SARS-CoV-2 Membrane protein-specific antibodies from critically ill SARS-CoV-2 infected individuals interact with Fc-receptor expressing cells, but do not neutralize the virus

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Running head: Enhanced FcR activation by anti-M IgG from critically ill COVID-19 patients

Conflicts of Interest: None.

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Abstract

The membrane glycoprotein (M) of SARS-CoV-2 is one of the key viral proteins regulating virion assembly and morphogenesis. Immunologically, the M protein is a major source of peptide antigens driving T cell responses, and most individuals who have been infected with SARS-CoV-2 make antibodies to the N-terminal, surface-exposed peptide of the M protein. We now report that although the M protein is abundant in the viral particle, antibodies to the surface exposed N-terminal epitope of M do not appear to neutralise the virus. M protein-specific antibodies do, however, activate antibody-dependent cell-mediated cytotoxicity (ADCC) and cytokine secretion by primary human NK cells. Interestingly, while patients with severe or mild disease make comparable levels of M antigen-binding antibodies, M-specific antibodies from the serum of critically ill patients are significantly more potent activators of ADCC than antibodies found in individuals with mild or asymptomatic infection.

Key words: SARS-CoV-2 infection, COVID-19 disease, Antibodies, Natural Killer cells
**Introduction**

Infection with Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes the pandemic coronavirus disease 2019 (COVID-19). SARS-CoV-2 is a betacoronavirus and has a 30 kb positive single-stranded RNA genome [1]. The viral particle that contains the RNA genome is composed of four major virus encoded proteins, the Spike glycoprotein (S), the small membrane protein (E), the membrane protein (M) and the nucleocapsid protein (N) that are encoded in the last third of the viral genome (Supplementary Figure 1).

SARS-CoV-2 infection is associated with the development of robust humoral immunity to the S and N proteins, indeed the detection of antibody responses to these proteins are the basis of most diagnostic serological tests ([2, 3]). However, multiple other viral proteins, including the Cysteine-like protease [4], M and others have been reported to also elicit antibody responses [5, 6]. The M glycoprotein is the most abundant structural protein of the SARS-CoV-2 particle and is one of the key components for virion assembly and morphogenesis, mediating incorporation of the nucleocapsid into the newly formed virions and recruiting all other viral structural components to the ER-Golgi-intermediate compartment (ERGIC) where virus assembly and budding takes place [7-9]. Immunologically, M protein is a major source of peptide antigens driving T cell responses [10, 11]. M-specific antibody responses are detected in most COVID-19 patients [5, 12, 13], that chiefly target two regions of the M protein [13]. However, no studies of the function of these M-specific antibodies have been reported, even though the N-terminus of the M glycoprotein can be expressed at the surface of the virion and the infected cell [14] and so is a possible target for antibody immune surveillance, either by virus neutralisation or by recruiting effector leukocytes via antibody/Fc receptor interactions.

In this study, we report that antibodies to an N-terminal, surface exposed peptide of the M protein can be detected in most individuals who have been infected with SARS-CoV-2. Antibodies to this epitope did not neutralise the virus, however, they could stimulate antibody-dependent cell-mediated cytotoxicity by primary human NK
cells. Strikingly, serum antibodies from critically ill patients triggered FcγR-dependent activation more potently than antibodies from patients with only mild disease.

Materials and Methods

GST-M expression and purification

To express the surface exposed N-terminal region of the M and E proteins of SARS-CoV-2, oligonucleotides

GATCCCCATGGCAGATTCCAACGGTACTATTACCCTTCAACGGTAATAGTACCGTTG

were phosphorylated, annealed and then ligated into the pGEX-5x-1 (Cytiva, Merck Sigma Aldrich) vector digested with BamHI and EcoRI enzymes for expression of the M-protein N-terminal peptide. The oligonucleotides 5´-

GATCCCCATGGCAGATTCCAACGGTACTATTACCCTTCAACGGTAATAGTACCGTTG

TTGAACCAATGGAACATGGCAGATTCCAACGGTACTATTACCCTTCAACGGTAATAGTACCGTTG

were cloned into pGEX-5x-1 for expression of a GST fusion with three copies of the M epitope, and so increased epitope density when coated on plates for the ADCC assays. The GST fusion proteins were expressed in the Escherichia coli strain BL21 Star (DE3) pLysS (Thermo Fisher
Scientific) and purified using Glutathione Agarose beads (Merck Sigma Aldrich), following established protocols [15]. A construct encoding the M-protein epitope of the Omicron strain was prepared by site-directed mutagenesis using the oligos 5’-CCCATGGCAGGTCCAACGGT-3’ and 5’-ACGTTGGAACCTGCCATGGGG-3’ to introduce the M3 D>G mutation and 5’-GCTCCTTGAAGAATGGAACG-3’ and 5’-TCGTTCAATTTCAAGGAGC-3’ for the M19 Q>E change. The integrity of all plasmid constructs was verified by sequencing.

