

THE ROLE OF EXTRACELLULAR VESICLES IN PEDIATRIC COVID-19 PATIENTS WITH ASTHMA

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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**To my son,
who is the joy of my life,**

**To my husband,
who is the source of my courage,**

**To my mom and dad,
who are the reasons I am who I am today.**



ABSTRACT

THE ROLE OF EXTRACELLULAR VESICLES IN PEDIATRIC COVID-19 PATIENTS WITH ASTHMA

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SARS-CoV-2, the causative agent of coronavirus disease 2019 (COVID-19), has infected millions of people, and asthma was initially considered a risk factor for COVID-19. Although numerous studies on asthma and COVID-19 have been conducted, the role of plasma-derived extracellular vesicles (EVs) in COVID-19 patients with asthma remains unknown. In this study, we assessed the influence of EVs from healthy controls, severe and mild COVID-19 pediatric patients with or without asthma during acute and convalescent periods on healthy naïve CD4⁺T cells and monocytes. While plasma cytokines and anti-SARS-CoV-2 antibodies were similar between the groups with and without asthma, immune responses varied depending on the severity of COVID-19. In the severe acute group, whereas all cytokines increased, IFN γ , CD4⁺T cell counts, and monocyte numbers decreased. Stimulating healthy cells with EVs from severe acute patients led to increased PDL1 expression, Th2 and Treg cell proportions, decreased IFN γ secretion, Th1, and Th17 cell ratios. Patient EVs also reduced proinflammatory cytokine secretion from monocytes. Severe acute patient EVs caused a decline in healthy CD4⁺T cell and monocyte populations. Overall, our results indicate immunological responses and EV-related outcomes depending on the severity of COVID-19 rather than the presence of asthma, immunosuppression seen in severe acute COVID-19 and potential contribution of EVs to this immunosuppressive pattern in severe cases.

Keywords: CD4⁺T cells, COVID-19, extracellular vesicles (EVs), monocytes, SARS-CoV-2

ÖZET

ASTIMI OLAN PEDIATRİK COVID-19 HASTALARINDA EKSTRASELÜLER VESİKÜLLERİN ROLÜ

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Moleküler Biyoloji ve Genetik

Doktora Tezi

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2019 koronavirüs hastalığının (COVID-19) nedeni olan SARS-CoV-2, milyonlarca insanı enfekte etti ve astım, başlangıçta COVID-19 için bir risk faktörü olarak kabul edilmişti. Astım ve COVID-19 üzerine çok sayıda çalışma yapılmıştır. Ancak, astımı olan COVID-19 hastalarında plazmadan elde edilen hücre dışı keseciklerin (EV'ler) rolü hala bilinmiyor. Bu çalışmada, akut ve iyileşme dönemlerinde sağlıklı kontrollerden, astımı olan ve olmayan, ağır ve hafif COVID-19 pediatrik hastalarından elde edilen EV'lerin sağlıklı saf CD4⁺T hücreleri ve monositler üzerindeki etkisini değerlendirdik. Plazma sitokinleri ve anti-SARS-CoV-2 antikorları astımı olan ve olmayan gruplar arasında benzerlik gösterirken, bağışıklık yanıtları COVID-19'un şiddetine bağlı olarak değişmiştir. Şiddetli akut grupta, tüm sitokinler artarken, IFN γ , CD4⁺T hücre ve monosit sayıları azalmıştır. Bu sonuçlar, ağır vakalarda immünosüpresif bir patern olduğunu düşündürmektedir. Ağır akut hastalardan alınan EV'ler ile sağlıklı hücrelerin uyarılması, PDL1 ekspresyonunun, Th2, Treg hücre oranlarının artmasına ve IFN γ salgılanmasının, Th1, Th17 hücre oranlarının azalmasına yol açtı. Hasta EV'leri ayrıca monositlerden proinflamatuvar sitokin salgılanmasını da azalttı. Ağır akut hasta EV'leri, EV ile uyarılan sağlıklı PBMC'lerde CD4⁺T hücresi ve monosit popülasyonlarını azalttı. Genel olarak, sonuçlarımız astım varlığından ziyade COVID-19 şiddetine bağlı olan immünolojik yanıtları ve EV ilişkili sonuçları, şiddetli akut COVID-19'da görülen immünosüpresyonu ve EV'lerin şiddetli vakalarda bu immünosüpresif modele potansiyel katkısını göstermektedir.

Anahtar Kelimeler: CD4⁺T hücreleri, COVID-19, hücre dışı veziküller (EV'ler), monositler, SARS-CoV-2

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ABBREVIATION

Angiotensin-converting enzyme 2	ACE2
Airway epithelial cells	AECs
Airway hyperresponsiveness	AHR
Allergen-specific immunotherapy	AIT
Absolute lymphocyte count	ALC
Alanine aminotransferase	ALT
Absolute neutrophil count	ANC
Antigen-presenting cells	APCs
Acute respiratory distress syndrome	ARDS
Airway smooth muscle	ASM
Aspartate aminotransferase	AST
Bronchoalveolar lavage fluid	BALF
Childhood asthma control test	C-ACT
Coxsackie B virus	CB
CC-chemokine ligand 17	CCL17
Canadian Healthy Infant Longitudinal Development	CHILD
Chinese Medical Association	CMA
Childhood Origins of Asthma	COAST
Chronic obstructive pulmonary disease	COPD
C-reactive protein	CRP
Cytotoxic T lymphocytes	CTLs
Convolutd membranes	CVs
Damage-associated molecular patterns	DAMPs
Dendritic cells	DCs
Double membrane vesicles	DMVs

Envelope	E
Early-type bronchoconstrictor response	EAR
Endoplasmic reticulum	ER
Endoplasmic reticulum-Golgi intermediate compartment	ERGIC
Erythrocyte sedimentation rate	ESR
Environmental tobacco exposure	ETS
Extracellular vesicles	EVs
Fetal bovine serum	FBS
Forced expiratory volume in one second	FEV1
Green fluorescent protein	GFP
Gastrointestinal tract	GIT
Granulocyte monocyte colony stimulation factor	GM-CSF
genomic RNA	gRNA
Glucose-regulated protein 94	GRP94
Genome-wide association studies	GWAS
human angiotensin-converting enzyme 2	hACE-2
Hepatitis A virus	HAV
Hemoglobin	Hb
Healthy control	HC
Hepatitis C virus	HCV
House dust mites	HDM
Hepatitis E virus	HEV
Human immunodeficiency virus	HIV
Human rhinovirus	HRV
Inhaled corticosteroids	ICSs
Interferon	IFN γ
Immunoglobulin E	IgE
Interleukin 4	IL4

Intraluminal vesicles	ILVs
Interquartile Range	IQR
Interferon regulatory factor 1	IRF1
International Study of Asthma and Allergies in Childhood	ISAAC
International Society of Extracellular Vesicles	ISEV
Interferon-stimulated genes	ISGs
Inhibitory regulatory T cell	iTReg
Kawasaki disease	KD
Late-phase response	LAR
Lactate dehydrogenase	LDH
long non-coding RNAs	lncRNAs
Leukotriene C4	LTC4
Leukotriene receptor antagonist	LTRA
Membrane	M
Magnetic cell sorting	MACS
Myelin and lymphocyte protein	MAL
Monocyte colony stimulation factor	M-CSF
Myeloid derived suppressor cells	MDSC
Middle East respiratory syndrome coronavirus	MERS-CoV
Mean fluorescence intensity	MFI
Major histocompatibility complex	MHC
micro-RNAs	miRNAs
Multisystem Inflammatory Syndrome in Children	MIS-C
Minimal Information for Studies of Extracellular Vesicles	MISEV
Metalloproteinase	MMP
Multiplicity of infection	MOI
messenger RNAs	mRNAs
Multivesicular bodies	MVBs

Multivesicular endosomes	MVEs
Nucleocapsid	N
neutralizing antibodies	nAbs
NOD-like receptor family CARD domain-containing five	NLRC5
Nitrogen dioxide	NO ₂
Non-structural proteins	NSPs
Open reading frames	ORFs
Pathogen-associated molecular patterns	PAMPs
Peripheral blood mononuclear cells	PBMCs
plasmacytoid dendritic cells	pDCs
Phospholipase D 2	PLD2
Particle matter	PM
para-Nitrophenylphosphate	pNPP
Pattern recognition receptors	PRRs
Receptor binding domain	RBD
Replication organelle	RO
Respiratory syncytial virus	RSV
Replication-Transcription Complex	RTC
Real-time reverse transcription-polymerase chain reaction	RT-PCR
Spike	S
Severe acute respiratory syndrome coronavirus	SARS-CoV
SARS CoV-2 specific peptide pool	SMNO
Single nucleotide polymorphisms	SNPs
Sulfur dioxide	SO ₂
single-stranded RNA	ssRNA
Central memory T cells	TCM
T cell receptor	TCR
Terminal effector memory T cells	TEMRA

Effector memory T cells	TEM
Transmission Electron Microscopy	TEM
Transforming growth factor	TGF- β
Follicular helper	Tfh
T helper	Th
Transmembrane protease serine 2	TMPRSS2
Tumor necrosis factor	TNF
Tissue-resident memory	TRM
transfer RNAs	tRNA
Transcriptional regulatory sequences	TRSs
Tumor susceptibility gene 101	TSG101
Thymic stromal lymphopoietin	TSLP
Toxic shock syndrome	TSS
Urban Environment and Childhood Asthma	URECA
Untranslated region	UTR
Vascular cell adhesion molecule-1	VCAM-1
White blood cell	WBC

1. CHAPTER 1

INTRODUCTION

1.1.Asthma Overview

Asthma is the most frequently observed chronic respiratory condition in the pediatric population. The incidence of asthma varies among the different regions, with developing countries reporting rates between 3% and 5%, while industrialized countries have observed rates above 20%. This respiratory condition affects individuals across all age cohorts and has a prevalence of around 14% among pediatric population worldwide (1). Asthma is characterized by the presence of acute broncho-obstruction and accompanied by the cardinal symptoms including cough, chest tightness, shortness of breath, and wheezing. The symptoms and degree of airflow limitation exhibit variability across individuals and the severity of asthma severity, may also fluctuate over time. While there exist several effective treatment options such as inhaled, oral, and parental medications, there is not a curative therapy for asthma yet. Asthma exerts a significant burden on healthcare systems, on personal and family life in terms of treatment costs and quality of life (2).

The asthma symptoms manifest as a result of chronic inflammation of the airways, triggered mostly by the inhalation of allergens (3). This inflammation leads to increased sensitivity of the airways, structural changes in the airway walls (airway remodeling), and excessive production of mucus. Asthma is characterized as a heterogeneous condition with different endotypes due to the complexity and diversity of symptoms and pathophysiology (3).

The evidence that inflammation is the main component of asthma is eosinophilic infiltration, neutrophils, mast cell degranulation, thickening of the sub basal membrane, loss of epithelial cell integrity, mucous obstruction of the bronchial lumen, and hyperplasia of goblet cells (4). The primary mechanism underlying asthma is inflammation of the airways, which serves as the central focus for therapy interventions.

