Covid-19: the immune response to a “perfect storm”

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Note: highlighted in italic are information for non-biologists

Introduction

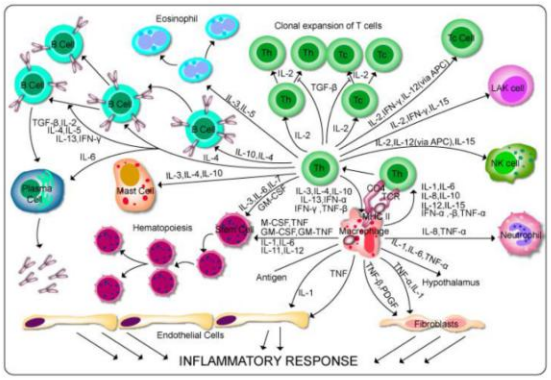
The need to understand how to contain Covid-19 pandemic, through the development of vaccines and treatments, and to unveil how our self-defense machine, i.e. our immune system, responds to SARS-CoV-2 (CoV-2) infection, appears extremely urgent. Related to this last point, one issue is that, to date, immunological studies have been performed in hospitalized patients, which, frequently, underwent critical conditions. Noteworthy, an exacerbated immune response characterizes Covid-19 severe clinical cases, with lymphopenia and eosinopenia, activation of cytokine-secreting cells, consequent cytokine storm, leading to acute respiratory distress syndrome (ARDS), tissues damage, disseminated intravascular coagulation and multiorgan failure. However, it is now clear that our immune system employs all its army and weapons to fight CoV-2 and eradicate Covid-19[1].

Mediators of inflammation: acute phase reactants and the cytokine storm

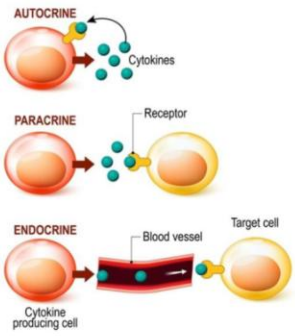
Acute phase proteins, raising at early stages of the inflammatory process, due to infectious and non-infectious agents, may be diagnostic and prognostic markers of human disease[2]. They include: C-reactive protein (CRP), alanine transaminase (ALT), lactate dehydorgenase (LDH), creatin kinase (CK). In Covid-19 severe patients, these molecular indicators, together with cardiac troponin I, procalcitonin, increased prothrombin time, ferritin, D-dimer (*which is a degradation product of fibrin, digested by the plasmin enzyme)*, fibrinogen, aspartate transaminase, increased erythrocyte sedimentation rate, were constantly reported at diagnosis and correlated with the severity of the disease. Importantly, patients carrying these indicators and with dismal prognosis were older than less severe patients and likely affected by other pathologies (cardiovascular disease, diabetes, hypertension, cancer etc.)[3].

Figure 1

a



b



c

Type 1 cytokines (2, 5)					Type 2 cytokines (7–10)		TNF (12, 15)	IL-1 (11)	IL-17 (13, 14)
γc	βc	gp130	IL-12	Others	IFNs	IL-10			
IL-2	IL-3	IL-6	IL-12	Growth hormone	IFN-α	IL-10	TNF	IL-1α	IL-17A
IL-4	IL-5	IL-11	IL-23	Prolactin	IFN-β	IL-19	LTα3	IL-1β	IL-17B
IL-7	GM-CSF	IL-31	IL-27	Erythropoietin	IFN-γ	IL-20	LTα1β2	IL-1RA	IL-17C
IL-9		Oncostatin M	IL-35	Thrombopoietin		IL-22	OX40L	IL-18	IL-17D
IL-15		Leukemia inhibitory factor		M-CSF		IL-24	CD40L	IL-33	IL-17E (IL-25)
IL-21		Ciliary neurotrophic factor		SCF		IL-26	RANKL	IL-36α, β, γ	IL-17F
		Cardiotropin-1		TSLP		IL-28A[a]	TWEAK	IL-36RA	
		NNT-1/BSF-3		IL-13		IL-28B[a]	APRIL	IL-37	
		Leptin				IL-29[a]	BAFF	IL-38	
		G-CSF					LIGHT[b]		

