48h later cells were either mock-infected or infected with SARS-CoV-2 MOI=5 for a further 24h. For some experiments, lysates were digested overnight at 37°C with EndoH or PNGaseF (NEB) according to manufacturer’s instructions. In some experiments, MG132 (10µM), Leupeptin (200µM) or cycloheximide (CHX, 2.5µg/ml) inhibitors were added 18h prior to harvest. Samples were then analysed by SDS-PAGE followed by western blotting for proteins of interest. Membranes were stained with primary antibodies (anti-MICA/B, BAMOMAB, BAm01, 1:2000; anti-B7-H6, Abcam, ab121794, 1:2000; anti-ULBP-2, R&D Systems, AF1298, 1:2000; anti-GFP, Fl, SC-8334, Santa Cruz Biotechnology, 1:2000; anti-Spike, Genetex, 1A9, 1:2000; anti-Actin, Sigma, 1:2000), followed by anti-mouse, anti-rabbit or anti-goat HRP-conjugated secondary antibody, then developed using Supersignal West Pico, and imaged in a Syngene XX6.

Immunofluorescence

Cells were plated on coverslips, then infected with SARS-CoV-2. After 24h, cells were fixed with 4% paraformaldehyde, then stained with nucleocapsid mAb (1C7), followed by anti-mouse AF594, Phalloidin-AF488, and DAPI. Coverslips were washed, then mounted in prolong gold anti-fade (Thermofisher), before being imaged on a Zeiss LSM800 confocal microscope.

Flow cytometry

Cells were dissociated using HyQtase, then stained with primary antibody for 30 min at 4°C. Following washing, they were incubated with secondary antibody, again for 30 min at 4°C. Cells were washed, fixed in 4% paraformaldehyde, and data collected on an Attune cytometer (Thermofisher). In one experiment (Fig 1A), cells were fixed and permeabilised (Cytofix/Cytoperm, BD) before staining. Antibodies used were against HLA-ABC (W632; AbD Serotec), NCR3LG1/B7-H6 (MAB7144, Biotechne R&D Systems), Nectin 1 (R1.302; Biolegend), MICA (AMO1-100; BAMOMAB), MICB (BMO2-100; BAMOMAB), ULBP2 (BUMO1;
ELISA

To measure levels of spike antibodies, an ELISA using the spike trimer (Acro Biosystems) was used according to manufacturers instructions. Each sample was measured in duplicate, and compared to a standard curve. A pre-pandemic serum was included in each assay to define the cut-off.

Plasmids and transfections

Lentivirus plasmids encoding each SARS-CoV-2 ORF individually, with a C-terminal twin-strep tag, were obtained from Addgene, and have been validated for expression previously\(^45\). Plasmids were midiprepped (Nucleobond Xtra Midi; Machery-Nagel), and transfected into 293T cells using GeneJuice (Merck) according to manufacturers’ instructions.

