Title: Spectrum of innate and adaptive immune response to SARS-CoV-2 infection across asymptomatic, mild and severe cases – a longitudinal cohort study

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The ratio between monocytes and NK may represent a prognostic marker of disease development in COVID-19. Individuals with asymptomatic SARS-CoV2 infection have a high frequency of NK cells associated to a transient IgA response to the infection.

Background: SARS-CoV-2 is a novel coronavirus, not encountered before by humans. The wide spectrum of clinical expression of SARS-CoV-2 illness suggests that individual immune responses to SARS-CoV-2 play a crucial role in determining the clinical course after first infection. Immunological studies have focussed on patients with moderate to severe disease, demonstrating excessive inflammation in tissues and organ damage.

We have studied the individual response to SARS-CoV-2 of asympomatic, mild and severe COVID-19 patients in order to investigate the role of innate and adaptive immunity in determining the clinical course after first infection.

Methods: To understand the basis of the protective immune response in COVID-19, we performed a longitudinal follow-up analysis of innate and adaptive immunity in 64 adults with a spectrum of clinical presentations: (28 healthy SARS-CoV-2-negative contacts of COVID-19 cases; 20 asymptomatic SARS-CoV-2-infected cases; 8 patients with mild COVID-19 disease and 8 cases of severe COVID-19 disease).

Results: Our data show that high frequency of NK cells and early and transient increase of specific IgA and, to a lower extent, IgG are associated to asymptomatic SARS-CoV2 infection. By contrast, monocyte expansion and high and persistent levels of IgA and IgG, produced relatively late in the course of the infection, characterize severe disease. Modest increase of monocytes and rapidly declining antibodies are detected in mild COVID-19.

Conclusions: The importance of innate NK cells and the short-lived antibody response of asymptomatic individuals and patients with mild disease suggest that only severe COVID-19 may result in protective memory established by the adaptive immune response.

Keywords: COVID-19, antibodies, NK, monocytes, adaptive immune system, innate immune response.

Abbreviations: SARS-CoV-2: Severe Adult Respiratory Syndrome-Coronavirus-2, COVID-19: Coronavirus Disease 2019, ACE2: Angiotensin Converting Enzyme-2, MBCs: memory B cells, MLR: Monocyte-Lymphocyte ratio, NLR: Neutrophil-Lymphocyte ratio, MNKR: Monocyte-NK ratio, IFN-γ: interferon-gamma, HLH: Hemophagocytic Lymphohistiocytosis, TGF-beta: transforming growth factor-beta
Introduction

SARS-CoV-2 is a novel coronavirus, not encountered before by humans. Thus, everyone is susceptible to infection as the virus rapidly spreads in the current Coronavirus disease 2019 (COVID-19) pandemic. A wide spectrum of clinical expression of SARS-CoV-2 infection occurs, ranging from asymptomatic to mild upper respiratory tract illness, or moderate to severe disease with respiratory distress and multi-organ failure requiring intensive care and organ support[1]. This variability of disease severity suggests that the individual immune responses to SARS-CoV-2 play a crucial role in determining the clinical course after first infection. Understanding the pathogenesis of COVID-19 disease requires in-depth study of underlying immune responses[2].

In COVID-19, lymphopenia is common and correlates with severity of clinical disease[3]. Lymphopenia is caused by the reduction of both CD4+ and CD8+ T cells. Surviving T cells are functionally exhausted and reduced T-cell count predicts an unfavourable clinical course[4]. T cells able to react to SARS-CoV-2 peptides can be demonstrated in healthy individuals, probably because of a cross-reactivity with previous infections by other coronaviruses and are expanded in individuals convalescent from COVID-19[5].

Antibodies to SARS-CoV-2 are produced in large amounts in patients with severe disease, two-three weeks after the occurrence of first symptoms[6]. The role of antibodies in viral elimination is supported by the successful use of convalescent plasma in patients with severe COVID-19[7]. Neutralizing antibodies are directed against the Receptor Binding Domain contained in the S1 subunit of the Spike protein[8,9]. Whilst immune responses to novel antigens encountered for the first time, are first dominated by antibodies of IgM isotype, followed by IgG[10], the kinetics and protective or deleterious nature of the antibody responses to SARS-CoV-2 remains to be defined. A recent study indicated that the IgA response to SARS-CoV-2 may be rapid, strong and persistent[11]. The observation that patients with severe COVID-19 disease have very high antibody levels led to the suggestion that antibodies to SARS-CoV-2 may be damaging or ineffective rather than protective[12,13], as was reported from very sick patients with Middle East respiratory syndrome (MERS)[14].