Figure 1. a) Overview of the complexity of the inflammatory response. b) Schematic representation of cytokines action. c) Table representing cytokines families. Abbreviations: APRIL, a proliferation-inducing ligand; BAFF, B cell–activating factor; BSF-3, B cell–stimulating factor 3; CD40L, CD40 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; IL-1RA, IL-1 receptor antagonist; IL-36RA, IL-36 receptor antagonist; LTα1β2, lymphotoxin α1β2; LTα3, lymphotoxin α3; M-CSF, macrophage colony-stimulating factor; NNT-1, novel neurotrophin 1; SCF, stem cell factor; OX40L, OX40 ligand; RANKL, receptor activator of NF-κB; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; TWEAK, TNF-related weak inducer of apoptosis. (Adapted from: Lin & Leonard, Annu Rev Immunol, 2019).

Cytokines are non-antigen specific proteins, produced by a variety of cells (e.g. pericytes, astrocytes, epithelial endothelial and muscle cells), including immune cells, to answer a specific stimulus, to communicate each other and with tissues and organs (figure 1a).

Upon cytokine induction, a cell may proliferate, differentiate or even die. Cytokines may act in an "autocrine" manner, that is they may give instructions to the cell which has produced the cytokine itself, a "paracrine" manner, when the cytokine action is exerted on neighbouring cells, or "endocrine", when they act on districts far from their source of production (figure 1b). Cytokines produced by immune cells are commonly named lymphokines and belongs to different families according to their structure, receptors and signaling mechanisms (figure 1c)[4]. They include interleukins (IL), interferons (IFN), chemochines (CC) and tumour necrosis factors (TNF) and are released by a plethora of immune cells, including B and T lymphocytes, Natural Killer (NK) cells, macrophages,

monocytes, dendritic cells, neutrophils. Covid-19 severe patients recurrently presents what is called “a cytokine storm syndrome”, with clinical manifestations overlapping the secondary haemophagocytic lymphohisiocytosis (sHL), a hyperinflammatory syndrome, usually triggered by viral infections, characterized by unremitting fever, hyperferritinaemia, cytopenias[5] and ARDS, occurring in the 50% of cases[6]. A typical cytokine profile detected in Covid-19 patients shows high levels of IL-2, IL-6, IL-7, granulocyte colony stimulating factor (GCS-F), IFN-γ inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1-α (MIP 1-α), and TNF-α[7,8]. A recent transcriptome analysis (*i.e. the identification and analysis of all the RNAs produced within a cells)* from bronchoalveolar lavage fluid (BALF) and peripheral blood mononuclear cells (PBMCs) in a number of Covid-19 patients specimens has revealed that, in BALF, pathways related to viral replication are activated while in PBMCs the major transcripts output is represented by immune related pathways (figure 2)[9].