NK activation assays

PBMCs from healthy donors were thawed from liquid N\(_2\) storage, rested overnight in RPMI supplemented with 10% FCS, and L-glutamine (2 mM). For assays investigating viral inhibition of NK activation, PBMC were stimulated overnight with IFN-α (1,000 U/ml) to provide a baseline level of activation against which inhibition could be seen. For ADNKA this stimulation is not required, and so cells were used unstimulated\(^23\). To confirm that phenotypes were due to direct effects on NK cells, NK cells were purified by depletion, using the human NK cell purification kit (Miltenyi) according to manufacturer’s instructions. Target cells were harvested using TrypLE Express (Gibco), preincubated for 30 min with the relevant antibody or serum preparations, then mixed with effectors at an effector:target (E:T) ratio of 10:1 (PBMC) or 1:1 (purified NK cells) in the presence of golgistop (0.7 μl/ml, BD Biosciences), Brefeldin-A (1:1000, Biolegend) and anti-CD107a–FITC (clone H4A3, BioLegend). Cells were incubated for 5 h, washed in cold PBS, and stained with live/dead Fixable Aqua (Thermo Fisher Scientific), anti-CD3–PECy7 (clone UCHT1, BioLegend) or anti-CD3–BV711 (Clone SK7, Biolegend), anti-CD56–BV605 (clone 5.1H11, BioLegend), anti-CD57–APC (clone HNK-1, BioLegend) or anti-CD57–PECy7 (clone HNK-1, Biolegend), and anti-NKG2C–PE (clone 134591, R&D Systems). In some experiments, cells were also
fixed/permeabilized using Cytofix/Cytoperm (BD Biosciences) and stained with anti-TNFα–BV421 (clone MAb11, BioLegend) and anti-IFNγ–APC (clone 4S.B3, BioLegend). Data were acquired using an AttuneNxT (Thermo Fisher) and analyzed with Attune NxT software or FlowJo software version 10 (Tree Star). Individual assays were run in technical triplicate. To ensure inter-assay variation did not affect results, a donor serum demonstrating moderate ADCC activity against SARS-CoV-2 was included as a positive standard in every assay, while a serum collected before 2020 was included as a negative control in every assay. Where sera were tested at a range of dilutions, the area under the curve (AUC) was calculated using Graphpad Prism 9. This value was then normalised to the AUC for the standard serum in that particular assay. In all experiments, serums were additionally tested against mock infected cells to control for non-specific activation of NK cells by serum components. An example gating strategy is shown in figure 4-supplement 1, and involved gating on NK cells defined as CD56 positive, CD3 negative. This strategy will capture all ‘classical’ NK cells, however it will miss the recently described CD56 negative NK cell population. Since these are only present at substantial levels in specific conditions such as chronic HIV infection, and older people with HCMV or EBV infection, and have not been described to expand during SARS-CoV-2 infection, they are unlikely to play a major role in the NK response examined herein.

Serums

Serums were collected from healthy donors vaccinated with the BNT162b2 vaccine, a minimum of 3 weeks after the first dose, or 1 week after the second. A pre-vaccine sample was taken from every donor, and an ELISA for SARS-CoV-2 RBD performed as previously described, to determine pre-exposure to live SARS-CoV-2. Longitudinal serums from naturally infected individuals have been described previously, clinical characteristics are in Supplementary File 3. Those labelled ‘mild’ had severity scores of 0, 1, 2 or 3, and ‘severe’ was 4 or 5, as described previously.

Virus neutralisation assays

600PFU of SARS-CoV-2 was incubated with appropriate dilutions of serum, in duplicate, for 1h, at 37°C. The mixes were then added to pre-plated VeroE6 cells for 48h. After this time, monolayers were fixed with 4% PFA, permeabilised for 15 min with 0.5% NP-40, then
blocked for 1h in PBS containing 0.1% Tween (PBST) and 3% non-fat milk. Primary antibody (anti-nucleocapsid 1C7, Stratech, 1:500 dilution) was added in PBST containing 1% non-fast milk and incubated for 1h at room temperature. After washing in PBST, secondary antibody (anti-mouse IgG-HRP, Pierce, 1:3,000 dilution) was added in PBST containing 1% non-fat milk and incubated for 1h. Monolayers were washed again, developed using Sigmafast OPD according to manufacturers’ instructions, and read on a Clariostar Omega plate reader. Wells containing no virus, virus but no antibody, and a standardised serum displaying moderate activity were included as controls in every experiment. NT50 were calculated in Graphpad Prism 9.

**Antibody depletions**

Anti-spike or anti-nucleocapsid antibody was depleted from sera using magnetic bead conjugated spike trimer protein or nucleocapsid protein (Acrobiosystems). Beads were resuspended in PBS+0.05% BSA, then 50µl serum was mixed with 150µl beads. Mixtures were incubated on a rotating mixer at 4°C overnight. Serum diluted in buffer alone was used as a control. Magnetic beads were then removed using a 3D printed magnetic stand. All values given in assays are corrected for this initial 4-fold dilution. Levels of anti-spike antibody were measured using a spike trimer ELISA (Acrobiosystems).

