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De novo natural anti-M alloantibody emergence in severe Coronavirus Disease 2019

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1 Table and 1 Figure

Abstract

The immune response is a key player in the course of SARS-CoV-2 infection, and is often seriously dysfunctional in severe Coronavirus Disease 2019. The hyperinflammatory status has been described to be accompanied by the appearance of autoantibodies. In a lethal COVID-19 infection, we observed the emergence of a *de novo* natural alloantibody which targeted the M antigen from the MNS blood group on red blood cells (RBC) without evidence of any cross-reaction with SARS-CoV-2 antigens. This IgM lambda alloantibody was unmutated and unswitched. Here, we describe for the first time the emergence of a bystander *de novo* natural alloantibody against RBCs in a severe COVID-19 patient, highlighting the extra-follicular humoral response reported in these cases.

Keywords:

COVID-19, transfusion, alloantibody, M-antigen

The immune response is often seriously dysfunctional in severe Coronavirus disease 2019 [1-7]. In particular, patients with a severe form do not resolve the course of the disease despite prolonged and high neutralizing antibody titers against SARS-CoV-2. Herein we report a severe SARS-CoV-2 infection that showed the emergence of a *de novo* natural alloantibody targeting the M antigen from the MNS blood group found on red blood cells (RBC). This IgM alloantibody was unmutated and unswitched, highlighting the bystander extra-follicular humoral response reported in severe COVID-19, due to the hyperinflammatory status, as described by the appearance of autoantibodies [2-5].

A 54-year-old, hypertensive, diabetic and obese patient was diagnosed positive for SARS-CoV-2 by RT-PCR two days after the onset of respiratory symptoms. Nine days later, the patient was admitted and placed under mechanical ventilation in the intensive care unit (ICU) for severe acute respiratory distress syndrome (ARDS) (Figure 1A). Blood work revealed an exacerbated inflammatory response, typical of COVID-19 [8-9]: CRP levels at 324 mg/L, ferritin at 3245 ng/ml, neutrophil-to-lymphocyte ratio at 11, and increased levels of proinflammatory cytokines (Table 1). On day 16, due to refractory ARDS despite muscle blockers, prone position and high doses of steroids, the patient was placed under extracorporeal membrane oxygenation (ECMO). Hemoglobin fell from 8.7 g/dL to 6.8 g/dL and 2 packed RBC transfusions were administered. On day 22, following bilateral non-reactive mydriasis, a cerebral CT scan showed a massive hemorrhagic stroke leading to death.

Surprisingly, a systematic indirect antiglobulin test (IAT) before transfusion on day 16 revealed the presence of an anti-M antibody in the patient's blood, despite his M-negative phenotype, with positive agglutination at 37 and 4°C. The absence of agglutination after dithiothreitol treatment of serum indicated the presence of an isolated IgM, without additional IgG. This anti-M antibody targeted the M antigenic variant of the MNS system, carried on glycophorins A and B and predominantly present (76%) in white and black populations [10]. Anti-M alloantibodies can cause hemolytic disease of the fetus and newborn, and hemolytic transfusion reactions.

Since the patient had no history of any blood transfusions, this alloantibody was considered as natural. It apparently appeared *de novo* in the context of the active immune response as it was not detected on day 14 (Table 1). Indeed, anti-SARS-CoV-2 antibody titers increased 5-fold for the anti-nucleoprotein IgG index, and about 45-fold for anti-spike protein IgG and IgM index. Proinflammatory cytokines remained elevated and flow cytometry on circulating lymphocytes showed increased plasmablasts, and a more marked rise in switched memory B cells and marginal zone-like $CD27^{+}IgD^{+}IgM^{+}$ B cells (>4-fold), typically found in severe COVID-19 [1,2].

Analysis of the immunoglobulin mRNA B cell repertoire by RACE-repertoire sequencing [11], revealed a predominant IgM lambda clone on day 16, with unmutated heavy and light chain variable regions. Complementarity-determining regions 3 (IGH and IGL CDR3; Figure 1B-1C) did not express the variable heavy chain 4-34 (VH4-34) segment, frequently found in lupus and COVID-19 patient autoantibodies [2]. This clone was absent on day 9, and this immunoglobulin heavy chain variable region rearrangement was not detected among IgG sequences, suggesting its *de novo* emergence without any class switching. The similarities

with the anti-M IgM, detected by IAT, strongly indicates that the predominant IgM lambda clone, found by sequencing, corresponds to this antibody.

To determine whether the anti-M IgM could recognize SARS-CoV-2 antigens, we screened the predominant IgM lambda clone against the SARS-CoV-2 data base [12]; none of the published sequences matched. We then looked for cross-reactivity between M antigen and the viral antigens by adsorbing this antibody on M^+ RBC, and tested the eluate on the three available anti-SARS-CoV-2 immunoassays. The results were negative. This clearly indicates that the patient mounted a bystander anti-M immune response related to his SARS-CoV-2 infection.