Traditionally, asthma is divided into allergic and non-allergic types based on the presence of Immunoglobulin E (IgE) antibodies to certain allergens. Both are characterized by airway infiltration of T helper (Th) lymphocytes. The immunopathophysiology of asthma includes both innate and adaptive immune responses. The inflammatory cells involved in the pathogenesis of asthma are, in particular, eosinophilic cells, mast, neutrophils, and T lymphocytes (5). One of the most important mechanisms in the pathophysiology of asthma is the regeneration of the airway. This remodeling has been associated with extracellular matrix accumulation and it can lead to irreversible fibrosis and airway narrowing in case of the persistence of the inflammation. These architectural changes include increased mass of the smooth muscle layer of bronchi by hypertrophy and hyperplasia, subepithelial collagen deposition, goblet cell hyperplasia, thickening of airway mucosa and increase in vascularity (6) **(Figure 1.1.)** (7). In addition, at the cellular level, many inflammatory cells are attracted to the respiratory tract through chemokines and various cytokines, such as IL3, IL4, IL5, IL13 and granulocyte-macrophage colony-stimulating factor (GM-SCF) (8).

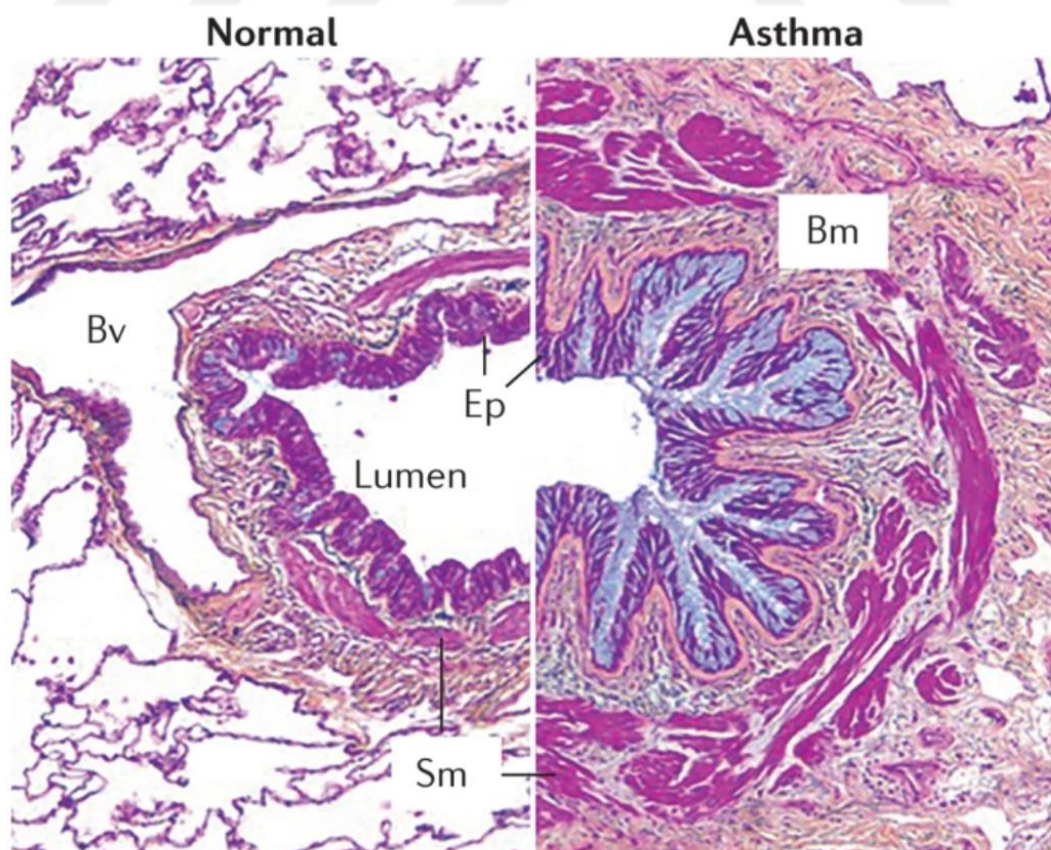


Figure 1.1. Differentiation of the airway in asthma. Comparison of the cross section of an airway affected by severe asthma (right) with that of a healthy airway (left). Asthma is characterized by inflammation of the mucosal lining, which is primarily caused by activated eosinophils, mast cells, and T lymphocytes. This inflammation occurs in the setting of an altered airway structure, which includes changes such as mucous metaplasia, an increase in smooth muscle, fibrosis, and angiogenesis. Bm; basement membrane, Bv; blood vessel, Sm; smooth muscle, Ep; epithelium. (Adapted from Ref. 7).

1.2. Epidemiology

Asthma often manifests during early infancy; however, it may thereafter enter a period of remission, occasionally resurfacing in adulthood. The significant majority of the individuals diagnosed with asthma experience the first episode of wheezing prior the age of 6 years (9,10). Mostly, wheezing which is experienced at the early stages of childhood tends to resolve, but asthma that persists into adulthood is more likely to last. The prevalence of wheezing exhibits a gender disparity, with a higher occurrence observed in boys compared to girls. However, this pattern undergoes a transition throughout adolescence, where asthma becomes more prevalent in females than in males. The prevalence of asthma has increased significantly in recent years in various age groups and coincides with an increase in sensitization and allergic conditions, including atopic dermatitis and allergic rhinitis, and urbanization trends. Annually, asthma claims the lives of around 250,000 individuals, representing a proportion of 1 in every 250 global fatalities (11).

Currently, there is a lack of internationally standardized metrics for assessing the occurrence of asthma, as well as the likelihood of getting asthma within a defined period. According to a research conducted in the United States, the prevalence of asthma during the initial year of life was seen to be 3%. Subsequently, this figure decreased to 0.9% in the age range of 1 to 4 years, and further declined to 0.1% beyond the age of 15 years. According to a cohort research conducted in New Zealand, it was shown that 27% of children at the age of 9 had experienced wheezing at least once, while 4.2% of them were on asthma treatment (9). The findings from cohort studies conducted in both the United States and Australia demonstrate a similarity in the observed statistics. Specifically, the age at which wheezing was initially recorded was

found to be highest during the initial year of life and then stabilized during the adolescent years (12).

According to the findings of the International Study of Asthma and Allergies in Childhood (ISAAC), the prevalence of symptoms during a 12-month period varied among different nations. The reported rates ranged from 3% to 5% in countries such as Indonesia, China, and Greece, while exceeding 20% in Canada, Australia, New Zealand, and the United Kingdom (13). The occurrence of childhood asthma in Western nations exhibited either a stable or slightly declining trend across the span of seven years between the ISAAC surveys. However, a notable increase was seen in several non-Western countries with initially low prevalence rates. The plateau observed in Western nations may indicate that the population has reached its genetic potential, wherein all children who are prone to developing asthma already manifest the condition. The rise in low-prevalence countries is associated with economic growth and Westernization in these countries (10). Further investigation is required to get a comprehensive understanding of the underlying processes that contribute to shifts in prevalence rates. These mechanisms include modifications in microbial colonization within the lung and gastrointestinal tract, exposure to chemicals and pollutants, as well as differences in dietary patterns associated with Western-style lifestyles (14). There is a strong correlation between early life allergic sensitization and pediatric asthma in industrialized cultures, although this association is not as prevalent as in many developing countries, where non-atopic asthma tends to be more common (14).

1.3. Pathogenesis and Etiology

Asthma is a complex inflammatory condition affecting respiratory tract, distinguished by increased responsiveness of the airways, transient restriction of airflow, and excessive production of mucus (15). The presence of chronic airway inflammation is the distinct feature, often accompanied by structural changes in the airway wall. Although structural alterations predominantly manifest in the large bronchi, it also influences the smaller bronchi and bronchioles when asthma progresses to severe form (16). Chronic inflammation is often accompanied by structural alterations that are commonly referred to as remodeling (17). The prevalence of asthma is notably elevated in the pediatric population due to their increased exposure to allergens, such as air pollution and tobacco smoke and viral infections. Additionally, their immune systems

are not adequately trained to respond effectively when stimulated by these allergens (11). The understanding of asthma as a complex disorder with several risk factors contributing to its development has been well-established for a considerable period. The genetic nature of asthma was initially shown by research conducted on twins. Basically, it can be stated that genetic predispositions play a significant role in the pathogenesis of asthma, with 60% inheritance rates (18).

1.3.1. Genetics

Large-scale genome-wide association studies (GWAS) conducted in both pediatric and adult populations have discovered asthma-related single nucleotide polymorphisms (SNPs) that exhibit high statistical significance (19). The findings have emphasized the significance of genetic variations in genes known to have a role in asthma, such as HLA-DQ, SMAD3, TSLP, IL1RL1/IL18R1, and IL33 (19). The collective risk associated with these genetic variations is estimated to account for approximately 10% of the inheritance and prevalence of asthma.

The GSDMB-ORMDL3 locus, located on chromosome 17q21, has shown the most consistent replication in GWAS of asthma. Implicated genes suggest that changes in epithelial barrier function contribute to asthma. Within the Avon Longitudinal Study of Parents and Children, this specific genetic locus presented the highest level of risk for ongoing wheezing in children (20). Single nucleotide polymorphisms (SNPs) located in the ch17q21 region have consistently shown a correlation with severe acute asthma exacerbations that need the use of oral steroids and/or hospitalization.

Epigenetic changes have a crucial role in controlling the production of cytokines and transcription factors that are important for the differentiation of T cells. Furthermore, epigenetic processes, such as DNA methylation, might impact pediatric asthma by controlling the levels of IgE and other asthma-related genes (ALOX15, CAPN14, and POSTN) (21,22).

1.3.2. Indoor Allergens

The correlation between the development of asthma and the exposure to airborne allergens is well-known. A notable elevation in the likelihood of developing asthma is observed in those who are sensitized to aeroallergens before the age of five (23). Indoor allergens such as house dust mite, mice, cockroach, animal dander, and fungus are particularly important in childhood asthma.

In a study involving children, it was shown that to be sensitized to dust mites at ages 1 and 2 increased the likelihood of experiencing wheezing in adolescence by 3.3 and 6.4 times, respectively (24). It was clearly demonstrated that children who were exposed to house dust mites (HDM) are at risk for asthma (25). In addition, the exposure to cockroaches is a stronger trigger for sensitization compared to other indoor allergens (26). However, the relationship between exposure to furry pets and the likelihood of developing atopic conditions is inconsistent. Surveys that rely just on the presence of pets in the home may not be enough to accurately quantify exposure to cat and dog allergies, which are quite common (27). In general, there is no evidence to suggest that exposure to pet allergens increases the chance of developing allergies. In fact, one research found that exposure to cats may lessen the risk of developing asthma (28).

There is a correlation between both qualitative and quantitative assessments of fungal exposure and an increased susceptibility to allergies and asthma. The involvement of sensitivity to *Alternaria* has been linked to higher rates of asthma, regardless of sensitization to other airborne allergens. The exposure to fungi elevates the likelihood of severe asthma exacerbations that might potentially be life-threatening, presumably due to the production of IL-33 (29). Patients with severe asthma who require frequent hospital stays often have mold sensitivity (30).

Conversely, the Urban Environment and Childhood Asthma (URECA) birth cohort was shown that higher levels of mouse, cat, and cockroach allergens in early childhood were shown to be associated with a lower risk of developing asthma (31).

1.3.3. Outdoor Allergens

Exposure to grass pollen in the environment is a significant factor that might worsen children asthma, leading to emergency room visits. This has been recently verified by

a comprehensive review (32). Insufficient information exists about the impact of early life exposure to pollen on the development of pediatric asthma (33).