**Ethics**

PBMC were extracted from apheresis cones obtained from the Welsh Blood Service (WBS) via an ad-hoc agreement or from blood samples from healthy volunteers and stored in liquid N₂ until use. Informed consent, and consent to publish, was obtained for use of healthy volunteer PBMC for this project, including those from WBS. This was ethically approved by the Cardiff University School of Medicine Research Ethics Committee (SMREC) nos. 20/55 and 20/101. Recruitment of healthy volunteers after vaccination was covered by the Cardiff University School of Medicine Research Ethics Committee under reference no. 18/04.
References


Fig 1. SARS-CoV-2 remodels the Plasma Membrane Proteome. (A) AAT cells were infected with SARS-CoV-2 in biological triplicate (MOI=5). 24h later they were detached with trypsin, fixed and permeabilised, stained for Spike protein, and analysed by flow cytometry. (B) Schematic of plasma membrane profiling and analysis pathway. (C). Examples of temporal profiles of viral and cellular genes, fold change is compared to 0 h timepoint. Data points show mean ±SD. (D). Left – heat map of the 914 significantly changing proteins clustered by k-mean, colour indicates log₂ fold change compared to 0 h, right, Z-score normalised temporal profiles of proteins within each cluster.
Figure 1-Figure Supplement 1. Plasma Membrane Proteins are enriched in the PMP dataset. (A) Pie charts showing the proportion of proteins falling into previously defined annotation categories for plasma membrane proteins by left, protein abundance, or right, protein identifiers. (B) Pie chart showing the distribution of proteins defined by the Molecular Signatures Database (MSigDB) gene set “Hallmark Interferon Alpha Response” into the defined temporal clusters. (C). Example temporal profiles of type I interferon inducible genes upregulated in cluster 5, y-axis shows fold change compared to 0 h timepoint.
Figure 2. SARS-CoV-2 manipulates multiple classes of plasma membrane proteins. Enriched gene ontology and pathway annotations of proteins downregulated in temporal cluster 1 were condensed to groups of related terms, bar chart shows the enrichment score of these groups. Examples of annotation terms falling within each group are shown underlined with Bejamani-Hochberg adjusted P-values in parentheses, alongside four examples of proteins for each annotation.
Figure 2-Figure Supplement 1. NK ligands detected in the PMP Dataset. Temporal profiles of NK-cell ligands detected in the PMP dataset, y-axis shows fold change compared to 0 h timepoint.
**Figure 3. SARS-CoV-2 infection inhibits the expression of multiple NK ligands.** (A) AAT cells were either mock infected, or infected with SARS-CoV-2 for 24h (MOI=5), detached using TrypLE, then stained for the indicated NK cell receptors before being analysed by flow cytometry (bottom). Plots for the same proteins from PMP are included for reference (top). (B) AAT cells were infected with RAd-MICA, RAd-ULBP2, or RAd-B7-H6, then after 48h either mock infected, or infected with SARS-CoV-2 (SCV2) for a further 24h (MOI=5). Lysates were either kept undigested, or digested with EndoH (E), or PNGaseF (P), then western blotted for the indicated proteins. Mature cell surface glycoproteins are resistant to EndoH, while immature (ER resident) forms are digested and therefore run with a smaller mass following EndoH digestion. (C) AAT cells were infected with RAd-MICA or RAd-B7-H6, then after 24h either mock infected, or infected with SARS-CoV-2 (SCV2), and treated with MG132 (M) or Leupeptin (L). 24h later lysates were made, and analysed by western blot for the indicated proteins. (Note: The additional band seen when staining for actin in the presence of SARS-CoV-2 may be non-specific)
Figure 3-Figure Supplement 1. (A) CACO2 cells were either mock infected, or infected with SARS-CoV-2 for 24h (MOI=5), detached using TrypLE, then stained for the indicated NK cell receptors before being analysed by flow cytometry.
Figure 3-Figure Supplement 2. Modulation of NK activity is dependent on virus replication.