We next investigated if this antibody was observed after SARS-CoV-2 infection in other patients. IAT conducted independently of the MNS phenotype in 571 French COVID-19 convalescent donors (median age 34 years, 66% men), collected at least four weeks after recovery in the context of specific passive immunotherapy [13], and in 30 COVID-19 patients at ER admission, failed to retrieve any anti-M antibodies.

From a clinical point of view, if pre-transfusion IAT is performed as required, this alloantibody should be readily detected, and could be considered by the local protocols for the choice of packed RBCs. Furthermore, the risk of hemolytic disease of the newborn, proven only in the presence of anti-M IgG [10], can be evaluated through isotype determination in pregnant female patients. Most importantly, this case illustrates the impact of marked inflammation on the immune response in severe COVID-19 patients. The impairment of T-B cooperation and germinal center formation could lead to an inefficient humoral response due to diminished IgG switch, hyper-somatic mutations, and lack of

memory B cells [1]. Indeed, we did not observe a preferential increase in anti-Spike protein IgG levels between day 9 and day 16 compared to IgM in our patient (Table 1).

This impairment leads to activation of the extrafollicular B cell pathway [2], characterized by direct low-affinity antibody production, possibly with multireactive or autoreactive features similar to systemic lupus erythematosus [3]; autoantibodies in COVID-19 have been described and target RBCs particularly [3-5]. Here we show for the first time, the emergence of a bystander *de novo* natural alloantibody against RBC in a severe COVID-19 patient, with no cross-reactivity with SARS-CoV-2 antigens. The increased number of circulating Marginal Zone–like B cells on day 16 and the characteristics of the anti-M antibody are in agreement with the concept of an inappropriate extrafollicular response [2] that could favor the emergence of auto- and natural alloantibodies. Evidence via IAT and recognition of a glycophorin allogeneic M form by this antibody may be incidental, although it should be considered in transfusions or pregnancies.

This antibody was not retrieved in convalescent donors, indicating that it may be transient and/or restricted to severe forms of COVID-19. Interestingly, it has been described that anti-M antibodies disappeared more rapidly from the blood [14]. One should remember that only a quarter of patients are M-negative [10] and that self-tolerance mechanisms control autoimmune responses. Altogether, this reduces the probability that such an alloantibody might emerge and remain detectable in convalescent patients.

Conflict of interest

Authors have no competing interest to disclose

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figure and table caption

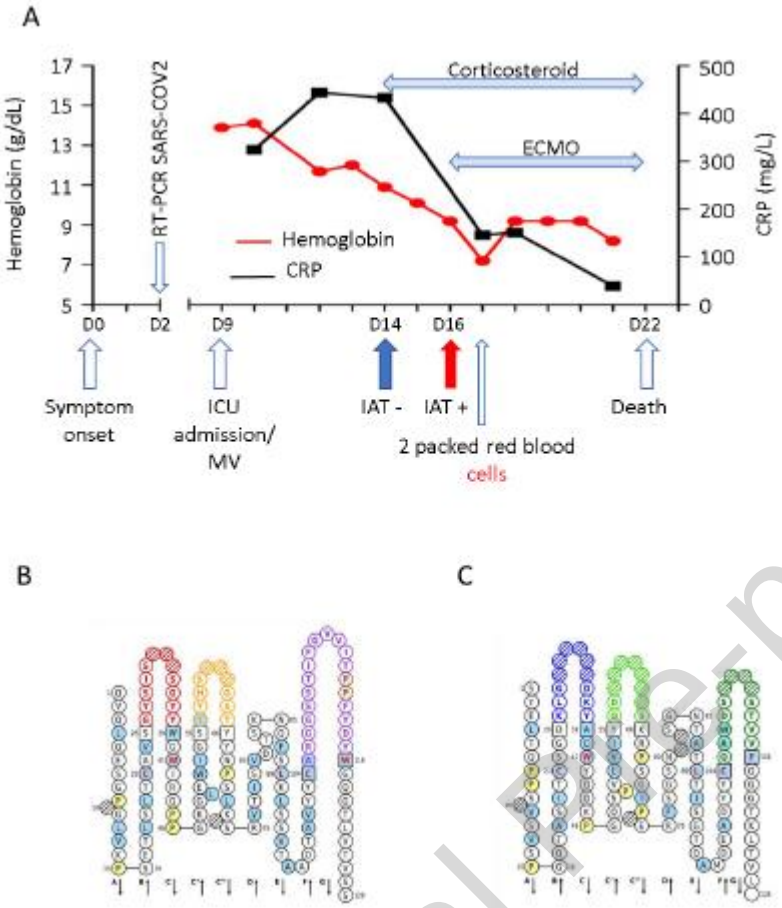

Figure 1: Graphical summary of patient clinical course (see text for details) and sequences of the two major mu heavy chain and lambda light chain clones.