Study subjects

Samples used in this study were provided by the Biobank Hospital Universitario Puerta de Hierro Majadahonda (HUPdHM)/Instituto de Investigación Sanitaria Puerta de Hierro-Segovia de Arana (IDIPHISA) (PT17/0015/0020 in the Spanish National Biobanks Network). The use of these samples was approved by the Research Ethics Committees of the CSIC (173/2020) and the HUPdH. Experiments were carried out following the ethical principles established in the Declaration of Helsinki. Analysis of the antibody reactivities to Spike, RBD, NP and MPro, as well as clinical information of these individuals has been published previously [16].

Affinity purification of SARS CoV 2 M-specific antibodies

Approximately 20mg of purified GST or GST-M protein were coupled to CNBr-activated sepharose using standard techniques [17]. Convalescent plasma samples, obtained as described previously [18], were treated with calcium chloride, centrifuged to remove precipitate and then run over the GST and GST-M columns. After extensive washing M-specific antibodies were eluted using 0.2M Glycine pH2.7 and the fractions
were neutralised using 1.5M Tris pH8.8. Eluted fractions were analysed by SDS-PAGE and for IgG antibody reactivity to SARS CoV 2 Spike and M proteins by ELISA and western blot (a representative ELISA experiment is shown in Supplementary Figure 3). M-specific antibody containing fractions with no reactivity to Spike were pooled, dialysed against PBS and filter-sterilised.

**PBMC isolation and culture**

Cryopreserved peripheral blood mononuclear cells (PBMCs), isolated by centrifugation on Ficoll-Paque, were thawed in warmed RPMI 10% FBS and incubated with 25 U/ml Benzonase (Merck #70746) for 10’ at RT. After two washes, the cells were incubated overnight in RPMI 10% FBS to recover and used in NK activation experiments the next day.

**ELISA assay**

SARS-CoV-2 Spike, E, M-specific antibodies were analysed using a previously described ELISA protocol [4]. Briefly, recombinant Spike [19], GST, GST-M, GST-M3 or GST-E antigens were plated overnight in Thermo NUNC MaxiSorp plates at 3 μg/ml in BBS at 4º. After 3 washes with PBS-T, wells were blocked overnight in PBS 1% Casein (PBS-C) at 4º. Then a range of serum dilutions (1/50 to 1/350) were added in PBS-C and incubated at RT for 2 hours. Bound IgG antibodies were revealed by adding 100 μl/well of AffiniPure Rabbit Anti-Human IgG, Fcy fragment specific secondary antibody (Jackson Labs). After incubation for 1 h at room temperature, the plates were washed with PBS-T three times and incubated at room temperature in the dark with 50 μl/well of 1-Step Ultra TMB (Thermo Fisher) (typically for 3 min). Fifty microliters of stop solution
(2 M H₂SO₄) was then added to each well, and the OD (at 450 nm) of each well was
determined using a microplate reader.

SARS-CoV-2 Neutralisation assay

Polyclonal anti-M antibodies purified from seven donors (#1, #2, #3, #095, #509,
#800 and #972) and a human IgG control antibody were serially diluted in DMEM + 2%
FBS + 2% pre-immune human serum containing media to achieve 2x final
concentrations of 30, 10 and 3.3 µg/ml of each of the antibodies. A dilution of SARS-
CoV-2 virus sufficient to infect one out of hundred cells (moi = 0.01) was prepared in
DMEM + 2% FBS containing media. 20 µl of 2x antibody dilutions were mixed in a 1:1
ratio with 20 µl of virus dilution and they were incubated for 1 hour at 37°C. Each
mixture was prepared in triplicate wells. After the incubation period, 60 µl of DMEM +
2% FBS was added to each well and the virus-antibody mixtures were added onto
monolayers of Vero-E6 cells that had been plated the day before in 96-well plates, and
they were incubated at 37°C, 5% CO₂. Twenty-four hours later cells were fixed with a
4% formaldehyde solution for 20 min and after extensive washes the infection efficiency
was determined by quantitation of SARS-CoV-2 N protein by automated
immunomicroscopy [19].