Figure 2

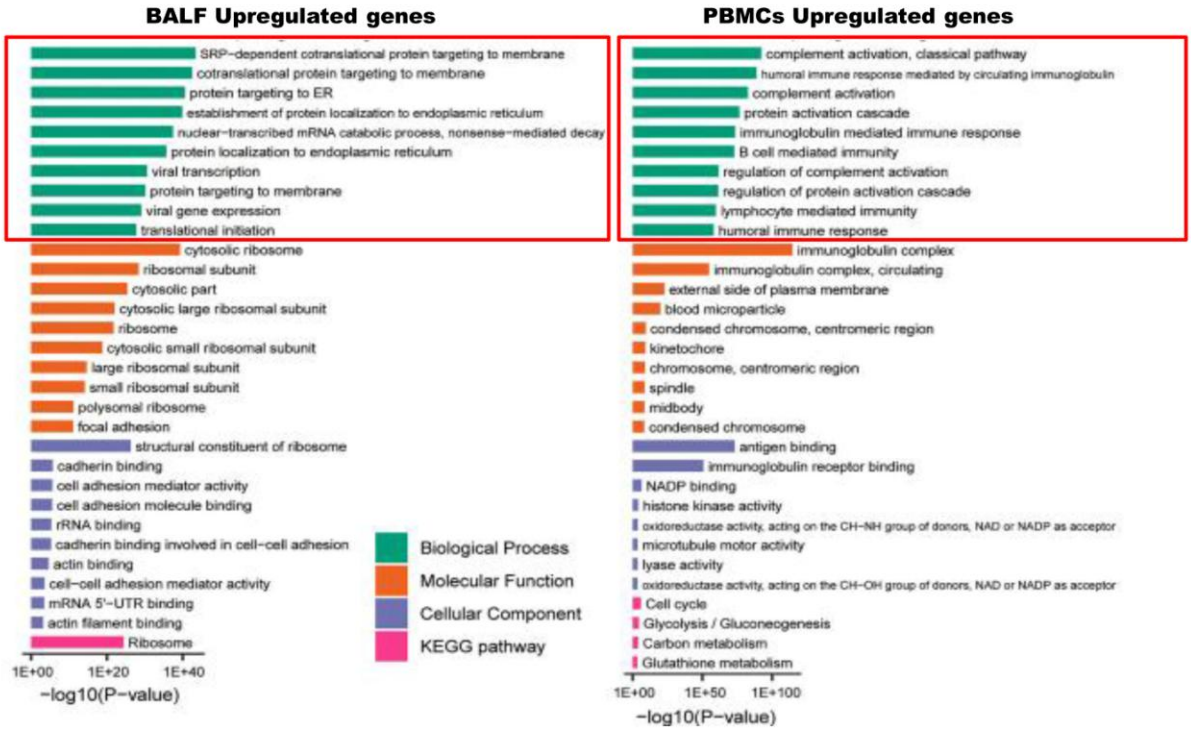


Figure 2. Panels of upregulated genes in BALF and PBMCs of Covid-19 patients, grouped by different criteria. Highlighted by red boxes are genes belonging to the same biological process. *The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway is a collection of maps representing the current knowledge of the interaction, relation and reaction networks for cellular, genetic, environmental processes, organisms, human disease and drug development.*(Adapted from: Xiong et al., Emerg Microbes Infect, 2020).

Interferons: beneficial or detrimental?

Interferons are cytokines produced by tissue cells, white blood cells and, also, tumour cells. *Usually, their production is activated by viral particles, therefore, interferons primary function is to repress the replication of infected cells, but also to potentiate the immune response by inducing the expression of genes belonging to the Major Histocompatibility Complex (MHC; Human Leucocyte Antigen, HLA, in humans) and by activating immune cells, like macrophages and NK cells.* Interferons are classified into three subtypes, alpha (α), beta (β) and gamma.(γ). Interferons α and β belong to the type 1 subclass, whereas interferon γ, represents the type 2 subclass. Recently, another