A) X-axis: evolution in days. Left Y-axis: Hemoglobin levels (red curve). Right axis: C-reactive protein (CRP) levels (black curve). Time of symptom onset, Intensive Care Unit (ICU) admission/mechanical ventilation (MV), indirect antiglobulin test (IAT), Red blood cell transfusion; and death are indicated by vertical arrows below the x-axis. Duration of corticosteroid (8 days) therapy and extracorporeal membrane oxygenation (ECMO, 5 days) are shown by horizontal bi-directional arrows within the graph.

B) IgM heavy chain VH4-38-2 (0/11/4) DH3-3 (4/13/7) JH4*02 amino acid (AA) IMGT/ Collier de perles corresponding to 22.6% of G/A/M repertoire and C) Lambda light chain

Vλ3-1 (2//2) Jλ2 AA IMGT/ Collier de perles representing 5.5% of the Kappa/Lambda repertoire found by high-throughput sequencing (HTS). Amino acids are shown as one-letter abbreviations.

Table 1: Hematologic parameters, cytokine blood concentrations and serologic status at ICU admission (Day 9) and prior to ECMO (Day 16).

Parameter (Reference range)	Day 9	Day 16
Complete Blood Count		
Hb (14.0-18.0 g/dL)	**12.8**	**8.7**
Plt (150-450 x10^9/L)	373	**513**
WBC (4-10 x 10^9/L)	7.45	**19.34**
Neut (2-7 x10^9/L)	6.81	**16.39**
Lymph (1.5-4 x10^9/L)	**0.62**	1.05
Mono (0.2-0.8 x10^9/L)	0.31	0.60
B Lymphocytes (cells/µL)		
CD19+ (90-660)	150	350
CD19+ Transitional (1.7-13.8)	3	**0**
CD19+ Plasmablast (0.2-5)	**14**	**52**
CD19+ Naïve (44-84)	**98**	**147**
CD19+ MZ-like (4.8-32)	10	**46**
CD19+ Switch Memory (1.9-13.4)	13	**69**
T Lymphocytes (cells/µL)		
CD3+ (700-2508)	**371**	**497**

CD4+ CD8- (464-1721)	**241**	**305**
CD4- CD8+ (135-852)	**104**	**62**
Cytokines (pg/mL)		
IFN-α2 (<2-33)	7	13
IFN-γ (<5-75)	14	38
IFN-λ1 (<36-309)	191	55
IFN-λ2/3 (<2-98)	82	75
IL-1β (<7-61)	20	21
IL-6 (<2-17)	**317**	**94**
IL-8 (8-37)	**186**	**281**
IL-10 (<3-16)	**21**	**18**
IL-12p70 (1-25)	4	5
IP-10 (32-344)	**4012**	**1679**
TNF-α (4-40)	16	23
Anti-SARS-CoV-2 serology		
Anti-S IgM (positive ≥ 1.1 index sample/Calibrator)	**2.02**	**93.60**
Anti-S IgG (positive ≥ 15 AU/mL)	8.96	**396.00**
Anti-N IgG (positive ≥ 1.4 index sample/Calibrator)	1.13	**6.69**

Complete blood count, B and T lymphocyte subsets, cytokines and anti-SARS-CoV2 antibodies in the patient at ICU admission (D9 after first COVID-19 symptoms) and before ECMO (D16). Values outside the normal range and positive results of serology are in bold.

Abbreviations: Hb: hemoglobin; Plt: platelets; WBC: white blood cells; Neut: neutrophils; Lymph: lymphocytes; Mono: monocytes; MZ-like: Marginal zone like.

Indicated cytokine ranges are 5-95 percentiles laboratory reference values from a pool of 10 healthy volunteers.

Other indicated ranges are reference ranges.

B cell subpopulations were defined as followed: CD45+CD19+CD38+CD24+= Transitional B cells; CD45+CD19+CD38+CD24-= Plasmablasts; CD45+CD19+CD27-IgD+ = Naïve B cells; CD45+CD19+IgD+CD27+ = MZ-like B cells; CD45+CD19+CD27+IgD- = Switched Memory B cells.

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CRediT authorship contribution statement

AD and HP identified the patient, performed blood typing, IAT tests on serum patients and serum adsorption; TD and BF provided clinical care, and reported clinical data; XL conceptualized the study; XL and JF designed the study; RJ performed flow cytometry phenotyping and cytokine dosages and analyzed the data; VP performed IG sequencing and analyzed the data; SH performed the serological assays versus SARS-CoV-2 and analyzed the

data; SLC supervised the IAT tests on COVID-19 convalescent donors; RJ, XL and JF wrote the manuscript. All authors contributed to the article and approved the submitted version.