Recent research suggests that asthmatic children are prone to have worsened symptoms that need hospitalization when exposed to outdoor fungal spores, compared to other outdoor allergens (34). Moreover, there is a strong correlation between elevated levels of outdoor fungal/mold exposure during peak periods with the worsening of asthma symptoms and increased mortality rates in adults (35,36). The effect of indoor and outdoor allergens in childhood asthma has been demonstrated in **Figure 1.2** (37).

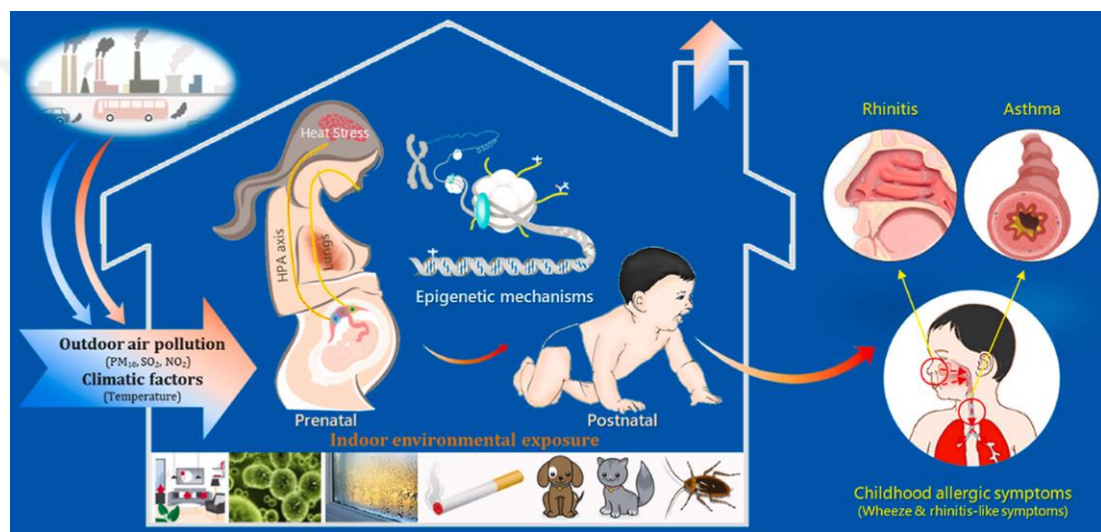


Figure 1.2. Overview of the effect of the indoor and outdoor allergens in childhood asthma. The onset of allergy symptoms is significantly influenced by the exposure to the home environment allergens throughout early life. Elevated exposure to PM and SO₂ during the first three years of life increases the likelihood of developing chronic allergy symptoms. Subsequent exposure to outdoor and indoor allergens increases the likelihood of developing asthma and rhinitis. Indoor and outdoor exposures have distinct impacts on the initiation and progression of allergies. (Adapted from Ref. 37).

1.3.4. Environmental Tobacco Exposure

Exposure to environmental tobacco exposure (ETS), is significantly increased the likelihood of developing asthma throughout early stages of life. Maternal smoking throughout both the prenatal and postnatal periods had a substantial impact on the occurrence of asthma at all stages of development (odds ratio 1.18-1.70). Furthermore,

there was a clear correlation between fathers who smoke and a notably higher likelihood of asthma in children aged 5-18 (odds ratio of 1.39) (38).

1.3.5. Air pollution

Studies on the epidemiology of air pollution and asthma have found that there is a higher risk of worsening lung diseases when exposed to air pollution in the short term, as well as an increased likelihood of developing or worsening asthma when exposed to air pollutants over a long period of time. Multiple pollutants, such as ozone, nitrogen dioxide (NO₂), particle matter (PM), sulfur dioxide (SO₂), and others, have been identified as culprits, even when present at levels below the current National Ambient Air Quality Standards (39).

Global warming has been implicated in the augmentation of both the length and strength of the pollen season (40). Pollutants also intensify both immediate and delayed immune responses to different allergens and contribute to the progression of diseases by increasing the initial sensitivity to allergens. The heightened contact with allergens, in conjunction with pollution, synergistically amplifies the allergic reaction.

1.3.6. Respiratory viruses

Respiratory viruses have an impact on the development of wheezing and asthma, either on their own or in combination with atopy. Respiratory syncytial virus (RSV) and human rhinovirus (HRV) are the predominant respiratory viruses linked to wheezing in young children. In addition, influenza has been linked to the worsening of existing medical conditions (41). In a particular study, the presence of influenza A virus was found in 2.6% of children who were admitted to the hospital, and in 14.1% of patients with asthma flares who were treated on an outpatient basis (42).

The Childhood Origins of Asthma (COAST) research found that HRV driven wheeze is linked with a higher likelihood of developing atopic asthma in the future, similar to the risk associated with allergen sensitization. This was observed in follow-up assessments conducted at ages 7 and 13 (43). Asthma exacerbations triggered by HRV also increase the probability of dust mite sensitization (44). Conversely, wheezing

caused by RSV during infancy was linked to non-atopic asthma at the age of 7. Approximately 50% of infants who experienced wheezing caused by RSV developed long-lasting asthma when monitored for up to 7 years (45). Nevertheless, a research found no correlation between wheezing caused by RSV during the first three years of life and the likelihood of developing asthma at the age of 13 (23).

1.3.7. Microbiome Exposures

The hygiene hypothesis suggests that our early-life microbial environment plays a crucial role in the formation of our immune system and protection against atopic disorders and asthma. Over the past ten years, several studies have examined the protective benefits of growing up on a farm compared to living in rural areas or cities (46,47). These farm life research have provided a convincing situation in favor of the hygiene idea. Living in close contact with domestic animals throughout early life seems to prevent the development of atopic asthma, since it exposes individuals to beneficial microbial agents (47). The widely acknowledged concept posits that the microbial variety present in the farm environment elicits defensive immune responses. Gender also seems to exert an influence on the effects of exposure, since females raised on a farm had a lower overall occurrence of asthma compared to males, as indicated by a lower cumulative incidence rate (48).

Another study indicating that a decrease in the variety of microorganisms in the stool at one month old may be used to predict the occurrence of atopy at two and six years old (49). The incidence of asthma is higher in children delivered by cesarean section, suggesting that differences in microbial colonization patterns may play a role compared to children delivered vaginally (50). Furthermore, the presence of older siblings has been found to have positive impacts on the neonatal microbiota, in addition to the benefits of vaginal birth (51).

Arrieta et al. discovered in the Canadian Healthy Infant Longitudinal Development (CHILD) project that the initial 100 days of life play a critical role in the creation of the microbiome and the susceptibility to asthma and atopy (52). The emphasis on biodiversity throughout early life has prompted efforts during pregnancy and early infancy to avoid atopy and asthma. However, none of these therapies have demonstrated

effectiveness thus far. Possible predisposing factors for the development of the asthma was summarized in **Figure 1.3** (7).

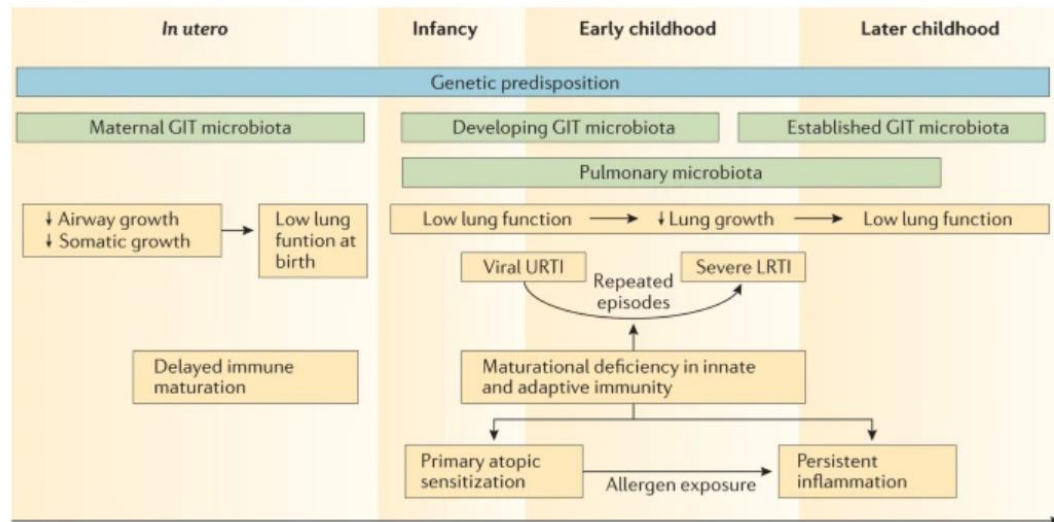


Figure 1.3. Development stages of childhood asthma. This diagram illustrates most of the variables that contribute to the likelihood of developing asthma at different stages of life. The likelihood of developing asthma at birth is determined by genetic predispositions, compromised lung function, and delayed immunological development. Postnatal factors that increase the risk of asthma include insufficient lung growth leading to decreased lung function, the timing of acquiring certain components of the lung's microbiota, recurrent viral infections in the upper respiratory tract that spread to the lower airway and cause severe infections, developmental deficiencies in the immune system that increase the risk of severe infections and promote allergic sensitization, and repeated exposure to allergens leading to ongoing inflammation in the airways. The maternal gastrointestinal tract (GIT) microbiota is believed to impact the activation of the fetal immune system, and the formation of the newborn GIT microbiota after birth is regulated by early-life exposures. Even if each pathway contributes to the overall risk, the risk of asthma increases when a child experiences multiple risk pathways at the same time (Adapted from Ref. 7).

1.4. Mechanism and Endotypes of Asthma

Asthma has significant variations regarding the clinical presentation and triggers for allergic reactions among the individuals (53). Through extensive study on the fundamental molecular processes or endotypes of asthma, the asthma population has recently been categorized into two groups: "Type-2-high" and "Type-2-low" asthma (54). "Type-2-high" asthma is distinguished by the excessive production of interleukin

(IL)4, IL5, and IL13, increased levels of eosinophils, and total IgE, which includes both allergic and eosinophilic asthma (55,56). "Type-2-low" asthma, which includes neutrophilic, paucigranulocytic, and obesity-related asthma, refers to a condition where there are no apparent elevation in Type-2 biomarkers or eosinophils (56,57) (**Figure 1.4**) (58).

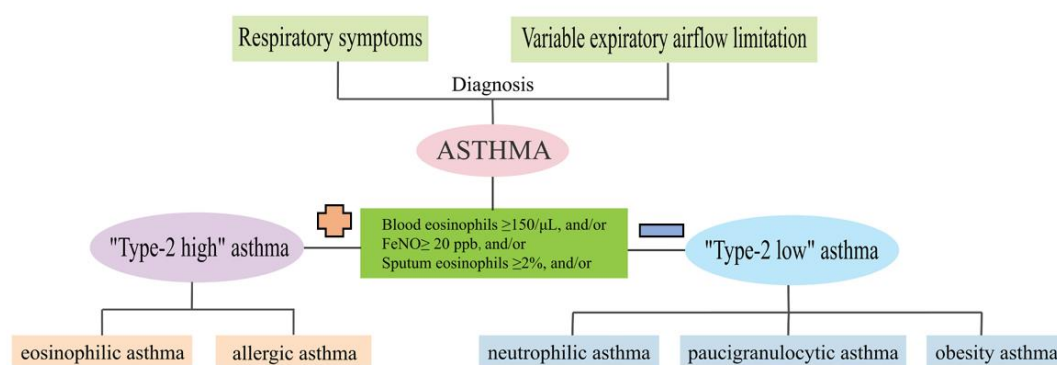


Figure 1.4. Endotypes and phenotypes of asthma (Adapted from Ref. 58).