Whilst immunological studies to date have focussed on patients with moderate to severe COVID-19 disease[15], studies from across the clinical spectrum are required to better understand immune responses to SARS-CoV-2 infection. We performed a longitudinal study of innate and adaptive immune populations in the peripheral blood of adults with asymptomatic SARS-CoV-2 infection, and those with mild and severe COVID-19 disease and their healthy contacts.

Methods:

Ethical approval
Ethical approval was obtained from the Medical Research and Ethics Committee at Sapienza, University of Rome. The study was performed in accordance with the Good Clinical Practice guidelines, the International Conference on Harmonization guidelines, and the most recent version of the Declaration of Helsinki.

Study sites: 1) Bambino Gesù Children Hospital 2) Policlinico Umberto I Hospital.

Patients.

Sixty-four adult patients were enrolled in the study:

- **28** healthy controls: Contacts of SARS-CoV-2 confirmed cases who were confirmed as negative by qPCR and were studied as control group;
- **20** asymptomatic cases (defined as contacts of COVID-19 cases but with no symptoms during the duration of study time but tested positive for viral RNA). Asymptomatic patients were quarantined and monitored for 14 days, and quarantine ended when two consecutive nasopharyngeal swabs showed negative results[16];
- **8** patients with Mild COVID-19 disease (defined by positive SARS-CoV-2 nasopharyngeal swab qPCR test, with symptoms such as fever, myalgia and fatigue without obvious chest HRCT findings for COVID-19 did not require hospitalization);
- **8** cases of Severe COVID-19 disease from Policlinico Umberto I Hospital who were hospitalized for respiratory disease with bilateral lung infiltrates at HRCT highly suggestive of COVID-19 interstitial pneumonia, and P/F $\geq$ 300 mmHg (defined as the ratio of arterial oxygen tension [PaO2] to inspiratory oxygen fraction [FiO2]).

Clinical Characteristics.

Severe adult COVID-19 patients (8 patients) were older (mean age, 65 years, range 30-90) than mild-symptoms adult COVID-19 patients (8 patients, mean age, 55.2 years, range 48-64). Sex ratio with a prevalence of male patients was similar in the two groups (severe: M/F 6/2; mild: M/F 5/3). During the study period, disease activity was regularly assessed, and COVID-19 patients continued their therapies according to the standard of care. Four out of eight severe cases were treated with anti-IL-6R monoclonal antibody (tocilizumab). All hospitalized COVID-19 patients were discharged and none died. Twenty patients, grouped as SARS-CoV-2 asymptomatic patients had a mean age of 40.4 (range 27-64) and a sex ratio (M/F) equal to 4/16. Twenty-eight contacts SARS-CoV-2-negative had a mean age of 40.8 years (range 27-68) and an 8/20 M/F sex ratio (Supplementary Table 1).

Flow-cytometry and antibodies
Four leukocyte profiling panels computing 7- to 9-surface marker antigens for monitoring the major leukocyte subsets as well as characteristics of T-cell, B-cell, monocytes and NK cells subsets were designed (Supplementary Table S2). Results of immune-profile of analyzed patients are reported in Supplementary Table 3-10.

1 ml of total blood (EDTA) was incubated with the lysing solution Pharm Lyse (BD) to lyse red blood cells. Then, cells were divided in four equal aliquots and stained with the appropriate combination of fluorochrome-conjugated antibodies (Table E1) to identify immune cell subsets according to standard techniques. For the staining of Figure E7, heparinized PBMCs of three healthy donors were isolated by Ficoll Paque™ Plus 206 (Amersham Pharmacia Biotech) density-gradient centrifugation. Cells were then stained with antibodies against CD19, CD24, CD27, CD38, IgM, IgG, IgA and IgD (Supplementary Table 1). Cells were acquired on a BD FACSlyric™ (BD Biosciences). Data were analyzed with FlowJo ver. 10 (Treestar). Dead cells were excluded from analysis by side/forward scatter gating.