AAT cells were mock infected, or infected with SARS-CoV-2 (MOI=5), or with heat inactivated SARS-CoV-2. After 24h, cells were dissociated with trypLE. (A) cells were stained with the indicated antibodies before being analysed by flow cytometry. (B) Cells were mixed with PBMC and incubated for 5h in the presence of golgistop and CD107a antibody, before staining for CD3, CD56, CD57 and Live/Dead Aqua. Cells were gated on live NK cells, and the percentage of cells positive for CD107a calculated. Assays were run in technical triplicate, and are representative of two biological repeats. One-way ANOVA **p<0.01.
Figure 4. SARS-CoV-2 infection restricts the expression of multiple NK ligands, inhibiting NK activation. (A) AAT cells were infected with RAd-MICA, RAd-B7-H6, or RAd-GFP, then after 24h either mock infected, infected with SARS-CoV-2 (SCV2), or treated with Cycloheximide (CHX). 24h later cells were lysed and analysed by western blot for the indicated proteins (Note: The additional band seen when staining for actin in the presence of SARS-CoV-2 may be non-specific). (B) 293T cells were transfected with expression plasmids for Nsp1 or Nsp14, or GFP as a control. 24h later, cells were detached with HyQtase and stained for the indicated proteins. (C-E) AAT cells were either mock infected, or infected with SARS-CoV-2 for 24h (MOI=5), detached with TrypLE and mixed with interferon stimulated PBMC for 5h in the presence of golgistop, golgiplug, and CD107a antibody, before staining for CD3/CD56 and Live/Dead Aqua. Cells were then fixed, permeabilised, and stained for TNFα and IFNγ. Cells were gated on live NK cells, and the percentage of cells positive for CD107a, TNFα and IFNγ calculated. Individual assays were run in technical
triplicate, with data shown from four assays using different donors. Kruskal–Wallis, *p<0.05, **p<0.01.
Figure 5. SARS-CoV-2 inhibition of NK activation can be overcome via ADNKA. AAT cells were either mock infected, or infected with SARS-CoV-2 for 24h (MOI=5), detached using TrypLE, then mixed with PBMC in the presence of golgistop, CD107a antibody, and serum from donors who were seronegative or seropositive for SARS-CoV-2. After 5h, cells were stained for CD3, CD56, CD57, and live/dead aqua, fixed, permeabilised and stained for IFNγ and TNFα, then analysed by flow cytometry. (A-F) assays were performed using 1% serum, and the percentage of CD57+ NK cells positive for CD107a (A, D), TNFα (B, E), and IFNγ (C, F) were calculated. Individual donors were performed in technical triplicate (A-C), 2-way ANOVA **p<0.01, ****p<0.0001. Assays from multiple donors were also compared (D-F), Kruskal–Wallis **p<0.01, ***p<0.001. (G-H) assays were performed using a serial 3-fold dilution of serum, starting from 3.3%, then the area under the curve (AUC) calculated. (G) longitudinal sera from donors who experienced mild (black) or severe (grey) were used. (H) In addition to AUC for ADNKA activity, sera were tested for their ability to neutralise SARS-CoV-2 infection of VeroE6 cells, and the NT50 calculated, then compared to the AUC for ADNKA; Spearman rank correlation analysis is shown.
Figure 5—Figure Supplement 1. ADNKA is dependent on virus replication, and occurs through direct action on NK cells. (A-C) AAT cells were mock infected, or infected with SARS-CoV-2, or with heat inactivated SARS-CoV-2 (MOI=5). After 24h, cells were dissociated with trypLE. (A-B) Cells were mixed with PBMC (A) or purified NK cells (A-B) and 1% sera that was seropositive or seronegative for SARS-CoV-2. Cells were incubated for 5h in the presence of golgistop and CD107a antibody, before staining for CD3, CD56, CD57 and Live/Dead Aqua. Cells were gated on live CD57+ NK cells, and the percentage of cells positive for CD107a. Assays were run in technical triplicate. Two-way ANOVA ****p<0.0001. (C) cells were stained for spike or nucleocapsid. (D) Vero or Caco2 cells were infected with SARS-CoV-2. After 24h, cells were dissociated with trypLE and stained for spike or nucleocapsid, before being analysed by flow cytometry.