Asthma is characterized by two distinct endotypes, each associated with certain types of immune responses and structural changes in the airways. These responses involve Th2, T follicular helper (Tfh), Th17, and Th1 cells. The fundamental mechanism of type 2 inflammation involves the activation of Th2-pathways, which results in the overproduction of the cytokines IL4, IL5, and IL13. IL5 stimulates eosinophil activation, maturation, and recruitment, whereas IL4 and IL13 contribute to goblet cell metaplasia, airway smooth muscle (ASM) contractility, and airway hyperresponsiveness (AHR) (59). Tfh cells play a crucial role in controlling the growth of B cells and the transformation of immunoglobulin classes, especially inside germinal center structures (60). The generation of IL21 and IL4 by these cells is crucial for the stimulation of B cells and has a significant impact on regulating IgE production (61,62). On the other hand, "Type-2-low" asthma is linked to atypical Th17 or Th1 cell immunological reactions. Th17 cells that produce the IL17 family of cytokines have a role in promoting inflammation and airway remodeling in the context of neutrophilic inflammation. Additionally, these cells are responsible for the development of resistance to corticosteroid treatment in individuals with asthma (63). The activation of

Th1 cells promotes the production of interferon (IFN) γ and tumor necrosis factor (TNF) α , which in turn adds to the inflammation of neutrophils (64). Additional Th cell subsets, namely Th9 and Th22, have been identified by some research groups as being responsible for the production of IL9 and IL22, respectively. These cytokines are known to be related with airway inflammation and increased AHR in asthma (65,66) (**Figure 1.5**) (58). The relevance of these subgroups to asthma etiology remains uncertain.

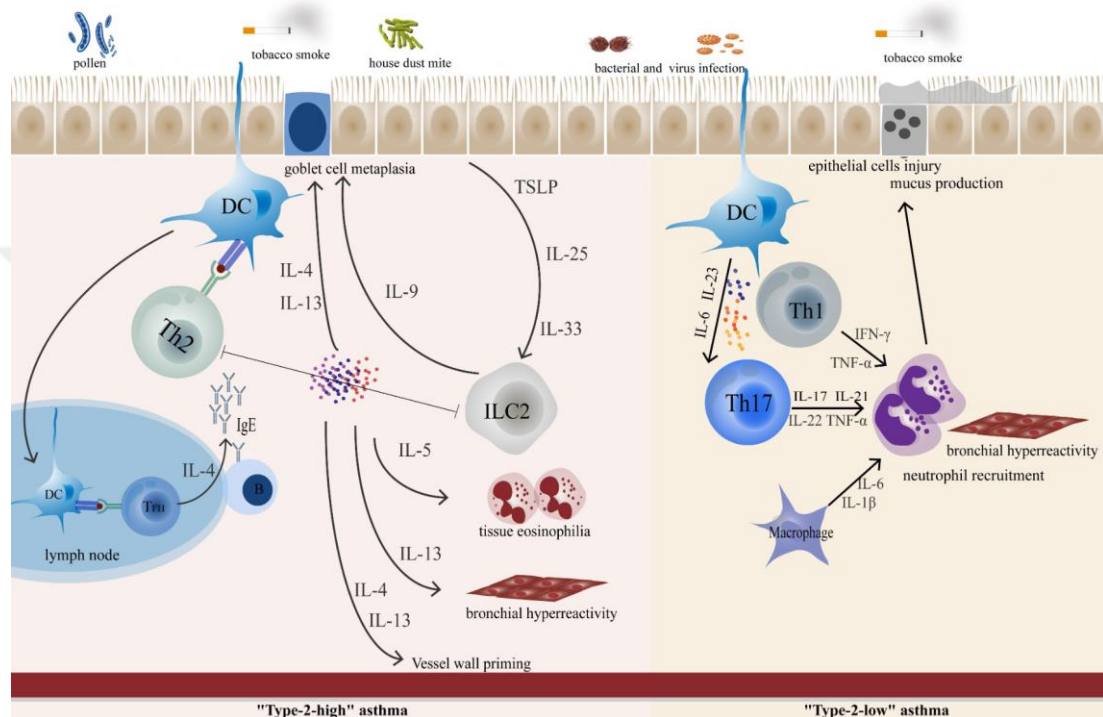


Figure 1.5. Development of Type-2 high asthma. TLPS, IL-25, and IL-33, which are produced by epithelial cells, can activate innate lymphoid cells-2 (ILC2) in individuals with "Type-2 high" asthma. Epithelial cells secrete cytokines that enhance the activity of dendritic cells, induce the differentiation of CD4⁺T cells, and facilitate the polarization of Th2 cells. T follicular helper (Tfh) cells regulate the production of immunoglobulin E (IgE) by releasing interleukin 4 (IL4) to B cells that are specific to allergens. IL9 generated from ILCs, which stimulates the transformation of goblet cells. IL5 stimulates eosinophil inflammation, whereas IL13 and IL4 contribute to goblet cell metaplasia and bronchial hyperresponsiveness. Th17 and Th1 are crucial in the development of airway neutrophil inflammation and airway remodeling in Type-2-low asthma. (Adapted from Ref. 58).

1.4.1. Type-2-high asthma

1.4.1.1. Allergen sensitization and mechanism of Type-2-high asthma

Allergen sensitization involves a connection between specialized antigen-presenting airway dendritic cells (DCs) and T lymphocytes. Processing of allergen into short peptides and the preferential major histocompatibility complex (MHC) class II presentation of these processed peptides to the T cell receptors of naïve T cells are required (67) (**Figure 1.6**) (7). Efficient communication of allergens also needs the collaborative connections between DCs and T cells, which occur in nearby lymphoid clusters. This leads to the development of T cells into different types of TH2 cells. The TH2-type T cells release pro-allergic cytokines, including IL3, IL4, IL5, IL9, IL13, and granulocyte-macrophage colony-stimulating factor (GM-CSF). These cytokines trigger IgE, mast cell, and eosinophilic responses that are specific to allergic asthma. Asthma-related allergens, including dust mite, cockroach, animal, and fungal allergens, possess enzymatic characteristics that allow them to permeate the epithelial barrier and directly interact with mucosal DCs (68). During this period, dormant DCs undergo a transformation to exhibit a variety of cell adhesion and co-stimulatory chemicals. Naïve T cells identify these chemicals and engage with DCs to form an immunological synapse that aids in the presentation of allergens (69). While only a small number of allergen-specific TH2 cells move to the B cell follicle to start the process of changing immunoglobulin class from IgM to IgE (70), the rest of the cells move to the airway mucosa due to chemoattractant molecules. This triggers the T2-type inflammatory response and the simultaneous release of pro-allergic cytokines (71).

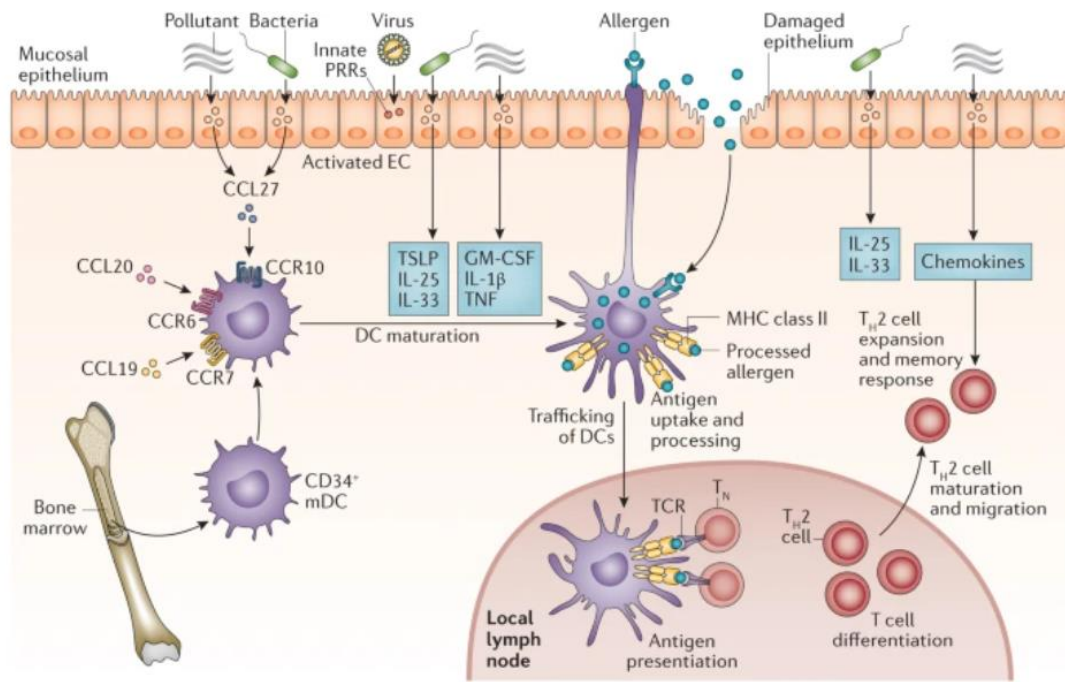


Figure 1.6. Allergen sensitization of the respiratory system in Type-2 asthma. The airway epithelium is disrupted by infectious agents as well as pollutants, resulting in the initiation of danger signals and the activation of innate signaling receptors. This signaling prompts airway epithelial cells (ECs) to release chemokines, resulting in the migration of immature dendritic cells (DCs) to the mucosal epithelium. These dendritic cells (DCs) detect danger signals by using pattern recognition receptors (PRRs), which triggers their development into fully functional antigen-presenting myeloid-type DCs. The activated DCs detect and process allergens either by extending their cellular processes into the airways or by capturing allergens that have entered the epithelium. Allergen loaded DCs subsequently induce T cell differentiation by moving to nearby lymph nodes, where they engage with naïve T cells through the T cell receptor (TCR), major histocompatibility complex (MHC) class II, and co-stimulatory molecules. The activation of DCs and the maturation and migration of T helper 2 (TH2) cells into the mucosa are affected by several cytokines and chemokines produced by epithelial cells, such as IL25, IL33, CC-chemokine ligand 17 (CCL17), and CCL22. CCR; CC-chemokine receptor, GM-CSF; granulocyte–macrophage colony-stimulating factor, mDC; mucosal DC, TNF; tumour necrosis factor, TSLP; thymic stromal lymphopoietin (Adapted from Ref. 7).

After being sensitized, subsequent exposure of the airways to an allergen leads to an early-type bronchoconstrictor response (EAR) that is driven by mast cells. This response lasts up to 90 minutes and involves the release of histamine, prostaglandin D2,

and leukotriene C4 (LTC4), which is then converted to LTD4 and LTE4 (72). The EAR is followed by a late-phase response (LAR) that unfolds over 3–12 hours, and is connected to infiltration and activation of leukocytes (particularly eosinophils) with increased LTC4 production, TH2 cytokine release from mast cells and T cells and an increase in airway responsiveness (73). While EAR and LAR have been examined in both people and animal models to analyze the allergic processes of asthma, it is important to note that these two responses do not coincide with the long-lasting inflammation that is often shown in the majority of asthma patients. Consequently, EAR and LAR do not provide a sufficient mechanistic framework within which to explore exacerbations other than those produced by allergen exposure (74).