Serum samples
Sera were obtained from the sixty-four patients included in the study. Some of the samples were collected at different time points. Included in this study were 151 serum samples obtained from subjects with available clinical records. In particular: forty-four sera from SARS-CoV-2 negative contacts, fifty-one from SARS-CoV-2 asymptomatic patients, forty-one from COVID-19 mild patients and fifteen from COVID-19 severe patients. All sera were kept on ice after collection and then stored at -80°C.

Serological assays
The Euroimmun Anti-SARS-CoV-2 ELISA IgG and IgA assays (Euroimmun, Luebeck, Germany) were performed on serum samples according to the manufacturer’s instructions. The recommended serum samples dilutions used were 1:100; in samples in which the IgA or IgG quantity was not detectable (overflow), we used 1:1000, 1:3000, 1:6000, 1:25000 dilutions. These ELISA assays provide a semi-quantitative in vitro determination of human antibodies of the immunoglobulin classes IgG and IgA against the SARS-CoV-2. The microplate wells are coated with recombinant S1 structural protein. The results are evaluated by calculation of the ratio between the extinction of samples and the extinction of the calibrator. The ratio interpretation was as follows: <0.8 = negative, ≥ 0.8 to < 1.1 = borderline, ≥ 1.1 = positive.

Statistical analysis
We performed the unpaired, two-tailed Mann-Whitney \( U \)-tests. A \( p \leq 0.05 \) was considered to be statistically significant.

**Results:**

**Innate immunity**

The PBMCs of patients with asymptomatic infection, mild and severe disease and their healthy contacts were compared. We correlated the immunological findings with the clinical course and studied the dynamic changes of cells of innate and adaptive immune response.

By flow-cytometry, we confirmed that the Monocyte-Lymphocyte ratio (MLR) increases in advanced COVID-19 cases, when T cells, normally representing the major lymphocyte population in the peripheral blood, are reduced[15] (Figure 1a and b). Previous studies indicated that neutrophils and macrophages infiltrate the lungs and are expanded in the peripheral blood of Intensive Care Unit (ICU)-admitted patients[17]. The increase of circulating neutrophils and monocytes, along with lymphopenia, explains why the Neutrophil-Lymphocyte ratio (NLR) and MLR are significantly higher in patients with severe COVID-19 disease.

T-cell frequencies are preserved in asymptomatic individuals and in patients with mild disease. Thus, in order to investigate whether other lymphocyte populations change in relationship to monocytes, we excluded T cells from the first analysis (Figure 1c and Supplementary Figure 1). Besides B cells discussed below, the CD3\(^-\) gate includes monocytes that can be distinguished by their larger size measured by the high FCS, and NK cells. NK cells express the markers CD7 (Figure 1c) and CD56 (Supplementary Figure 1s).

We found that the frequency of NK cells was reduced and that of monocytes increased in patients with severe COVID-19. Similar alterations of NK and monocytes were observed in the group of patients with mild disease (Figure 1d and e). For this reason, we calculated the Monocyte to NK ratio (MNKR), which appeared to be altered not only in the severe cases, but also in patients with mild disease (Figure 1e). The differences between asymptomatic, mild and severe COVID-19 remained significant when we included all the samples collected from the patients at different time points (Figure 2a), suggesting that the reduction of NK and monocytes and the high MNKR was not an incidental finding observed in a particular moment of the infection, but rather is a characteristic of the disease. Each individual maintained his typical NK and Monocyte frequency throughout the time of follow-up (Figure 2b). We confirmed the importance of the frequency of NK cells by the retrospective analysis of 77 patients with severe COVID-19. Cases who did not need ICU treatment had a significantly higher number of NK cells (CD56\(^+\) cells calculated in CD3\(^-\) lympho-monocyte gate) than ICU patients (Figure 2c). In addition, the percentage of NK cells
increased in those individuals who recovered from severe disease, whereas it remained low in patients with fatal COVID-19 (Figure 2d). These results are corroborated by the observation that ICU patients had lower perforin+ NK cells compared to non-ICU patients[18].