Figure 6. Multiple SARS-CoV-2 proteins mediate ADCC. (A) Plots of viral proteins detected in the PMP analysis. (B) 293T cells were transfected with plasmids expressing the indicated SARS-CoV-2 proteins for 48h, then mixed with PBMC in the presence of golgistop, CD107a antibody, and 1% serum from donors who were seronegative or seropositive for SARS-CoV-2. After 5h, cells were stained for CD3, CD56, CD57, and live/dead aqua, then analysed by flow cytometry. Assays were performed in technical triplicate. Data is shown from two donor serums. **p<0.01, ****p<0.0001, 2-way ANOVA. (C) 293T cells were transfected with plasmids expressing SARS-CoV-2 nucleocapsid or spike (or empty vector; light grey) for 48h, or AAT cells were infected with SARS-CoV-2 (MOI=5) (or mock infected; light grey) for 24h, then cells were detached with TrypLE, stained for nucleocapsid or spike, and analysed by flow cytometry. (D) AAT cells were infected with SARS-CoV-2 (MOI=5) for 24h, then cells were fixed in paraformaldehyde and stained for the indicated targets. Images are shown as maximum intensity projections of Z-stacks.
Figure 7—Figure Supplement 1. Anti-spike antibodies bind to SARS-CoV-2 infected cells. AAT cells were mock infected (light grey), or infected with SARS-CoV-2 (black; MOI=5) for 24h, then detached with TrypLE, stained with the indicated human anti-spike antibodies, then analysed by flow cytometry. UL16 represents a negative control antibody targeting a protein from a different virus (HCMV)\textsuperscript{23}.
Figure 7. Monoclonal anti-spike antibodies bind infected cells strongly, but only weakly activate ADNKA. (A-B) AAT cells were either mock infected, or infected with SARS-CoV-2 (MOI=5), or (C) 293T cells were transfected with an expression plasmid for spike. After 24h (A-B) or 48h (C), cells were detached with TrypLE and mixed with PBMC and the indicated antibodies, or a serum from a moderate case of COVID-19. Cells were incubated for 5h in the presence of golgistop, golgiplugs, and CD107a antibody, before staining for CD3, CD56, CD57 and Live/Dead Aqua. Cells were gated on live CD57+ NK cells, and the percentage of cells positive for CD107a calculated. Assays were run in technical triplicate. 2-way ANOVA ***p<0.001, ****p<0.0001.
Figure S7—Figure Supplement 2. Monoclonal anti-spike antibodies only weakly activate ADNKA. AAT cells were either mock infected, or infected with SARS-CoV-2 (MOI=5) for 24h, detached with TrypLE and mixed with PBMC and the indicated antibodies, or a serum from a moderate case of COVID-19. Cells were incubated for 5h in the presence of golgistop, golgiplug, and CD107a antibody, before staining for CD3, CD56, CD57 and Live/Dead Aqua. Cells were then fixed, permeabilised, and stained for TNFα and IFNγ. Cells were gated on live CD57+ NK cells, and the percentage of cells positive for TNFα (A) and IFNγ (B) calculated. Assays were run in technical triplicate. 2-way ANOVA *p<0.05, ***p<0.001, ****p<0.0001.