Triplicate wells infected in parallel in the absence of antibodies were used to set
the maximal infection efficiency (No antibody; set as 100%), while wells infected in the
presence of 30, 10 and 3.3 µg/ml of a Spike-specific neutralising monoclonal antibody
[20] were used as positive controls.

ELISA-based NK activation assay
This assay was based on that described by Chung et al [21]. Briefly, GST-M3, and negative control GST, antigens were plated overnight in Thermo NUNC MaxiSorp plates at 3 μg/ml in BBS at 4º. After 3 washes with PBS-T, wells were blocked overnight in PBS 1% Casein (PBS-C) at 4º. Then convalescent patient sera were added in a 1:50 dilution in PBS-C and incubated at RT for 2 hours. After 3 washes in PBS-T and 3 more in PBS, 50-100,000 PBMC were added per well in 100 μl of RPMI medium containing 10% FBS, 5 μg/ml Brefeldin A (Biolegend #420601) and 1 μg/ml anti-CD107a-APC antibody (Biolegend H4A3). After a 5-hour incubation, cells were transferred to a U-bottom 96-well plate and stained with anti-CD3-FITC (Biolegend UCHT1) and anti-CD56-PC5 (Beckman N901) antibodies in PBA for 30’. After a 5-minutes fixation in 4% PFA, cells were incubated with anti-MIP1β-PE antibody (BD D21-1351) in 0.25% Saponin. Stained cells were analysed using a Cytoflex flow cytometer (Beckman Coulter).

Statistical analysis

Statistical analysis was performed with Graph Pad Prism 8 Software (GraphPad Software, www.graphpad.com). Unless otherwise indicated, the Mann–Whitney U test was used to test for statistically significant differences and two-tailed P values are indicated.
Results and discussion

Antibody responses to the N-terminus of the M glycoprotein can be detected in SARS-CoV-2 infected individuals

The coronavirus M protein, the most abundant glycoprotein in the virus particle, has three domains: a short N-terminal ectodomain, three transmembrane domains, and a large interior C-terminal domain. Since previous work with SARS-CoV-1 showed that infected individuals made antibody responses to the N-terminal domain of M [22], we explored whether individuals infected with SARS-CoV-2 also made antibodies to the M protein ectodomain. Our experiments focused on this epitope since it will be expressed on the surface of the virion and the infected cell, unlike the majority of the M-protein that will be inside the membrane of the viral particle [23]. For these experiments we expressed the N-terminal amino acids of the predicted M ectodomain as GST-fusion proteins in E. coli (Supplementary Figure 2A). In parallel, we also prepared a construct expressing the N-terminal amino acids of the E glycoprotein as a GST fusion protein. When sera from COVID-19 convalescent patients were analysed by ELISA, antibodies to the M protein N-terminal peptide were detected in most COVID-19 infected individuals tested, but not in samples collected prior to the pandemic (Figure 1A). ROC analysis of these data showed that detection of M glycoprotein antibodies to this single peptide discriminated between infected and non-infected individuals with around 90% efficiency (Figure 1B). It should be noted that the sequence of this region of the SARS-CoV-2 M protein is very different from the equivalent region of common coronavirus M glycoproteins [23] and Supplementary Figure 2B), suggesting that detection of this epitope by antibodies could avoid possible cross-reactive immunity that might give rise to false positives. No antibodies to the equivalent region of the E glycoprotein were detected in these experiments (Figure 1A). The inability to detect specific antibody responses to the N-terminal portion of the E-glycoprotein might reflect either low expression of this protein or the immunodominance of another region, but it is interesting to note that antibodies to SARS-CoV-1 E were also not detected in convalescent patient sera [24] nor in hamsters immunised with parainfluenza virus type 3 recombinants expressing this protein [25], suggesting that this region of this protein may not be very immunogenic.
Although in general, the plasma of patients suffering severe disease contains higher levels of SARS-CoV-2 specific antibodies than in mildly affected patients [26], as seen for anti-Spike antibodies in this cohort (Supplementary Figure 3A), no significant differences in levels of M-specific antibodies were detected between age and sex-matched cohorts of patients with severe or mild COVID-19 disease (Figure 1C). In contrast to a previous report [13], we did not observe significantly lower antibody reactivity to the M peptide epitope in samples obtained later after infection compared to samples collected at earlier time points (Supplementary Figure 3B). However, it should be pointed out that these are samples from different individuals at different time points, which would make finding a correlation more difficult.