CD16 expression allows to differentiate three types of CD14+ monocytes (Supplementary Figure 2a and 3) in the peripheral blood, reflecting sequential stages of maturation and distinct functions[19,20]. Classical monocytes are the precursors of the other types and play an important role in the response to pathogens and in the resolution of inflammation. Intermediate monocytes secrete inflammatory cytokines in response to Toll-Like Receptor (TLR) stimulation and expand in the blood of patients with severe infections[21]. Non-classical monocytes contribute to the resolution of inflammation and maintaining vascular homeostasis and endothelial integrity[22]. We found that non-classical monocytes were significantly reduced in patients with severe COVID-19 when compared to SARS-CoV-2 negative contacts, SARS-CoV-2 positive asymptomatic and also mild COVID-19 disease patients (Supplementary Figure 2b). Intermediate monocytes were increased in the severe cases, but the difference reached statistically significance only when we considered all samples collected for the study (Supplementary Figure 2c). As the progression from the classical to intermediate stage is a dynamic step driven by infectious triggers[19], we compared the monocyte phenotype in the same patients at different time points during the course of the disease. Whereas intermediate monocytes were rare in the blood of contacts, asymptomatic and mild disease patients at all time points, transient increases were observed in patients experiencing severe disease (Supplementary Figure 2d).

In summary, we found that the MNKR reflects the clinical phenotype of the disease. Contact and asymptomatic patients had either higher representation of NK cells or a similar frequency of NK and monocytes (ratio around 1). The ratio was >1 in patients with mild disease and reached higher values in the severe cases. Thus, the equilibrium between two cell types of the innate immune system may play a role in the control of SARS-CoV-2 infection. Prevalence of NK cells is associated to asymptomatic infection, while increased frequency of monocytes to severe disease.

Adaptive immunity

T and B cells play key roles in response to viral infections. CD8+ T lymphocytes are crucial for the limitation of viral spread through their cytotoxic function. CD4+ T cells are indispensable for the expansion of CD8+ T cells and the generation of CD8+ memory T cells[23]. In addition, CD4+ T cells are necessary for the germinal centre (GC) response and the production of memory B cells (MBCs) and plasma cells[24].

A recent study reported that 82.1% of the severe COVID-19 cases had low circulating lymphocyte counts because of the reduced frequency of CD3+ T cells, both of CD4 and CD8 type[25]. In severe
COVID-19, not only the number of CD8+ T cell declines but also their function is impaired, in association with the increase of pro-inflammatory cytokines[18].

We observed an increase of activated, HLA-DR+ CD4 T cells in patients with mild and severe disease (Figure 3a and b). By contrast, HLA-DR+ CD8 T cells were increased only in patients with severe COVID-19 (Figure 3a and b). This finding was confirmed when we included all samples in the analysis (Supplementary Figure 4).

In a separate staining, we identified naïve and memory T cells, including central, effector and terminally differentiated (TEMRA) memory T cells (gating strategy in supplementary Figure 5).

In the CD4+ population of patients with mild and severe COVID-19, we found a reduction of recent thymic emigrants (CD45RA+CCR7+CD31+) and a consequent relative increase of CD31+ CD45RA+CCR7+ T cells. No other significant modifications of the cell distribution were observed and confirmed by the analysis of all patient samples (Supplementary Figure 6). In the CD8+ T-cell population, the most important change was the reduction of CD45RA+CCR7+ T cells, with modest alterations of the CD31+ and CD31- cell frequency. Total effector memory CD8+ T cells were increased only in asymptomatic cases (Figure 4 and Supplementary Figure 6), whereas in severe disease, CD8+ EM2 were increased and EM4 decreased. As reported before[5,26] CD8+ TEMRA were increased in patients with advanced disease (Figure 4b and Supplementary Figure 6).

Our data indicate that the circulating pool of T cells is not dramatically changed by SARS-COV-2 infection in asymptomatic individuals. In mild and severe cases, CD4+ T cells express HLA-DR. Activation of CD8+ T cells could be only demonstrated in the severe cases and was associated to the increase of highly cytotoxic EM2[27] and to the accumulation of exhausted TEMRA.