Figure 3

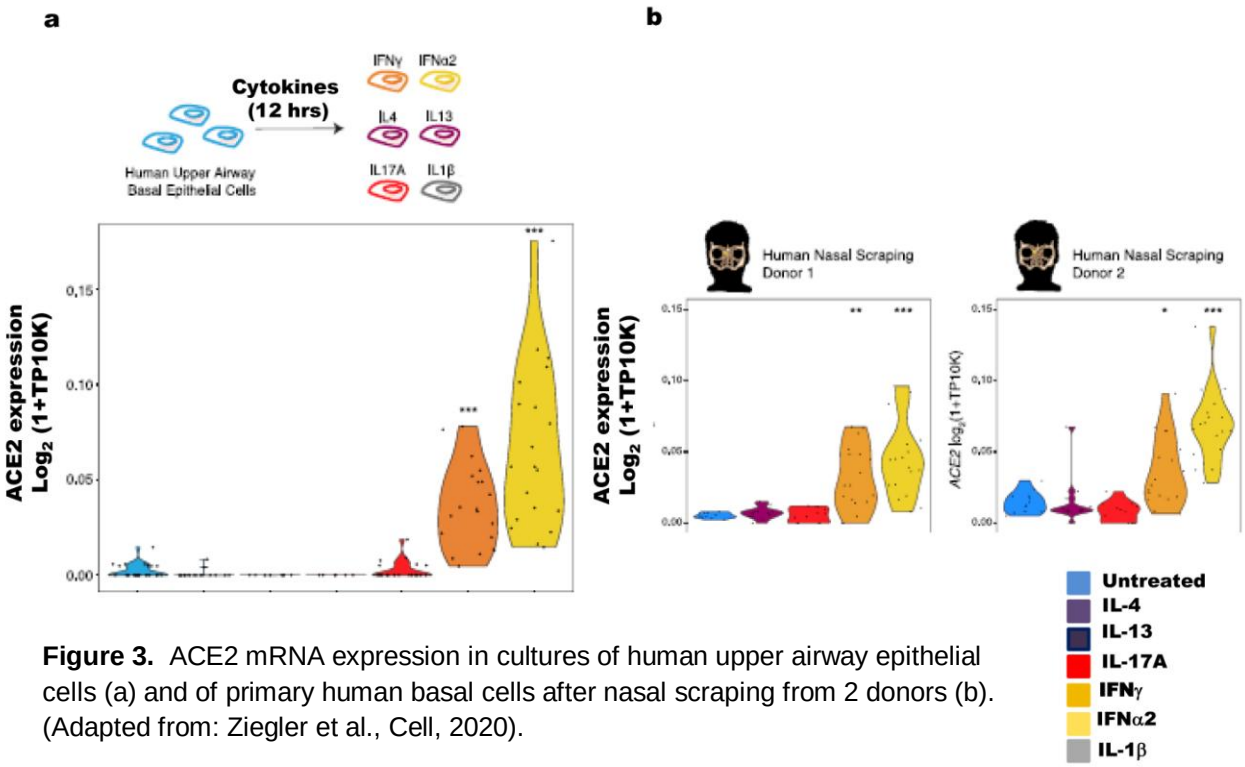


Figure 3. ACE2 mRNA expression in cultures of human upper airway epithelial cells (a) and of primary human basal cells after nasal scraping from 2 donors (b). (Adapted from: Ziegler et al., Cell, 2020).

interferon subclass has been identified, named type 3, called interferon lamba (λ), which, however, resembles type 1 interferons. Cov-2 has been demonstrated to weakly induce interferons[10]. Given their antiviral properties, interferons-based prophylaxis and therapies have been exploited. In particular, interferon α and β have been used to treat hepatitis C and B, and preliminary data show that they could be efficient in prevent CoV-2 infection[11]. Nevertheless, type 1 interferons-based therapy has considerable side effects, due to the ubiquitous expression of their receptors. Conversely, interferon λ is expressed on epithelial

and immune cells and it has been demonstrated to prevent viral dissemination from nasal epithelial cells to the upper respiratory tract[12]. Furthermore, interferon λ is a tissue-protective molecule rather than pro-inflammatory and stimulates both adaptive immunity and antibody production, essential for long term immunity. Therefore it may have beneficial effects on Covid-19 patients. Regarding the use of interferon λ in the clinics, this issue will be discussed in the next episode about Covid-19 therapies and vaccines. Interestingly, it has been proven that both type 1 and type 2 interferons may induce ACE2 expression in human upper airway epithelial cells and in human primary basal cells from nasal scraping of human donors[13] (figure 3). This finding suggests that CoV-2 may use interferons to enhance its infection ability. However, interferon γ-stimulated cells upregulate the guanylate binding protein 5 (GBP5), a protein that inhibits furin activity and viral spread from infected cells[14]. Whether interferons may be beneficial or detrimental for Covid-19 patients is a still debating issue and may depend on factors such as the time of infection, age, gender, co-morbidities.

CoV-2 and the Human Leukocyte Antigen system

The HLA system is constituted by surface proteins which recognize what is "self" from "non-self". These proteins are divided into two classes (I and II). Class I HLA proteins are expressed on all nucleated cells, whereas class II HLA proteins are present on specialized cells, called antigen presenting cells (APCs). Both present peptides to the immune system and are, therefore, the first robust defense against pathogens. The HLA system includes more than 220 genes located on the short arm of chromosome 6, with more than 27000 alleles (an allele is the alternate version of a gene, located at the same position on homolog chromosomes). Different HLA alleles have been demonstrated to confer different viral susceptibility to CoV-2 and disease outcomes, as occurs for SARS-CoV[15]. Recently, an "in silico" analysis (*i.e. based on mathematical predictive models, not on experimental evidences)* of the binding affinity of 145 HLA types for viral peptides of the entire CoV-2 proteome, revealed that HLA-A alleles show the best presentation capacity, whereas HLA-C the fewest. The comparison with proteomes of other human CoVs, revealed that the "best presenters" of conserved peptides, and potentially capable to confer cross-protective immunity, are the HLA-A*02:02, HLA-B*15:03 and HLA-C*12:03 alleles (*where A, B and C are the genes, the first number is the allele and the second number is the specific protein, according to the WHO Nomenclature Committee for Factors of the HLA System*). Conversely, the HLA-A*25:01, HLA-C*01:02 and HLA-B*46:01 alleles are the "least"