While the main focus in asthma pathophysiology has been on factors that promote inflammation, it is now becoming apparent that the failure to properly resolve inflammation also play a role in asthma. If the inflammatory response is not properly suppressed, mast cells and eosinophils may survive for an extended period due to the specific cytokine environment in the airway of individuals with asthma. Furthermore, a significant and novel concept in the understanding of asthma pathogenesis is the probable involvement of lipoxins and resolvins as agents that facilitate the natural resolution of inflammation inside the body. For example, lipoxin A4 has the ability to trigger programmed cell death in eosinophils and limit the activity of ILC2s and natural killer cells. Additionally, the synthesis of lipoxin A4 is diminished in the airways of individuals with asthma.

Allergic reactions at barrier surfaces contribute to the development of type 2 inflammation in the early stages of asthma. IL25, IL33, and thymic stromal lymphopoietin (TSLP) are secreted from bronchial epithelial cells when they expose to allergen triggers (75). IL25 is highly expressed in the lungs of sensitized mice, which leads to increased contraction of ASM and enhanced recruitment of eosinophils, as well as improved antigen-presenting capacity (76,77).

The levels of IL25 receptors were elevated in both myeloid cells and plasmacytoid dendritic cells (pDCs) in the airways of individuals with moderate asthma following exposure to the allergen (78). Patients with elevated levels of IL25 have increased AHR, larger numbers of eosinophils in the airway and blood, elevated serum IgE levels, greater subepithelial thickening, and increased expression of genes associated with Th2

immune response (79). IL33 triggers signaling in immune cells via its receptor IL-1RL1, resulting in the synthesis of type 2 cytokines and chemokines (80). Blocking the production of IL33, either as a preventive measure or as a treatment, reduced the severity of asthma exacerbations in mouse models (81). In individuals with allergic or eosinophilic asthma, the presence of IL-33 in bronchoalveolar lavage fluid (BALF) is associated with the severity of asthma (82). Anti-inflammatory cytokine IL-37 has the capacity to target many cells such as airway epithelial cells (AECs), Th cells, and DCs by effectively suppressing the pro-inflammatory effect of IL-33 (83). IL-37 suppressed the production of Th2 cytokines in allergen-stimulated monocytes from mice with normal IL-1RL1 expression, but not in animals lacking IL-1RL1. Additionally, IL-37 prevents the release of Th2 cytokines elicited by IL-33 (83).

TSLP is recognized as a key controller of the type 2 immune response to the respiratory barrier. It triggers the activation of Th2 cells, ILC2, mast cells, and other immune cells by stimulating the production of costimulatory molecules on DCs (84). Proposed is the utilization of accumulated shreds of evidence to enhance this perspective. Preliminary research has indicated that the levels of TSLP mRNA in the bronchial epithelium and submucosa of individuals with asthma are considerably higher compared to those without the condition (85). An extensive epidemiological investigation demonstrates that elevated levels of TSLP in the blood are linked to present asthma, worse lung function, the long-term occurrence of asthma episodes and difficulty breathing even after a decade (86). Furthermore, a subset of individuals who have elevated levels of TSLP expression exhibit resistance to hormones, suggesting that there may be a significant connection between TSLP and ILCs in the context of asthma with glucocorticoid resistance (87). This phenomenon may be attributed to the increased expression of TSLP-receptors in ILCs, which leads to a reduction in ILC apoptosis through the activation of STAT5 and the overexpression of Bcl-xl (B-cell lymphoma-xl) (87). Human thymic stromal lymphopoietin (TSLP) stimulates the development of Th17 cells with the central memory T cell characteristics by activating dendritic cells (DCs) (88). The expression of TSLP in human BALF was strongly correlated with the infiltration of neutrophils. Studies have demonstrated that medication targeting TSLP can effectively decrease exacerbations in individuals with severe, uncontrolled asthma who had lower levels of blood eosinophils and FeNO (89).

Furthermore, the evolution of asthma is influenced by the interactions between cytokines generated by primarily T cells and AECs. Increased expression of activin-A by AECs might potentially enhance the production of Th2 and Th9 cells, leading to an upregulation of type 2 inflammation (90). Additionally, IL-4 released by Th2 cells plays a role in coordinating the polarization of AECs (91). Moreover, there are epithelial markers that rely directly on the cytokine profile of individuals with asthma and might potentially contribute to the treatment (92).

1.4.1.2. Th2 cells and Type 2 cytokines

The Th2 driven adaptive immune response and ILC2 driven innate immune response are considered to be two distinct but interconnected pathways that contribute to the development of asthma. Both of these mechanisms result in the production of type 2 cytokines (93). ILC2s are largely stimulated by epithelial cell-derived "alarmins" which leads to their proliferation and activation (94,95). ILC2s exhibit a distinctive characteristic of producing a significant amount of IL9, which can stimulate the transformation of goblet cells and enhance the development and survival of mast cells (96). Th2 cells, which secrete IL4, IL5, and IL13 in response to antigens, are believed to have a significant impact on the development of asthma (97). Type 2 cytokines has distinct and overlapping actions on the airway, playing a crucial role in the pathological progression of allergic asthma. IL5 induces the formation of lung tissue and the presence of eosinophils throughout the body, whereas IL4 and IL13 contribute to the transformation of goblet cells and the development of airway hyperresponsiveness (98,99). These inflammatory and restructuring changes increase susceptibility to further deterioration, thereby promoting damage to the respiratory tract and subsequent restructuring (100). In addition, the interaction between Th2 cytokines and AECs may have a positive effect on the maintenance of a type 2 inflammatory milieu.

IL5, along with IL13 and granulocyte-macrophage colony-stimulating factor (GM-CSF), regulates the maturation, differentiation, and release of eosinophils (101). The levels of sputum eosinophils and IL5 were notably increased in asthmatic individuals who experienced a rapid loss in lung function (102). IL5 enhances the ability of inflammatory-like and lung-resident-like eosinophils to multiply, and this impact may be linked to the presence of IL5R in individuals with asthma (103). Eosinophils exhibit

a high level of IL5R α expression on their surface. The migration of eosinophils from the bone marrow and blood to the airway leads to airway inflammation and damage (104). These activated eosinophils are linked to the presence of endogenous AHR (airway hyperresponsiveness) and airway inflammation. This leads to the production of reactive oxygen species and granulins. Additionally, activated eosinophils interact with mast cells located within the epithelial layer of the airways to control the process of airway inflammation (105). Typically, eosinophils can be alive outside the body up to 48 hours. However, the presence of IL5 can extend the lifespan of eosinophils (106).

IL4 and IL13 both utilize a type II receptor, which can result in a range of pathophysiological alterations associated with airway blockage and increased airway hyperresponsiveness (AHR). IL13 and IL4 stimulate an increase in the expression of vascular cell adhesion molecule-1 (VCAM-1) on human endothelial cells and have a role in the formation of collagen in fibroblasts (107). Furthermore, they augmented the histamine-triggered movement of calcium ions, which was followed by an elevated production of messenger RNA for histamine H1 and cysteinyl leukotriene CysLT1 receptors. This led to the development of glucocorticoid-insensitive airway hyperresponsiveness in isolated human small airways (108). Additional data has substantiated that IL13 primarily facilitates the physiological processes of airway remodeling, such as the transformation of mucous cells, contraction of smooth muscle cells, the development of goblet cells, and their proliferation, as well as tissue fibrosis (109,110).

1.4.1.3. T follicular helper cells (Tfh)

Mounting empirical data indicates that Tfh cells, as opposed to Th2 cells, are responsible for promoting IgE responses in asthma. León and her colleagues discovered that Tfh cells are present in the draining mediastinal lymph nodes of the mice lungs after the initial sensitizing exposure to HDM. These cells then transform into Th2 cells and move to the lungs, where they have an impact following a subsequent exposure to HDM allergens (111). Additionally, research has demonstrated that pulmonary cDCs successfully stimulated the development of Tfh cells in asthmatic lungs. These induced Tfh cells then released IL4, which facilitated the formation of IgE antibodies (112,113). It is important to highlight that Tfh cells play a crucial role in the manufacture of IgE

antibodies. They are not only required for this process, but they are also capable of fulfilling this role even when conventional Th2 cells are absent or when their ability to carry out their job is compromised (112). Additional data indicates that a small and uncommon group of IL13 producing Tfh cells is crucial for the generation of high-quality IgE antibodies and the consequent allergic reactions triggered by allergens (62). The frequency of circulating CXCR5+CD4+T cells (also known as Tfh cells) was higher in individuals with allergic asthma. These cells showed enhanced production of IL4 and IL21, and their levels were positively associated with the total amount of IgE in the bloodstream (114). Upon stimulation by IL4 and/or IL13 secreted by Tfh cells, memory IgG⁺ B cells initiate the production of IgE (115). The connection between IgE and FcεRI on mast cells and basophils serves as an enhancer of the allergic cascade response, leading to heightened vascular permeability and enhanced cellular recruitment. This interaction also triggers the release of mediators, lipid mediators, and cytokines (116).

1.4.1.4. Th9

Th9 cells are a distinct subset of Th cells that primarily secrete IL9 as their characteristic cytokine. Th9 cell counts were elevated in the lung tissue, draining lymph nodes, and airways of mice models with asthma. However, the differentiation of Th9 cells and the allergic airway inflammation were suppressed by the administration of an anti-IL9 antibody (117). Transcriptomic study identified heightened IL9 expression in the peripheral blood cells of individuals diagnosed with allergic eosinophilic asthma (118). IL9 mostly originates from Th9 cells, with some contribution from ILC2 and Th2 cells, and plays a crucial role in promoting allergic airway inflammation. IL9 facilitates the development of asthma by stimulating and attracting mast cells and eosinophils, amplifying B-cell IgE synthesis, augmenting the generation of mucus by epithelial cells, and inducing airway hyperresponsiveness (90,119). Exposure to natural allergens increased the presence of Th2, Th9 cells locally and systemically, while reducing the number of Tregs cells. However, allergen-specific immunotherapy (AIT) suppressed the frequencies of Th2 and Th9 cells, as well as the production of transforming growth factor (TGF)-β and IL9 (120). Activin A, a member of the TGF-superfamily and closely associated with TGF-β1, plays a critical role as a pro-inflammatory mediator in allergic

responses. Additionally, it also controls the growth and functioning of Th9 cells (121). Musiol et al. discovered by focusing on the TGF- β pathway that it is possible to not only hinder the processes of airway remodeling but also specifically target pro-inflammatory Th2 and Th9 cells in the asthmatic model, without causing any negative effects on immunological tolerance (120). A further investigation discovered that activin-A had the capacity to imitate the capability of TGF- β 1 to induce Th9 polarization both in laboratory settings and inside living organisms. Importantly, the successful suppression of Th9 activity in living organisms relies completely on the suppression of TGF- β and activin-A (90).

1.4.2. Type-2 low asthma

The inflammation is primarily characterized by an augmentation in Th1 cells and the influx of distinctive cytokines such as IFN γ and TNF α . Early research has shown that Th1 has an inhibitory effect on asthmatic airway inflammation due to its ability to block the Th2 inflammatory response. Multiple investigations have demonstrated that IFN- γ suppresses the activity of Th2 cells in order to reduce the intensity of the inflammatory reaction in asthma (122). In addition, Mitchell et al. discovered that IFN- γ , when acting on the airway epithelium, suppresses the production of mucus, chitinases, and eosinophilia, regardless of Th2 cell activity (123).