We identified the different B-cell populations in the peripheral blood by staining with a combination of antibodies able to distinguish transitional, naïve, memory, atypical MBCs and plasmablasts (Figure 5a). In the CD27+ MBCs population, we separately analysed IgM+ and switched MBCs. The latter include IgG+ MBCs and IgG- MBCs. Most of the IgG- B cells correspond to IgA-expressing memory B and in minimal part to MBCs without detectable surface immunoglobulin (Supplementary Figure 7). The most significant findings were the reduction of total B cells and the increase of plasmablasts in the severe cases (Figure 5b). Among MBCs, we found an increase of IgM+ and a reduction of switched MBCs in asymptomatic and mild cases. In patients with severe disease, IgM+ MBCs were reduced and switched MBCs increased (Figure 5c), with a significant expansion of the IgG- switched population. IgG+ MBCs were reduced in the blood of patients with mild disease (Figure 5d). For this reason, the ratio between IgG+ and IgG- switched MBCs, that is >1 in the SARS-Cov-2 negative contacts and asymptomatic individuals, is significantly lower in patients with mild and severe COVID-19 indicating the expansion of MBCs.
that do not express IgG and most probably express IgA (Supplementary Figure 7). All the findings were confirmed by the cumulative analysis of all samples (Supplementary Figure 8).

B cells fight viruses by producing antibodies when they differentiate into circulating plasmablasts or tissue–resident plasma cells. The final stages of differentiation can be reached by B cells in the GCs, where either naïve or IgM+ MBCs[28,29] acquire somatic mutations and are selected for their increased affinity to the stimulating antigen[24]. T- and GC-independent antibody production is efficiently and rapidly triggered by TLR-mediated stimulation of MBCs[30,31].

IgG and IgA antibodies directed against the S1 domain of the SARS-CoV-2 Spike protein were measured in the entire study cohort. We found that antibodies are produced by all COVID-19 patients and also by SARS-CoV-2 positive asymptomatic individuals, with higher levels of IgG and IgA being detected in the serum of patients with severe disease (Figure 6a) as reported before[13,32].

In order to study the dynamic nature of the antibody response, we analysed its kinetics in 9 asymptomatic individuals, 8 patients with mild and 4 with severe disease. We found that asymptomatic patients secrete high levels of S1-specific IgA early after diagnosis. IgA rapidly declines and becomes undetectable after 5-7 weeks. IgG is low at most time points (Figure 6b).

The response of patients with mild disease has different kinetics, with IgG and IgA increasing later and remaining relatively low, with few exceptions (Figure 6c). IgG and IgA are produced late in patients with severe COVID-19, but in higher amounts[13,32]. IgA was always more abundant than IgG (Figure 6d).

Antigen-specific IgA and IgG were undetectable in 2 patients with severe disease and in 1 with mild disease. The inability to produce antibodies in isolated cases with severe and mild disease may be due to individual genetic variation or age: pt34 was 83-year-old with a severe disease (1 single sample was obtained), and pt14 was young but had a rapidly evolving severe disease (3 samples were analysed at different time points), that improved with treatment. Pt11 (data not shown) with mild disease was otherwise healthy, but had neurological symptoms and a positive nasopharyngeal swab PCR lasting for 8 weeks (6 samples were evaluated).

The most interesting and intriguing aspect of the immunoglobulin study was the observation that among the samples of asymptomatic individuals 17% (9 of 53 samples) were negative for IgG and 22.6% (12 of 53) were negative for IgA. This high percentage of negative samples cannot be explained by individual variations. As serum antibody titers rapidly decline in asymptomatic patients (Figure 6b), we correlated the level of SARS-CoV-2 specific antibody to the time from the first positive nasopharyngeal swab. We found that six serum samples with non-detectable specific IgA and IgG had been obtained from individuals diagnosed as positive for SARS-CoV-2 eight to sixteen weeks prior to serum analysis (Figure 6e-empty circles). Of the other asymptomatic
individuals, we had multiple samples. In one case, IgA and IgG were undetectable in the first sample obtained on same day of the first positive swab, but IgA and IgG rapidly increased in the second sample at day 18 after the first positive swab. The other values below the detection threshold refer to the late stages of the antibody kinetics from individuals with a previously detectable IgG and IgA response.

In summary, we observed that asymptomatic patients have an early serum IgA response that may be able to rapidly control SARS-CoV-2 in the respiratory mucosa, thus preventing a full adaptive immune response. The lack of the early IgA burst may result in a longer persistence of the virus and induction of the GC response in the local lymphoid tissue. The adaptive response becomes stronger and probably geographically diffuse in the lymphoid tissue draining the severely damaged respiratory epithelium in patients with severe disease.