presenters, where the latter is also associated with a more severe disease due to SARS-CoV infection[16]. However, this study has the limit to be based on predictive models; therefore, unless validated, it has not any clinical value. Regarding HLA class II proteins, HLA-DR deserves a special mention. HLA-DR is expressed on the surface of monocytes, such as dendritic and B cells and macrophages. HLA-DR presentation of pathogens-derived peptides to $CD4^+$ and $CD8^+$ T-cells initiates a specific immune response. Further, HLA-DR activates T-helper lymphocytes, playing a central role in the immune response against viral agents. HLA-DR molecules are underrepresented on the surface of $CD14^+$ monocytes upon CoV-2 infection, as occurs during sepsis. When the number of HLA-DR^+ monocytes drops, pneumonia evolves into severe respiratory failure, requiring mechanical ventilation[17]. This phenomenon may depend, at least in part, on the high quantity of IL-6 found in critically ill Covid-19 patients, as IL-6 downregulates the expression of HLA-DR[18]. IL-6 family if cytokines exerts many functions, including B cell activation, but are also involved in metabolic and neurotrophic control. In Covid-19 patients, IL-6 is produced mainly by $CD14^+$ monocytes and $CD4^+$ T lymphocytes[17]. A negative correlation exists between IL-6 levels and the number of HLA-DR molecules on the surface of $CD14^+$ monocytes and a correlation also exists between the absolute number of HLA-DR molecules on $CD14^+$ monocytes and the absolute number lymphocytes, which are frequently found decreased in whole blood cell counts (see below). Indeed, Tocilizumab, an IL-6 blocker, restores HLA-DR expression on monocytes and even increases the number of lymphocytes in whole blood[17].

T cells response to CoV-2

Once a respiratory virus enter into host epithelial airways cells, its peptides are presented by class I HLA to the cytotoxic $CD8^+$ T lymphocytes, which undergo clonal expansion, develop virus-specific effector and memory cells. Meanwhile, class II HLA, on the surface of APCs, activate $CD4^+$ T lymphocytes. Very recently, it has been demonstrated that a robust $CD4^+$ and $CD8^+$ T cells response occurs in 70-100% of cultured PBMCs from Covid-19 patients, with respect to CoV-2 unexposed individuals (figure 4a). Specifically, $CD4^+$ T lymphocytes were mainly activated when PBMCs were exposed to Spike (S) epitopes, less when Membrane (M) and Nucleocapsid (N) epitopes were used to stimulate Covid-19 PBMCs. $CD8^+$ cells were activated by M and S epitopes, with at least other 8 CoV-2 Open Reading Frames (ORFs) targeted (figure 4b)[19]. Furthermore, Covid-19 patients makes anti-S antibodies at a level corresponding to the magnitude of the activation of S-specific

$CD4^+$ T cells and also $CD4^+$ and $CD8^+$ T cell response were well correlated. *This observation perfectly fits with the role of $CD4^+$ T cells in helping B*