Increasing evidence indicates a correlation between Th1 cells and the development of "Type-2-low" corticosteroid-resistant asthma. The presence of AHR, neutrophilia, and airway remodeling remained constant in mice with heightened Th1 cells during allergic airway inflammation, and these animals demonstrated resistance to corticosteroids (124,125). Wisniewski and colleagues found that IFN γ ⁺CD4⁺T cells were highly present in the BALF of adult patients with severe asthma. They also observed Th1 airway inflammation in children with both allergic and non-allergic severe asthma (126). Both IFN γ and TNF α have a notable impact on ASM, which is responsible for the lack of response to corticosteroids in individuals with severe asthma. Rodney et al. employed human fetal ASM as a model in a laboratory setting and discovered that the interplay between TNF α and IFN γ signaling pathways might potentially contribute to the development of resistance to corticosteroids in ASM (127). Using a mouse model, the suppression of TNF α led to a reduction in substances associated with the remodeling

of the airways (128). Recent investigations have discovered a correlation between obesity-related asthma patients and the activation of Th1 cells, inflammation caused by neutrophils, and impairments in pulmonary function (129).

1.4.2.1. Th17 cells in Type-2 low asthma

Recent studies indicate that Th17 cells and their cytokines are associated with some cases of "Type-2-low" asthma, namely in severe asthma when neutrophils are the predominant cell type.

The process of Th17 cell development relies on the collaborative action of many prominent cytokines, namely IL23, IL6, and TGF β . The combination of IL6 and TGF β triggers the development of naïve T cells into Th17 cells, which plays a crucial role in autoimmune disorders (130). Th17 cells that have been activated secrete a higher amount of IL6, which in turn enhances the process of Th17 cell differentiation (130). IL23, belonging to the IL12 family of cytokines, is largely secreted by DCs, macrophages, B cells, and endothelial cells. It plays a crucial role in the survival and functional ability of Th17 cells. When IL23 is not present, Th17 cells that are activated by TGF β and IL6 have less proinflammatory effects, even if they produce more IL17 (131). IL23 amplifies eosinophil-mediated inflammation in asthma characterized by high levels of Type-2 immune response. Exposure to inhaled allergens in mice with asthma increased the production of IL23 in the cells lining the airways and the infiltration of eosinophils. However, when IL23 was blocked, it led to a decrease in airway inflammation and hyperresponsiveness (132). Furthermore, IL23 plays a crucial role in the recruitment of neutrophils. Wakashin et al. found that inducing the expression of IL23 in the airways of sensitized mice resulted in an elevated production of IL17A in response to antigens and an enhanced recruitment of neutrophils (133). Compared to individuals with good health, the activation of IL23 in neutrophils isolated from asthmatic patients significantly increased the production of IL17A and IL17F (134).

Th17 cells release IL17A, IL17F, IL21, IL22, and TNF α cytokines. IL17A and IL17F are thought to have a role in the development of neutrophilic inflammation in asthma. Studies using mice as experimental models of asthma indicate that IL17A promotes the recruitment of neutrophils in the bronchial area by activating CXCR2 signaling (135).

The asthmatic mice had elevated serum levels of IL17A compared to the wild type mice, suggesting that IL17A may facilitate the movement of neutrophils across the blood vessels (136). Sorbello et al. observed elevated levels of IL17F and IL17A in the nasal and bronchial lamina propria, as well as heightened expression of bronchial neutrophils in individuals with severe asthma (137). A research using immunohistochemistry on bronchial and nasal biopsies revealed a positive correlation between the levels of IL17F in the bronchial and nasal tissues, the number of neutrophils in the bronchial region, the frequency of asthma exacerbations, and the forced expiratory volume in one second (FEV1) (138). Multiple investigations have established a connection between IL17A and the structural changes in the airways and increased AHR. The study discovered that IL17A may stimulate the movement of mucus cells, regardless of STAT6 (139). A study measured the extent of blood vessel growth in the bronchial tubes, the number of IL17A cells, and the levels of angiogenic factors in the mucus of individuals with severe asthma, chronic obstructive pulmonary disease (COPD), and healthy individuals. The study discovered that IL17A promotes the remodeling of blood vessels in the bronchial tubes by stimulating the production of other factors that promote blood vessel growth. This process can cause congestion in the mucous membranes, leading to narrowing of the airways (140). Additional research has discovered that IL17F, but not IL17A, is responsible for airway inflammation in steroid-insensitive asthma models caused by toluene diisocyanate. Furthermore, it has been observed that the use of anti-IL17F improves toluene diisocyanate-induced airway hyperresponsiveness and airway neutrophilia, while also reducing the Th17 response (141).

IL21 stimulates Th17 cells by autocrine signaling, while TNF α is linked to airway inflammation in severe asthma. Patients diagnosed with neutrophilic asthma were shown to possess mucosa-associated invariant T cells that had a predisposition towards the Th17 subtype. These cells generated elevated amounts of IL17A and TNF α , while displaying reduced levels of IFN γ (142). Niessen et al. discovered that the levels of TNF signaling protein in sputum were considerably higher in individuals with neutrophilic asthma. Furthermore, they observed that elevated levels of sputum TNF signaling protein in patients with severe asthma were linked to decreased lung function and poorer management of asthma symptoms (143). Furthermore, the combination of TNF α and IL17 modified the proteome of bronchial epithelial cells to increase the presence of proteins that facilitate the movement of neutrophils (144).

Th17 related cytokines are also pertinent to asthma linked with obesity. The majority of late-onset asthma patients who exhibit characteristic symptoms of obesity-related asthma are predominantly female and have airway inflammation characterized by neutrophils and oxidative stress (145). In mice suffering from asthma linked with obesity, the presence of TNF α leads to an elevation in metalloproteinase (MMP)-9 levels and in situ gelatinase activity, which in turn contributes to the process of airway remodeling (146). Multiple studies have shown that individuals, both children and adults, who have asthma linked with obesity, have elevated levels of IL17A, IL21, and TNF α in their peripheral blood compared to non-obese patients (147,148). All T helper cell types involved in asthma was illustrated in **Figure 1.7. (7)**.

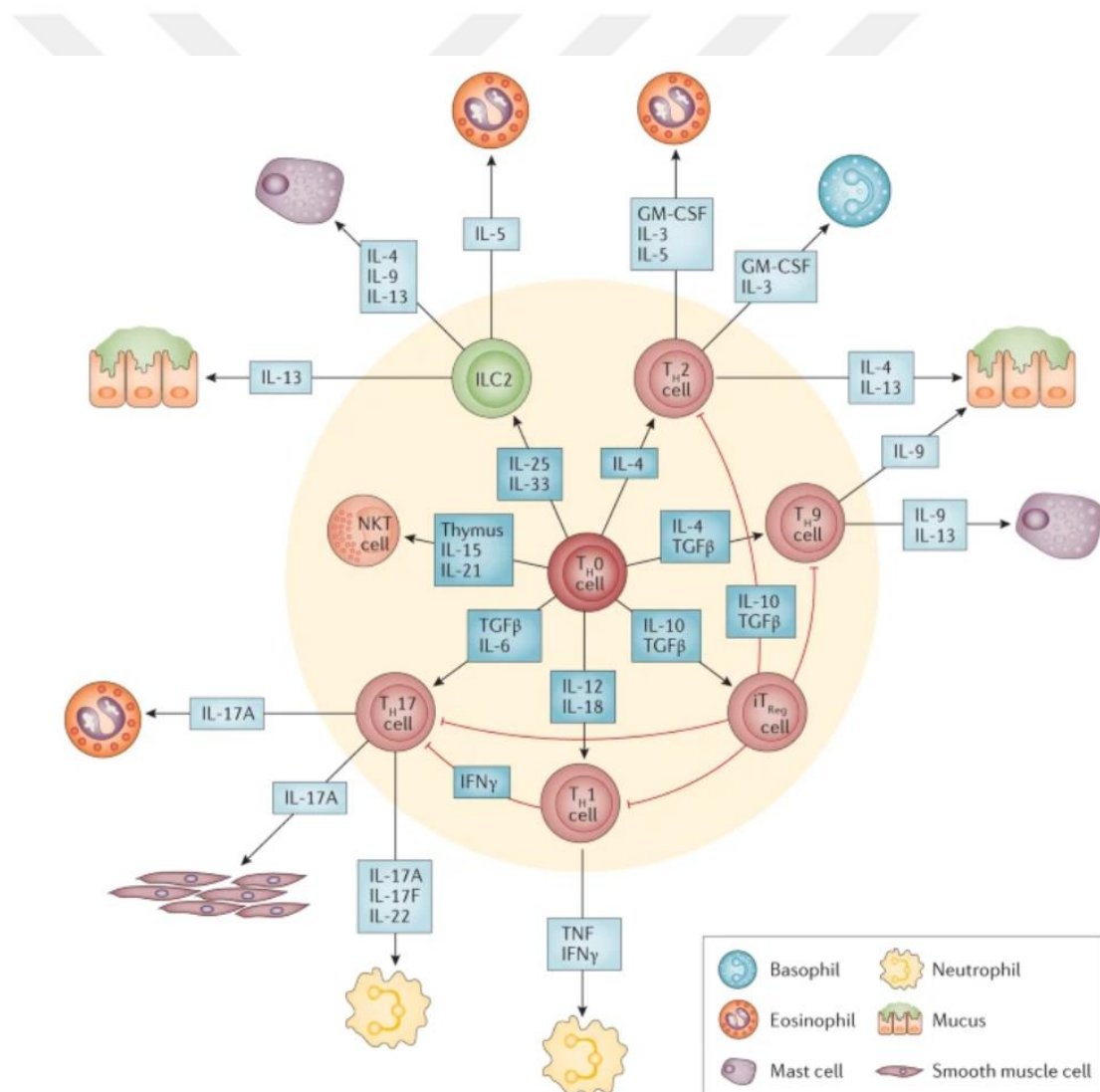


Figure 1.7. The participation of different T cell subtypes in the development and asthma endotypes. The immunological response leading to the development of asthma is initiated by

the collaboration between DCs and the thymic epithelium. The DCs and thymic epithelium induce the differentiation of several leukocyte subsets by a mix of cytokine signals and direct interaction. Prior to differentiation, these subsets either enhance or protect the airways against inflammatory responses associated with asthma. However, after differentiation, they produce cytokines that facilitate the progression of asthma. These cytokines exert an impact on different types of cells and the resulting inflammatory responses, leading to the development of allergic airway inflammation and increased sensitivity of the airways. GM-CSF stands for granulocyte–macrophage colony-stimulating factor. ILC2 refers to group 2 innate lymphoid cell. iTReg represents inhibitory regulatory T cell. NKT stands for natural killer T cell. TGF β stands for transforming growth factor- β . TH refers to T helper. TNF stands for tumor necrosis factor. (Adapted from Ref. 7).