Figure 8. The ADNKA response is dominated by non-spike antibodies following natural infection. Serums from individuals naturally infected with SARS-CoV-2 were depleted of anti-spike antibodies using spike trimer protein (A-E) or both spike trimer and nucleocapsid (G-H) conjugated to magnetic beads. (A) ELISA for spike trimer was used to measure levels of antibodies before and after depletion. (B, C) the ability of the original, or anti-spike depleted, serums to neutralise the ability of SARS-CoV-2 to infect VeroE6 cells was determined across a range of concentrations, then NT50 values calculated. Example plots (B) and NT50 values for multiple donors (C) are shown. (D-K) AAT cells were either mock infected, or infected with SARS-CoV-2 (D-H), or with SARS-CoV-2 from which ORF3 had been deleted (J, K) for 24h (MOI=5), detached using TrypLE, then mixed with PBMC in the presence of golgistop, CD107a antibody, and serial dilutions of serum, or monoclonal antibody. After 5h, cells were stained for CD3, CD56, CD57, and live/dead aqua, then analysed by flow cytometry for the percentage of CD107a positive CD57+ NK cells. AUC values were then calculated. Example plots (D, G, J) and AUC values for multiple donors (E, H, K) are shown. Kruskal–Wallis *p<0.05, **p<0.01, ****p<0.0001. (I) 24h after infection, cells were detached and stained for the indicated proteins after analysis by flow cytometry.
Figure 9. Vaccine responses focussed on spike are weak activators of ADNKA. Serums from donors that had been vaccinated against SARS-CoV-2 but were seronegative prior to vaccination were depleted of anti-spike antibodies using spike trimer protein conjugated to magnetic beads. (A) ELISA for spike trimer was used to measure levels of antibodies before and after depletion. (B, C) the ability of the original, or anti-spike depleted, serums to neutralise the ability of SARS-CoV-2 to infect VeroE6 cells was determined across a range of concentrations, then NT50 values calculated. Example plots (B) and NT50 values for multiple donors (C) are shown. (D, E) AAT cells were either mock infected, or infected with SARS-CoV-2 for 24h (MOI=5), detached using TrypLE, then mixed with PBMC in the presence of golgistop, CD107a antibody, and serial dilutions of serum. After 5h, cells were stained for CD3, CD56, CD57, and live/dead aqua, then analysed by flow cytometry for the percentage of CD107a positive CD57+ NK cells. AUC values were then calculated. All serums were additionally tested against mock infected cells to ensure no non-specific NK activation occurred (not shown). Example plots (D) and AUC values for multiple donors (E) are shown.
Note that (D) is plotted on the same scale as Fig 8D to enable easy comparisons. Neutralisation (F) and ADNKA (G) values are compared for samples categorised according to disease or vaccination status. Kruskal–Wallis *p<0.05, **p<0.001.
Figure Supplement 2. MS Method parameters.
Figure 1-Figure Supplement 3. Search Parameters for MS data processing
Figure 4—Figure Supplement 1. Example gating strategy for NK assays. Lymphocytes were gated on FSC/SSC, doublets excluded using FSC-A/FSC-H, and live cells selected on the basis of Live/Dead Aqua staining. The percentage of CD107a+ cells were determined in the CD56+/CD3- gate, or in the CD57+ gate of CD56+/CD3- cells.

Supplementary File 2. List of interferon inducible genes identified in the filtered PM dataset
Supplementary File 3. Clinical characteristics of patients giving longitudinal serum samples
Supplementary File 4. Monoclonal Anti-spike Antibodies used, data taken from51


Western Blot Source Data Information

Raw files for Figure 3B are provided as follows:

Data 1 = MICA, Data 2 = Actin, Data 3 = Spike (all samples from RAd-MICA experiment).

Data 4 = ULBP2, Data 5 = Actin, Data 6 = Spike (all samples from RAd-ULBP2 experiment).

Data 7 = B7-H6, Data 8 = Actin, Data 9 = Spike (all samples from RAd-B7-H6 experiment).

Raw files for Figure 3C are provided as follows:

Data 1 = MICA, Data 2 = B7-H6, Data 3 = Actin, Data 4 = Spike

Raw Files for Figure 4A are provided as follows:

Data 1 = MICA, Data 2 = B7-H6, Data 3 = GFP, Data 4 = Actin