Figure 4

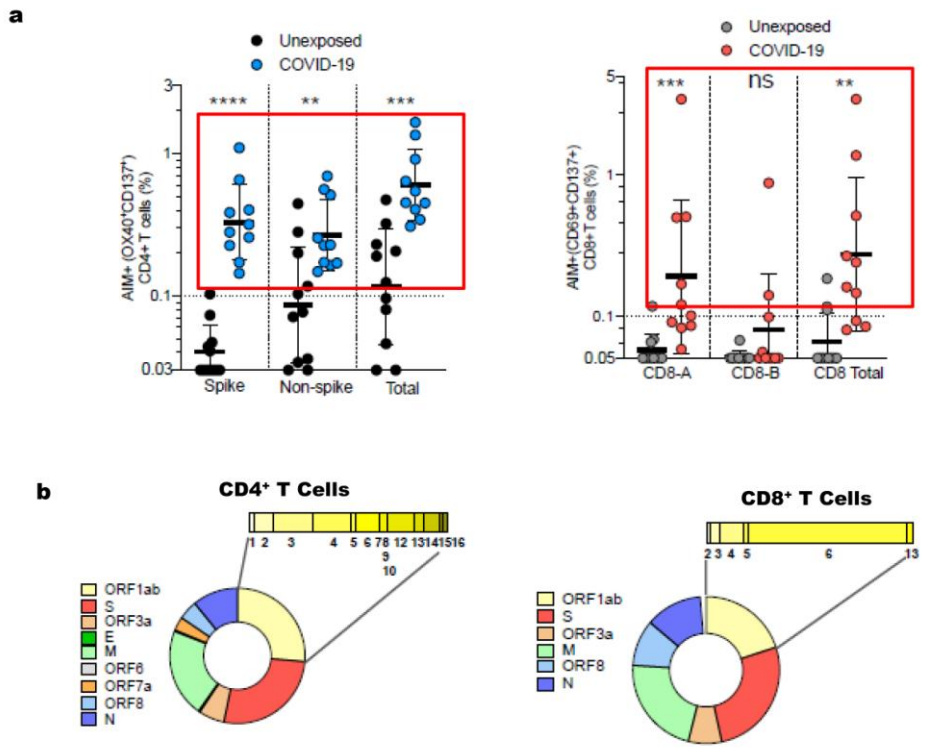


Figure 4. a) Magnitude of $CD4^+$ (left) and $CD8^+$ (right) T cell response in Covid-19 patients vs unexposed individuals. $CD4^+$ and $CD8^+$ T cells were isolated by FACS (*Fluorescence Activated Cells Sorting, which is a method to isolate distinct cell populations, on the basis of specific surface proteins, bound by related antibodies*) after a T cell receptor-dependent Activation Induction Marker (AIM) assay. b) Distribution of specific CoV-2 antigens recognized by $CD4^+$ (left) and $CD8^+$ (right) T cells. (Adapted from: Grifoni et al., Cell, 2020)

cells to produce antibodies and to orchestrate a $CD8^+$ T cell response. Finally, in line and supporting the results exposed in the previous paragraph, related to a cross-protective immunity, in the 40-60% of unexposed individuals, CoV-2 responding $CD4^+$ T cells were detected[19]. Interestingly, in contrast with other Coronaviruses, including SARS-CoV and MERS-CoV, which elicit mostly S, M and N-specific $CD4^+$ and $CD8^+$ responses, CoV-2 presents a broad panel of antigens able to stimulate $CD4^+$ and $CD8^+$ T cells response, including a variety of ORFs[19].

CoV-2 specific antibodies

Antibodies, synthesized by B lymphocytes and plasmacells, represent our humoral response to pathogens. They may indicate to our "garbage cells", basically macrophages, which cells are infected and have to be eliminated, they may activate the complement system, which lyses pathogens, or they may interfere with the binding of pathogens to the target cells, neutralizing their infectious activity. It is now well established that CoV-2 elicits

an antibody response in Covid-19 patients, with M immunoglobulins (IgM, *usually the first class of immunoglobulins appearing during a humoral response*) emerging in the acute phase of the disease and IgG at later time points[1]. Antibodies raise from 5 to 10 days after the emergence of symptoms (figure 5a). However, different timing of seroconversion has been reported, with IgM and IgG peaks at 17-19 and 20-22 days after symptoms, respectively, in some cases. IgA, which mediate mucosal immunity and may reduce the viral attachment to the mucosa epithelium, have been also reported in CoV-2 infected patients, with an onset intermediate to that of IgM and IgG (figure 5). Interestingly and in support of the great importance of humoral response to fight Covid-19, children, who seem