Discussion

Individual immune responses play an important role in determining the clinical course of SARS-CoV-2 infection in adults. Since the adaptive response requires time to build up after first encounter with a novel virus, the first line immediate responders are NK cells and natural antibodies, key components of innate immune system [33–37].

NK cells kill infected cells and produce IFN-γ with antiviral function[38]. ‘Natural antibodies’ are antibodies that have a protective role in the early phases of the response independent of any previous encounter with antigens [39–41]. In humans, natural antibodies are produced by innate IgM+ MBCs[31]. These antibodies, not yet been shaped by antigenic selection, carry few somatic mutations[28] and have broad reactivity[42]. We recently suggested that these antibodies might explain why most pediatric cases with laboratory-confirmed SARS-CoV-2 infection have either no or mild symptoms and recover within 1–2 weeks[43]. Cross-reactive antibodies found in children and adult never exposed to SARS-CoV-2[44] may correspond to natural antibodies. NK cells and natural antibodies contain the infection, whilst adaptive immune response develop to generate highly-specific memory T and B cells for clearing the virus and prevent re-infection[10].

In order to understand the basis of the immune response in COVID-19, we performed a global analysis of innate and adaptive immunity in patients selected across the spectrum of disease severity, ranging from SARS-CoV-2 positive asymptomatic individuals to patients with mild and severe COVID-19.

Our data show that the balance between NK cells and monocytes is a sensitive indicator of the individual reaction to the virus and is related to the clinical course of the disease. We found that when NK cells are reduced, monocytes increase. Since each individual included in our study
maintained typical NK and monocyte frequency throughout the time of follow-up, we utilized the ratio between peripheral blood monocyte and NK cells (MNKR) as a marker reflecting the clinical phenotypes of the disease.

In patients with severe disease, monocytes expand and secrete inflammatory cytokines[45]. At this point, inflammation may get out of control and cause immunopathology manifestations, supported by the observation that SARS-CoV-2 infection of respiratory epithelial cells results in aberrant transcriptional responses: antiviral IFN-I and III are not induced and, instead, a consistent chemokines signature is established[46]. Migration of monocytes to the infected lungs scales up inflammation resulting in further tissue damage[47,48]. Most of the described immune alterations are reminiscent of Hemophagocytic Lymphohistiocytosis (HLH), a condition often related to mutations of genes governing the cytotoxic lymphocyte machinery, where NK-cell deficiency leads to chronic expansion and activation of monocytes and causes the life-threatening condition known as cytokine storm[49–52]. As a consequence, therapeutic strategies counteracting the innate immune failure at earlier stages of virus infection and counteracting excessive pro-inflammatory cytokine levels have been successfully used in severe COVID-19[53,54]. Not only immune "suppressive, yet also immune-modulatory therapies may be advantageous, depending on the immunological situation of the patients[55].

We measured the specific IgG and IgA response to the S1 domain of the SARS-CoV-2 spike protein in the serum of all patients and controls in our study at different time points. We found that the clinical phenotypes of disease correspond to different antibody responses. We confirm that the highest levels of IgG and also IgA are produced by patients with severe disease relatively late during hospitalization[56]. Thus, a long and severe disease activates the adaptive immune response and is associated with the production of anti-SARS-CoV-2 antibodies and MBCs[57].

In asymptomatic patients, we show an early burst of IgA that may rapidly and effectively eliminate the virus in the respiratory mucosa and prevent a full adaptive immune reaction. Accordingly, IgG is produced in low amounts and becomes undetectable after 6-8 weeks. An intermediate response is observed in patients with mild disease without the early IgA peak and characterized by a late, but modest IgG and IgA production, suggesting a minor adaptive immune response. Further studies are necessary to establish whether specific memory persist and for how long after asymptomatic and mild disease.

The particular antigen-specific IgA/IgG profile associated with clinical outcome may reflect increased TGF-beta production induced by coronavirus-species[58]. Increased viral load may increase TGF-beta production, that – if produced locally in the lung, facilitates neutrophil attraction
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and induces specifically the isotype switch to IgA[59] - a situation that prompted the suggestion of anti-TGF-beta directed immunotherapies[60,61].