1.5. SARS CoV-2 Disease (COVID-19)

COVID-19 is caused by the SARS-CoV-2 virus and initially defined in Wuhan, China in December 2019. Coronaviruses belong to the family *Coronaviridae* and infect both people and animals. There are four coronaviruses that mostly infect humans, namely human coronavirus NL63 (HCoV-NL63), HCoV-229E, HCoV-OC43, and HCoV-HKU1. These viruses commonly infect the upper respiratory tract and result in symptoms similar to those of a common cold. Over the last twenty years, three zoonotic coronaviruses, namely severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2, have transmitted to humans from animal reservoirs (149–151). These three viruses can reproduce infection in the lower respiratory tract and lead to a potentially lethal condition known as acute respiratory distress syndrome (ARDS).

1.5.1. Biological Classification and Structure of SARS CoV-2

SARS-CoV-2, a positive-sense single stranded RNA virus that causes COVID-19, belongs to the *Coronaviridae* family and to the genus of *Betacoronavirus* (152). SARS-CoV-2 exhibits a sequence homogeneity of 79.6% with SARS-CoV and 96% with a bat CoV from the *β -coronavirus* family, which is known for its severe pathogenicity and potential lethality (153). The virus has a collection of structural proteins, including the membrane protein, nucleocapsid protein, envelope protein, and spike glycoprotein. It also has non-structural proteins, most of which are involved in the viral replication and transcription process, as well as accessory proteins. The structural proteins, together

with a lipid bilayer obtained from the host, combine to create an enveloped virion, also known as a virus particle. This virion is responsible for delivering the viral genomic RNA into the cell. The accessory proteins are not necessary for replication but frequently possess pathogenic activities (154–156).

1.5.2. Overview of SARS CoV-2

SARS CoV-2 has four structural proteins which are spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins. The spike protein is composed of two subunits: the S1 subunit, responsible for binding to the host entry receptor angiotensin-converting enzyme 2 (ACE2), and the S2 subunit, which facilitates the fusion to the membranes (157). Structural proteins and binding of the virus on to the host cells are illustrated in **Figure 1.8** (158).

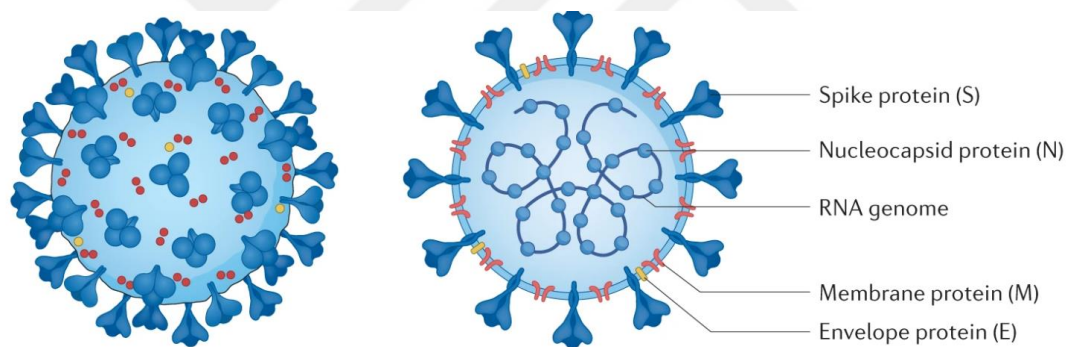


Figure 1.8. The molecular and cellular mechanisms underlying the development of SARS-CoV-2. The SARS-CoV-2 is composed of four structural proteins: spike protein (S), nucleocapsid protein (N), membrane protein (M), and envelope protein (E). (Adapted from Ref. 158).

1.5.3. Genetic Structure of SARS CoV-2

The genome of SARS-CoV-2 has a nucleotide length varying from 26.0 kb to 32.0 kb and has a variable number of open reading frames (ORFs) (159). Coronaviruses have a single-stranded RNA genome with a positive sense, which is unsegmented. This RNA genome has a 5'-cap and a 3'-poly(A) tail, enabling it to function as mRNA for the synthesis of replicase polyproteins through translation (160). The replicase gene,

known as Open Reading Frame 1a and ab (ORF1ab), is located in the viral genomic region that spans two-thirds of the initial 20 kb downstream from the 5'-end. This gene encodes the non-structural proteins (NSPs) called pp1a and pp1ab polyproteins. The pp1a non-structural protein encompasses NSP1 through NSP11, whereas the pp1ab non-structural protein consists of NSP12 through NSP16. The preceding 10 kb area before the 3'-end encodes a range of structural proteins, including surface (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. In addition, the structural genes contain nine accessory proteins, which are encoded by the ORF3a, ORF3d, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF14, and ORF10 genes (161). The genomic region located just before the 5'-end has two separate domains: a leader sequence and an untranslated region (UTR). These domains have the capacity of generating many stem loop structures that are necessary for the replication and transcription of the viral genome. The transcriptional regulatory sequences (TRSs) are located before each structural and accessory gene, and they are necessary for the proper expression of these genes. The presence of the structural component in the 3'-UTR is essential for the replication of viral RNA (**Figure 1.9**) (162).

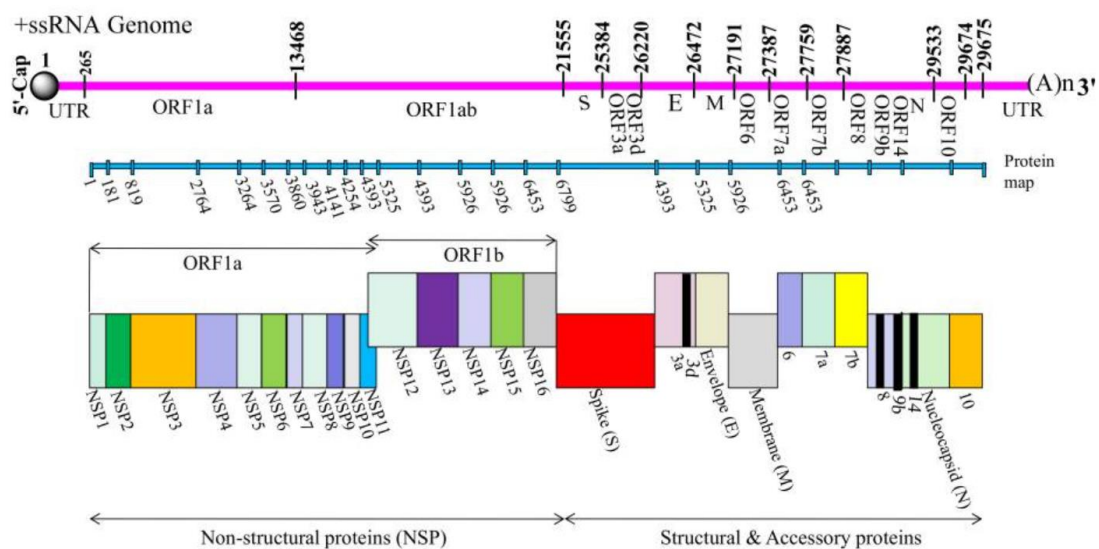


Figure 1.9. Illustration depicting the structure and genetic material of the SARS-CoV-2. Genomic organization of SARS-CoV-2. The genomic structure of SARS-CoV-2 exhibits a progressive arrangement of several non-structural, structural, and accessory genes as follows: 5'-cap leader sequence upstream of the UTR region encoding the replicase protein. S; Spike, E;

Envelope, M; Membrane, N; Nucleocapsid. The viral RNA genome's 3' end is preceded by structural genes, with accessory genes such as 3a, 3d, 6, 7a, 7b, 8, 9b, 14, and 10 interspersed inside the 3'UTR-poly (A) tail. (Adapted from Ref. 162).

1.5.4. Life Cycle of SARS CoV-2

The life cycle of SARS-CoV-2 involves the invasion of the cells by virus, expressing the genes of the virus, production of the offsprings, and eventually leaving the cells. It may be approximately categorized into the following five phases (**Figure 1.10**) (162).

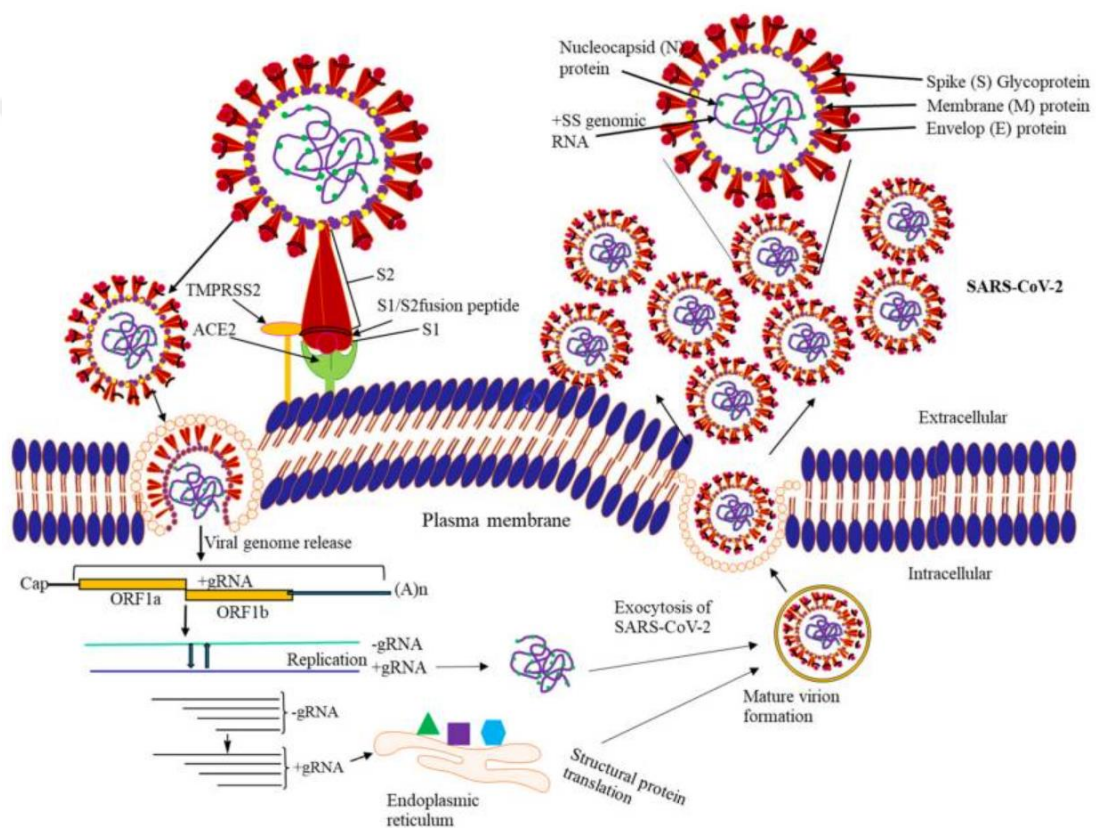


Figure 1.10. SARS-CoV-2 initiates its infection process by attaching to the ACE2 receptor on the host cell using its S protein (specifically the RBD/S1 region). This attachment triggers a structural change in the S2 subunit, allowing the virus to merge with the cell's plasma membrane. Following the release of single-stranded RNA (+ssRNA), translation promptly occurs, resulting in the production of non-structural polyproteins pp1a and pp1ab. These polyproteins undergo proteolytic cleavage and finally come together to form a functional replicase. The replicase enzyme catalyzes the production of a negative-sense intermediate, which is then duplicated to generate numerous copies of genomic RNA (gRNA) and a series of sub genomic RNA (sgRNA) by a process of discontinuous transcription. The sgRNA molecules

are translated into several types of structural and auxiliary proteins. These proteins are then assembled into a virion within the ERGIC (endoplasmic reticulum-Golgi intermediate compartment). Finally, the virion is released from the cell by a process called exocytosis. (Adapted from Ref. 162).