Figure 5

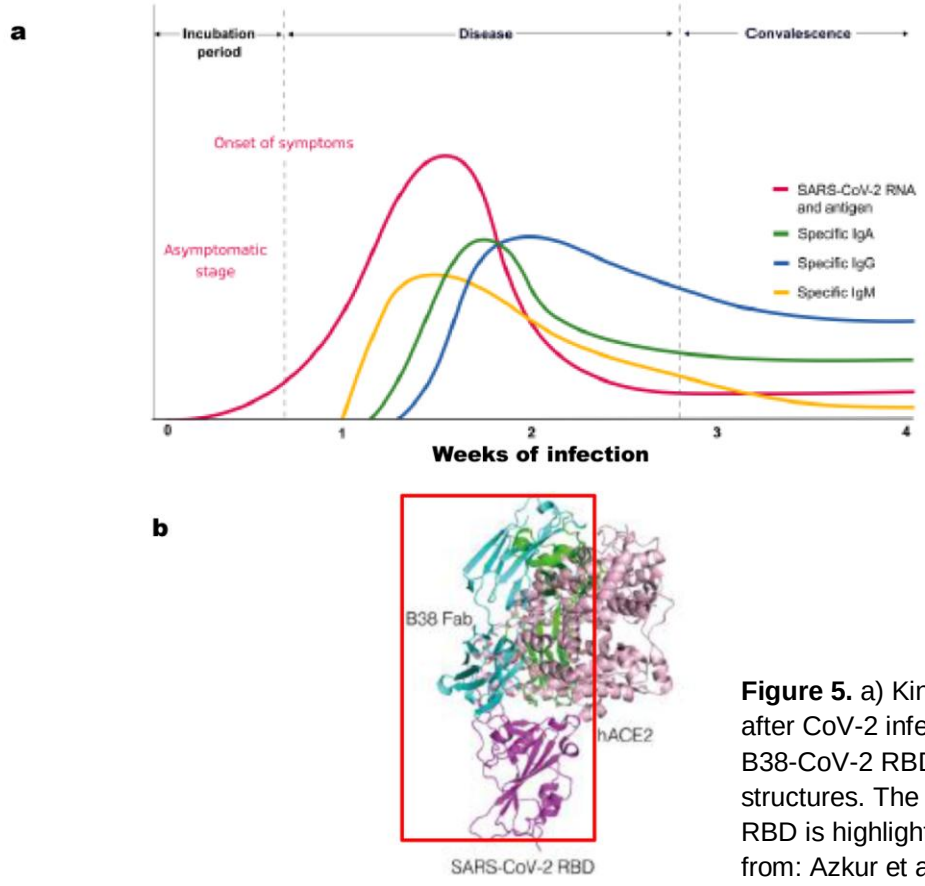


Figure 5. a) Kinetics of IgG seroconversion after CoV-2 infection. b) Superimposition of B38-CoV-2 RBD and ACE2-CoV-2 RBD structures. The B38 antibody bound to CoV-2 RBD is highlighted by a red box. (Adapted from: Azkur et al., Allergy, 2020; Wu et al., Science, 2020)

to exhibit low susceptibility to infection and milder symptoms, present high IgG levels within 1 week after the onset of the disease, with basically no IgM, indicating a very rapid seroconvesion from IgM to IgG. This may also account for an under-estimated number of asymptomatic pediatric patients[20]. Very recently, using S Receptor Binding Domain (RBD) of CoV-2 as a bait, B memory cells from recovered Covid-19 patients have been isolated and four antibodies produced by these cells have been demonstrated to specifically bind CoV-2 RBD, but not SARS-CoV RBD. Among them, two (named H4 and B38)

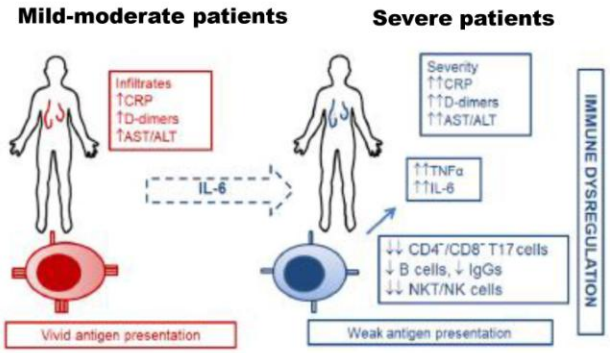
demonstrated specific neutralizing activity, that is, they impaired S binding to ACE2. Specifically, at the RBD-B38 interface 18 of the 21 aminoacids involved in RBD-ACE2 interaction are employed, explaining why B38 blocks the binding of CoV-2 RBD to ACE2[21] (figure 5b). Importantly, B38 and H4 antibodies have been proven to be protective against Covid-19 in a mouse model of the disease[21].