Finally, although the surface-exposed N-terminal peptide of the M protein is generally highly conserved between SARS-CoV-2 viruses (Supplementary Figure 2D) the sequence of the Omicron variant does differ in two amino acids. Therefore we also compared the reactivity of sera from patients, collected in March/April 2020 before the emergence of the Omicron VoCs, against M peptides corresponding to the SARS-CoV-2 Pre-Omicron and Omicron variants. These experiments revealed that, for most individuals the variation in sequence between the N-terminal M peptides had no significant effect in antibody recognition of this epitope (Figure 1D). However for 5 of 29 patients assayed (mainly those with mild disease), antibody recognition of the M peptide of the Omicron variant were somewhat reduced. Since the binding of these antisera to the Wuhan sequence or an M3 D>G mutant are not significantly different (Supplementary Figure 3C), this difference was probably due to the M19 Q>E change between Wuhan and Omicron. As the samples where antibody recognition of M from Wuhan and Omicron differed came from patients with mild disease it is not possible to assess whether this reduced antibody recognition might influence COVID-19 disease development.

Polyclonal M protein-specific antibodies, purified from convalescent sera, do not neutralise SARS-CoV-2 infection
The spike protein is the major target for virus neutralizing antibodies in SARS-CoV-2 infected patients [3]. However, the M-protein is highly abundant in the viral particle and prior studies with SARS-CoV reported that anti-M hyperimmune rabbit antisera could also neutralise SARS-CoV infectivity [27]. A SARS-CoV M protein-specific recombinant antibody, recovered from an antibody library prepared from lymphocytes of a convalescent SARS patient, was also reported to show significant neutralizing activity against SARS-CoV [28]. To assess whether M-specific antibodies contributed to neutralisation of SARS-CoV-2 in patients, anti-M antibodies from convalescent plasma samples of multiple donors were purified by affinity chromatography using GST-M protein coupled to Sepharose-4B (Supplementary Figure 4A). The composition (IgM, IgG etc) of the affinity-purified antibodies was not analysed, but SDS-PAGE analysis suggested that they were mainly of the IgG class (Supplementary Figure 4B). It’s interesting to note that although these antibodies were made in response to glycosylated M-antigen during viral infection, they are still able to bind the non-glycosylated M-protein produced in E coli. The yields of M-specific antibodies obtained corresponded to an estimated antibody concentration in the serum of these patients of around 10ug/ml, comparable to the concentrations of RBD and Spike-specific antibodies found in COVID-19 convalescent serum [29, 30]. Next the purified anti-M antibodies were assayed to test whether they could protect Vero-E6 cells from virus infection. A monoclonal antibody specific for Spike and able to neutralise virus infection was used as a positive control in this cell culture model [20, 31, 32]. As shown in Figure 2A, none of the antibody preparations significantly reduced the infection at any of the concentrations used, suggesting either that the frequency of neutralising M-specific antibodies in the plasma of COVID-19 convalescent patients is very low or that anti-M antibodies lack neutralization capacity, at least in this in vitro cell culture infection model. Since the interaction between Spike and ACE2 occurs far from the membrane at the top of the Spike protein [33-35], perhaps the distance from the very small extracellular domain of the M-protein to the binding site between SARS-CoV-2 RBD and ACE2 is too great for M-specific antibodies to block this interaction.
SARS-CoV-2 M protein-specific antibodies can activate NK cell antibody-dependent cell-mediated cytotoxicity (ADCC)

Since antibodies can mediate immune effector functions other than neutralisation and the M glycoprotein epitope could be exposed on virions and infected cells [14], we next studied whether M-specific antibodies could activate NK cell ADCC. For these experiments sera from COVID-19 convalescent patients who had developed either mild or severe disease, were analysed in ADCC assays along with a set of control serum samples that had been collected prior to the pandemic. These samples had been collected in March/April 2020, before the appearance of the delta and omicron variants, and so for these experiments the M-antigen used corresponded to the original SARS-CoV-2 virus (Wuhan strain). However, since NK cells ADCC depends on FcγRIIIA clustering, a GST fusion protein with three copies of the M-protein, N-terminal peptide was used to increase the density of epitopes coated on the plate. Natural Killer cells from PBMCs of healthy donors were used as effector lymphocytes and NK cell cytotoxicity (degranulation) and chemokine production (MIP-1β) were measured in parallel.