Innate MBCs are increased in asymptomatic and mild disease. Innate MBCs produce natural antibodies in response to TLR stimulation[30,39] but are also able to enter the GC where they remodel their antibodies to increase their affinity[28,62]. IgM+ MBCs are the precursors of most IgA+ and IgG+ switched MBCs[62] and give rise to IgA+ plasma cells at mucosal site[63]. We speculate that the early IgA burst of asymptomatic individuals may derive from the rapid activation of pre-existing innate or cross-reactive IgM+ MBCs that switched to IgA in the respiratory mucosa[11], as suggested also be the demonstration that moderate levels of IgM and IgA cross-reactive to SARS-CoV-2 are present in the blood healthy individuals never exposed to the infection[44]. In agreement with our hypothesis, neutralizing IgG MBCs isolated from COVID-19 patients may have no or very few somatic mutations[64]. In patients with severe COVID-19, IgM+ MBCs are reduced and we observed an increase of IgG+ MBCs probably expressing IgA (and thus reflecting the immune reaction in the lymphoid tissue associated to the respiratory tree for local protection. Circulating plasmablasts are increased only in the severe cases in correlation with their high antibody levels especially of IgA isotype.

Our analysis of circulating T cells shows that in asymptomatic individuals, SARS-COV-2 infection did not alter the T cell pool. In mild COVID-19, we only found an increase of activated CD4+ T cells, whereas in severe cases the activation of both CD4+ and CD8+ T cells might lead to clonal exhaustion[5].

SARS-CoV-2 has evolved in bats, which control the infection through their innate immune system, enriched for NK receptors and different types of INF type I genes[65]. Bats also produce antibodies that are highly diverse thanks to a repertoire of VH, DH and JH fragments that is much larger than that found in humans[66,67]. Antibodies do not undergo further improvement by introduction of somatic mutation. Thus, constitutive IFN type I secretion and ready-to-use antibodies may control viral infection in bats without the need of adaptive immune responses. For this reason, coronaviruses and other viruses remain endemic in bats, without damaging the host[65]. Asymptomatic humans may behave like bats, controlling the infection thanks to NK cells and antibodies. The adaptive immune response is strongest in patients with severe disease, following the extensive tissue damage caused by the uncontrolled inflammatory reaction.

Our data has important implications for the control of clinical disease and for public health. In COVID-19, it is indispensable to have prognostic markers early in the course of disease in order to promptly choose appropriate treatments[68]. Although the MLR can be used as an indicator of severe disease[69], the MNKR and the levels of specific IgA antibody in the serum may be more
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sensible early markers of disease evolution.

Herd immunity to a virus is established when the large majority of the population becomes immune and indirectly protects susceptible individuals. Memory T and B cells and long-lived plasma cells generated by the adaptive immune response are the prerequisite for herd immunity, because specific antibodies in the serum and immediately active memory cells prevent individual re-infection and arrest pathogen transmission. Our data suggests that herd immunity to SARS-CoV-2 cannot be achieved by natural infection. Social distancing and infection control will remain indispensable to limit spread of SARS-CoV-2, until effective vaccines able to induce strong and long-lasting B- and T-cell memory are available. Meanwhile, serology, especially when specific IgG is measured, may be not a useful tool to support health policy measures[70]. We may fail to detect past mild infections due to the rapid decline of IgG and we may under-evaluate persistence of viral circulation because asymptomatic individuals respond to the virus with IgA.

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Figure

Figure 1
**Figure 1:** (a) Flow-cytometry analysis of the monocyte to lymphocyte ratio (MLR) in the blood of three representative patients with asymptomatic, mild and severe disease. The lympho-monocyte gate was designed based on physical characteristics (FSC-A vs SSC-A). Lymphocytes were gated as FSC-A^{low} and CD3^{+} or CD3^{-} and monocytes as CD3^{-} FSC-A^{high}. (b) Scatter plot depicts the MLR in the sixty-four adult patients enrolled in the study (contacts n=28; asymptomatic n=20; mild n=8; severe n=8). (c) Gating strategy used to identify natural killer (NK) and monocytes inside the CD3^{-} cells in three representative patients with asymptomatic, mild and severe disease. NK cells were defined as CD3^{-}CD7^{+}FSC-A^{low} and monocytes as CD3^{-}CD7^{+}FSC-A^{high}. (d) Heatmaps of the percentage of NK and monocytes in contacts (indicated by the light green bar), asymptomatic (blue), mild (orange) and severe (red) patients. Percentage levels are reported as differential expression of color. (e) Plots indicate the frequency of NK, monocytes and the monocytes/NK ratio (MNKR) in our patients. (b and e) Midlines indicate mean. Statistical significances were determined using unpaired, two-tailed Mann-Whitney U-tests. *p<0.05, **p<0.01, ***p<0.001.
Figure 2: (a) Plots indicate the frequency of NK, monocytes and MNKR ratio in all analyzed samples. We show here all the results, including also those relative to multiple samples of the same patients collected at different time points.
(b) Heatmaps show the percentage of NK and monocytes in asymptomatic, mild and severe patients (Pt) measured at different time points. Percentage levels are reported as differential expression of red, blue and white. (c) Plot shows the percentage of NK cells in non-ICU \( (n=43) \) and ICU \( (n=34) \) patients. (d) Graphs show the evolution over time of NK cells percentage in patients with favorable and fatal outcome. (a and c) Midlines indicate mean. Statistical significances were determined using unpaired, two-tailed Mann-Whitney U-tests. *\( p \leq 0.05 \), **\( p<0.01 \), ***\( p<0.001 \).