Immune dysregulation in Covid-19: lymphopenia and eosinopenia

Many literature reports describe lymphopenia to occur in critically ill Covid-19 patients, with T lymphocytes, including T regulatory lymphocytes (Tregs), *which play a pivotal role in*

Figure 6

a



b

Immunologic changes	Covid-19
T cell responses	Lymphopenia in severe cases (< 20%). Initial lymphopenia is predictive of severe disease.
CD8⁺ T cells	Severe lymphopenia (< 5%) is observed in CD8+ T cells and can be a predictor of severe disease.
Eosinophils	Decreased circulating eosinophil numbers in 50 to 80% of the hospitalized patients.
Specific antibody levels	In the acute phase, virus-specific IgM increases followed by virus specific IgG during convalescence.
Cytokine storm	Innate and adaptive cytokines are released in high amounts linked to severe disease.
Acute phase reactants	High in severe cases. Initially high values are predictive of severe disease.

Figure 6. a) Schematic representation of immune responses occurring in mild-moderate (red) and severe (blue) Covid-19 patients. b) The table sums the principle features of immune e dysregulation, characterizing severe Covid-19 clinical cases. (Adapted from: Giamarellos-Bourboulis et al., Cell Host & Microbe, 2020; Azkur et al, Allergy, 2020)

limiting excessive immune responses to pathogens, being the most affected. In some cases, also NK cells decrease and B lymphocytes are reduced at the lower limit of the reference values[22]. The decrease in lymphocytes count, may be used as a diagnostic and prognostic marker for Covid-19. Indeed, patients with T lymphocytes greater than 20% at 10-12 days after the onset of symptoms were classified as mild-moderate; conversely, at that time point, patients with less than 20% T lymphocytes were classified as severe. At about 20 days after diagnosis, patients with more than 20% T lymphocytes were accepted as recovering; patients with a percentage of T lymphocytes between 5 and 20% were

classified at still high risk, whereas individuals with a percentage of T cells less than 5% were critically ill. The molecular mechanism underlying this phenomenon is still unknown. Interestingly, in PBMCs from Covid-19 patients an activation of the p53 pathway has been reported[9], suggesting that T lymphocytes decrease may be due to apoptosis. This possibility is also supported by the evidence that T lymphocytes may be susceptible to CoV-2 infection[23]. Lymphopenia may be also dependent on the viral load to which an individual is exposed. Low viral loads may induce an appropriate T and B cells response and appearance of neutralizing antibodies, leading to virus clearance. High viral loads may provoke an excessive immune response, with cytokines storm, decreased T lymphocytes levels and dismal prognosis[1]. Another parameter to be taken into account is the eosinophils count. Eosinophils act in adaptive immunity and produce antiviral molecules. Further, they serve as APCs against respiratory viruses. In Covid-19 patients, a constantly reduced level of eosinophils has been detected, when compared to pneumonia-affected individuals[24,25]. This phenomenon may depend on different factors: immune exhaustion, loss of eosinophilopoiesis *(i.e. block of eosinophils production from the bone marrow)* or recruitment to the site of infection, despite no accumulation of eosinophils has been found in autoptic specimens of CoV-2 infected lungs[26].

Conclusions

Figure 6 summarizes what happens in Covid-19 severe patients. Basically, critically ill individuals shows high levels of cytokines, especially IL-6, which lowers $CD14^+$ monocytes and lymphocytes count in a negative feedback loop, as $CD14^+$ monocytes and $CD4^+$ T lymphocytes are the major producer of IL-6 in Covid-19 affected individuals[17]. The low number of Tregs is not sufficient to counteract an excessive immune response, enhancing this immune dysregulation. The magnitude of the viral load is important to determine the severity of the disease, against which, however, we are able to elicit a robust humoral and T-cell mediated response[21,19]. Altogether, these findings establish strong premises for the set up of efficient therapies and vaccines.

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