Control, pre-pandemic serum samples stimulated neither NK cell degranulation nor chemokine production, whereas serum samples from individuals who had been infected with SARS-CoV-2 contained M-specific antibodies that stimulated both NK cell cytotoxicity and MIP-1β production (Figures 2B and C). However, although in ELISA the levels of antibodies binding to M antigen detected in patients with mild or severe disease were comparable (Figure 1C), in these functional assays the serum samples from critically ill patients were significantly more potent stimulators of NK cell degranulation and MIP-1β production. Since structural diversity between IgG subclasses and post-translational modifications of their Fc domains, particularly reduced core-fucosylation of the IgG Fc domain, are known to influence the strength of interactions between immune complexes and FcγRs [36, 37], it is possible that the enhanced NK cell activation triggered by M-specific antibodies from critically ill patients reflects a reduced Fc fucosylation of IgG anti-M antibodies in these patients, especially...
since anti-Spike antibodies with reduced fucosylation are already known to be a characteristic of patients who develop severe COVID-19 disease [38, 39].

Concluding remarks

The M protein of SARS-CoV-2 is an important source of peptide antigens recognised by CD4+ and CD8+ T cells [10, 11]. We now show that most SARS-CoV-2 infected individuals produce M-specific antibodies against a conserved, surface-exposed epitope at the N-terminus of this protein. Although these antibodies do not appear to neutralise SARS-CoV-2 infection efficiently, they are able to mediate Fc/FcγR interactions to drive the activation of NK cells, and likely other FcγRIIIA-expressing immune cells. Indeed, a striking finding of this study is that although the levels of M-antigen-binding antibodies are comparable between patients with severe and mild disease, M protein-specific antibodies present in the serum of critically ill patients are significantly more potent activators via Fc/FcγR interactions. High titres of anti-spike IgG with reduced fucosylation of the antibody Fc region, and so stronger Fc/Fc-receptor interactions, are associated with inflammation in COVID-19 disease [40, 41], and our data show that increased antibody binding to Fc-receptors is also a feature of anti-M glycoprotein antibodies in severe COVID-19 disease. Further studies of the biochemistry of the M-specific antibodies will be required to define the molecular basis of this enhanced activity.
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Figure legends

Figure 1

A. The serum of most SARS-CoV-2 convalescent patients contain antibodies reactive with a surface exposed N-terminal peptide of the M glycoprotein, but not the N-terminal peptide of the E-glycoprotein. The data presented represent absorbance values of serum samples from individual donors, normalised by reference to the reactivity of each serum against GST coated wells.
B. Receiver operating curve analysis shows that the detection of M-specific antibodies discriminates between seropositive (n=52) and seronegative (n=22) donors with around 90% specificity and sensitivity.

C. There are no significant differences in the levels of M-peptide binding antibodies between patients with mild (n=14) or severe (n=15) disease. The data presented represent absorbance values of serum samples from individual donors, normalised by reference to the reactivity of each serum against GST coated wells.

D. With few exceptions, the M-peptide binding antibodies present in patient serum collected in March/April 2020 bind equally well to the M-peptide epitope present in the α/β/δ and omicron variants of SARS-CoV-2. The dotted lines show the 95% confidence bands of the best-fit line obtained in an analysis of linear regression.

Figure 2

A. Affinity purified M-specific antibody preparations were assayed for neutralising activity against SARS-CoV-2 infection of Vero-E6 cells. None of the antibody preparations significantly reduced the infection at any of the concentrations used, while the neutralising Spike-specific mAb markedly reduced the infection compared to the No Ab condition. Data were analysed by ANOVA with Dunnet’s post-hoc test, using the no antibody group as control.

B. degranulation, and C. MIP-1β production by NK cells in PBMCs from healthy donors after stimulation by M-specific antibodies in sera of individual COVID-19 patients. Sera from critically ill COVID-19 patients with disease contained M-specific antibodies that were significantly more potent mediators of ADCC than those from patients with mild/asymptomatic disease. The data presented represent values for individual donors, normalised by reference to the reactivity of each serum against GST coated wells.
References


Figure 1

A. anti-M

B. anti-E

C. p<0.0001

D. p=0.001

AUC - 90%
(95% CI, 83-97)

Titre anti-M (Omicron VoC)

R² = 0.63
Figure 2

A. Viral replication (% of no antibody control)

- 30ug/ml
- 10ug/ml
- 3.3ug/ml

Donor 1: Donor 2: Donor 3: Donor 095: Donor 509: Donor 800: Donor 972: Human IgG Mab anti-Spike Infection

B. % NK cells CD107a⁺

Healthy Control Outpatients Critical

p<0.0001 p=0.0016 p=0.0256

C. % NK cells MIP-1⁺

Healthy Control Outpatients Critical

p<0.0001 p=0.0031 p<0.0001

Figure 2

165x221 mm (x DPI)