Figure 3: (a) FACS plots depict the gating strategy to identify CD3\(^+\), CD3\(^+\)CD4\(^+\), CD3\(^+\)CD8\(^+\) T cells in three representative patients (asymptomatic, mild and severe). HLA-DR expression in CD4\(^+\) or CD8\(^+\) T cells is shown in the relative plots. Frequency of HLA-DR\(^+\) cells are reported in the plots. (b) Plots show the percentage of CD3\(^+\), CD4\(^+\), CD8\(^+\) and HLA-DR\(^+\) T cells as single value. Mean is shown as midline. Statistical significances were determined using unpaired, two-tailed Mann-Whitney U-tests. *\( p \leq 0.05 \), **\( p<0.01 \), ***\( p<0.001 \).
Figure 4: (a-b) Results obtained by the analysis of CD4⁺ (Plots a) and CD8⁺ T cells (Plots b) are separately shown. Graphs show the percentage of naïve T cells (CD3⁺CCR7⁺CD45RA⁺) that either expressed or lack CD31 (CD31⁺ are
recent thymic emigrants and CD31− are revertant T cells). Effector memory T cells (CD3−CCR7−CD45RA−) can be separated in EM1, EM2, EM3 and EM4. TEMRA T cells are CD3−CCR7−CD45RA+. Midlines indicate mean. Statistical significances were determined using unpaired, two-tailed Mann-Whitney U-tests. *p≤0.05, **p<0.01, ***p<0.001.
Figure 5: (a) Viable lymphocytes were gated and then selected as CD19+ B cells in three representative patients with asymptomatic, mild and severe disease. The identification of the different B cell populations is shown in the empty
plots of the upper line. We identified transitional (CD24+CD38+), naïve (CD24+CD27+), memory (CD24+CD27+), atypical MBCs (CD24+CD38-) and plasmablasts (CD24+CD27+CD38+). In the CD27+ memory B-cell population based on IgM expression, we show IgM and switched (IgM-) MBCs. MBCs were also gated as IgM+, IgG+ and IgG-IgM+ MBCs. (b) Plots indicates the percentage of B cells, MBCs and plasmablasts. In c the frequencies of IgM and switched MBCs is show. Panel d we show the frequency of IgG-, IgG’IgM-MBCs. The ratio between IgG’ and IgG’IgM’ is also shown. Midlines indicate mean. Statistical significances were determined using unpaired, two-tailed Mann-Whitney U-tests. *p<0.05, **p<0.01, ***p<0.001.
Figure 6: (a) IgG and IgA specific for the S1 domain of the SARS-CoV-2 Spike protein were detected by an ELISA method at different time points and concentrations are shown in arbitrary units (AU). Data relative to all samples are shown. Midlines indicate mean. Statistical significances were determined using unpaired, two-tailed Mann-Whitney U-
tests. *p<0.05, **p<0.01, ***p<0.001. (b-d) Graphs show the levels (AU) of IgA (solid line) and IgG (dashed line) during the course of the disease in three asymptomatic (b), three mild (c) and three severe (d) disease patients. (e) Kinetics of antibody titers in asymptomatic patients. Time is indicated in weeks from the first positive nasopharyngeal swab. Empty circles represent patients of which we had only one sample and antibodies were undetectable.