1.5.4.1. Adherence of SARS CoV-2 on to the surface of the host cell

The S protein system is composed of three monomeric S polypeptides, forming a homotrimeric structure. The monomeric polypeptide consists of S1 and S2 subunits, each containing several functional domains and motifs. These subunits undergo various conformational modifications. SARS-CoV-2 binds to the ACE2 receptor on the host cells through its receptor binding domain (RBD) located in the S1 subunit of the spike glycoprotein. ACE2 is mostly expressed by epithelial cells in the lungs and small intestine, as well as in the kidney, heart, and other organs (163,164). The S2 domain of the S protein contains a heptad repeat region and fusion peptide, which facilitate fusing by conformational rearrangement (165). After the S protein of SARS-CoV-2 attaches to the ACE2 protein in the host, the spike protein is cleaved by furin protease at the S1/S2 cleavage site to prepare it for activation. Another cleavage occurs at the S'2 site, which is located next to a fusion peptide inside the S2 subunit. The S1/S2 site can also be cleaved by other proteases, including transmembrane protease serine 2 (TMPRSS2), cathepsin L, and perhaps additional proteases. Membrane fusion is likely initiated by subsequent cleavage at the S'2 site, leading to irreversible conformational alterations. This process allows for easier entry into the host cell cytoplasm (166,167). Aside from its role in infection and transmission across other species, the S protein is a critical target for neutralizing antibodies against the virus. Any significant mutation in this protein can result in substantial changes in the progression of the disease.

1.5.4.2. Viral entry and removal of the viral coat

After the fusion of the viral S protein with host ACE2, there are slight modifications in the structure, which allows the viral nucleocapsid to be released into the cell cytoplasm. Several host factors, such as type II transmembrane protease serine 2 (TMPRSS2) protease and cathepsin L, assist in this process.

1.5.4.3. Formation of the Replication-Transcription Complex (RTC)

After the release of the viral nucleocapsid, the positive-sense RNA acts as messenger RNA (mRNA) for the synthesis of polyproteins pp1a (440–500 kDa) and pp1ab (740–810 kDa), which are encoded by ORF1a and ORF1b, respectively. However, the expression of pp1a is 1.2–2.2 times higher than pp1ab due to differences in the effectiveness of frameshift between the ORF1a and ORF1b genes (168). The two polyproteins undergo self-catalytic cleavage, resulting in the production of 16 nonstructural proteins (nsps). These nsps collectively constitute the replication-transcription complex (RTC) responsible for synthesizing viral RNA. The functional RTC leads to the generation of a nested collection of sgRNAs by discontinuous transcription (154). RTC facilitates the formation of double membrane vesicles (DMVs), convoluted membranes (CVs), and tiny open double membrane spherules (DMSs). These structures collectively provide a suitable environment for the replication of the SARS-CoV-2 genome.

1.5.4.4. Viral RNA synthesis

The initiation of RTC triggers a cascade of molecular events that ultimately result in the production of many copies of viral RNA. The negative single-stranded RNA (ssRNA) molecules function as intermediary templates. During -ssRNA synthesis, the polymerase swaps templates at short motifs called transcription regulated sequences (TRS). This process results in the production of numerous 5'-nested set of negative sense sgRNAs. These negative sense sgRNAs are then employed as templates to generate a 3'-nested set of positive sense sgRNAs. Subsequently, they bind to the host ribosome and produce a range of structural and auxiliary proteins, which are used to construct numerous viral structures.

1.5.4.5. Assembly and Discharge of SARS-CoV-2 at the Molecular Level

The majority of the proteins, such as S, M, and E which are involved in the structure and function of the membrane are produced by ribosomes that are attached to the

endoplasmic reticulum. On the other hand, other viral proteins, like N protein, are synthesized by ribosomes that are not attached to any specific structure and are present in the cytoplasm of the host cells. These structural proteins also undergo further posttranslational modifications that alters their activities. The assembly of virions occurs at the site of the endoplasmic reticulum-Golgi intermediate compartment (ERGIC). The M protein acts as a scaffold and coordinates the formation of virions by interacting with other structural proteins, such as M-S and M-E. This interaction allows for the incorporation of molecules into the virions. In addition, the condensation of the nucleocapsid with the envelope is facilitated by M-N interactions, coupled with the involvement of the E protein (169). After the process of molecular construction, the offspring virions are carried in vesicles with smooth walls. Through the secretory route, they are then transferred to the plasma membrane and ultimately released through exocytosis, allowing them to disseminate to different areas of the body (170,171).

1.5.5. Transmission Routes of SARS CoV-2

The respiratory tract is the principal pathway for the entry of SARS-CoV-2 into the human body. The spreading of respiratory viruses is acknowledged to occur through four distinct transmission routes: droplet, aerosol, direct contact, and indirect transmission. SARS-CoV-2 was first identified as mostly transmitted by respiratory droplets excreted by an infected individual. Subsequently, it was conclusively demonstrated that aerosol transmission is the primary mechanism of transmission for SARS-CoV-2 (172,173). Airborne transmission includes both droplet and aerosol transmission. Droplets and aerosols are often differentiated based on their size, specifically at 5 μm . This size distinction allows for the identification of unique traits such as how efficiently they disperse, how long they remain in the air, and how they are deposited in the human respiratory tract (173). Direct contact transmission is the direct transfer of a virus from an infected person to a susceptible person, such as through contaminated hands. Indirect transmission is accepted when the virus is transmitted through contaminated surfaces or objects that act as carriers (174). The confirmed mode of transmission of SARS-CoV-2 is by direct contact. Nevertheless, the issue of whether SARS-CoV-2 may be transmitted indirectly through intermediary surfaces or fomites

(feces, excrement, etc.) (175). It is also possible that SARS-CoV-2 might also be transferred through contact with unprotected eyes (176).

SARS-CoV-2 (COVID-19) commonly spreads through intimate contact between individuals closer than 2 meters. This mode of transmission occurs via respiratory droplets expelled from an infected individual coughs or sneezes. Moreover, the virus has been detected in blood and feces, hence prompting inquiries into other modes of transmission (177). However, there is currently no evidence to support the transmission of SARS CoV-2 from contaminated surfaces to individuals. Emerging data indicates that SARS-CoV-2 can remain alive on surfaces for varying durations, ranging from a few hours to several days, depending on the characteristics of the surface (178). Possible transmission routes of SARS CoV-2 is shown in **Figure 1.11** (179).

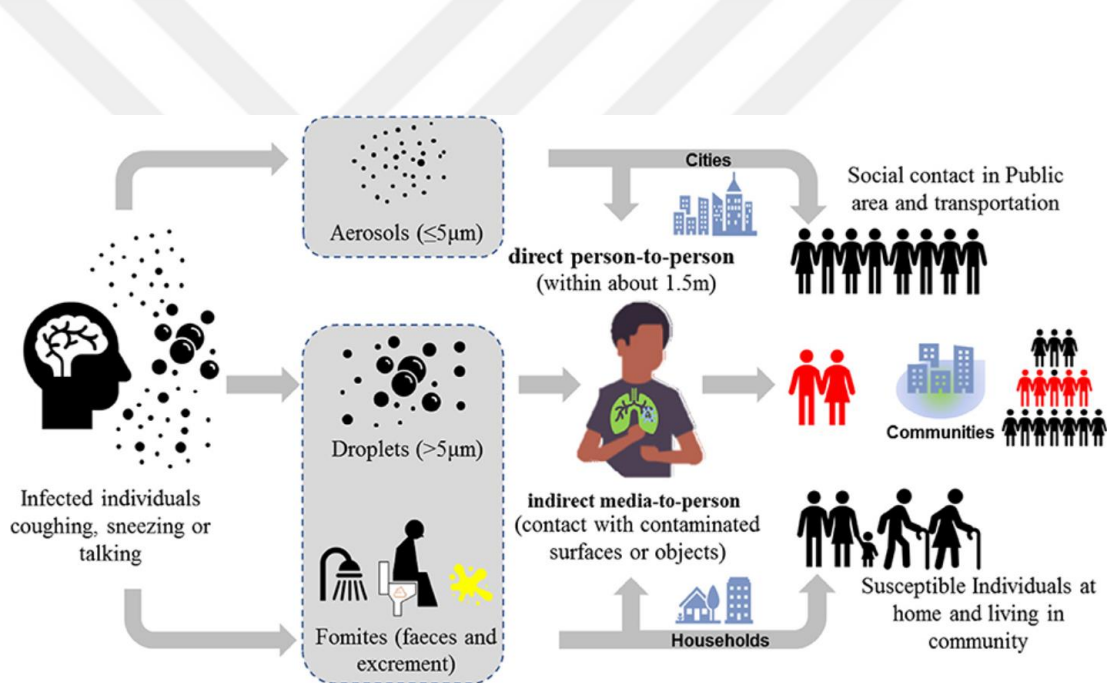


Figure 1.11. The transmission modes of COVID-19. SARS CoV-2 transmission occurs through two primary modes: (1) direct person-to-person contact and (2) indirect transmission through media-to-person contact. (Adapted from Ref. 179).

1.5.6. Clinical Presentations of COVID-19 in Children

Pediatric COVID-19 patients have exhibited less severe symptoms and lower probabilities of hospitalization and mortality in comparison to adults (180). Moreover, a significant proportion of juvenile COVID-19 cases exhibit no symptoms. The

documented prevalence of pediatric COVID-19 patients without symptoms varies from 15% to 65% (180,181).

The clinical manifestation of COVID-19 in children exhibits variation according to the age groups. The most frequent symptoms observed in children aged 9 years or less are fever (46%), cough (37%), headache (15%), diarrhea (14%), and sore throat (13%). Among those aged 10-18, there is a higher likelihood of experiencing symptoms often seen in adults with COVID-19, such as headache (42%), cough (41%), fever (35%), myalgia (30%), sore throat (29%), and shortness of breath (16%) (180). The occurrence of runny nose is often uncommon in children with COVID-19, with rates ranging from 10 to 22% (182). Anosmia is an uncommon occurrence in children (183), however it has been identified as the most influential factor in determining a positive COVID-19 test result (184).

The severe manifestations of COVID-19 in children have a clinical range that are comparable to that seen in adults. Children can exhibit several serious medical conditions including respiratory failure, myocarditis, shock, acute renal failure, coagulopathy, neurological complications such as encephalopathy, stroke, cerebral edema, Guillain-Barré syndrome, and multi-system organ failure (185,186). The hospitalization rate of pediatric COVID-19 cases in the USA has varied between 0.7% (2 out of 22 cases) and 3.8% (5 out of 20 cases) from May 2020 to February 2022. The mortality rate for pediatric COVID-19 patients was around 0.01% [2]. Woodruff et al. conducted a review of 3106 hospitalized children and found that almost 30% of them experienced severe cases of COVID-19, while 0.5% of them died during their hospitalization.

According to the classification which was reported by the Chinese Medical Association (CMA), Society of Pediatrics at the beginning of the pandemic (187), there are asymptomatic, mild, moderate, severe and critical presentations of the disease as described below in details (**Figure 1.12**) (188);

- 1) Asymptomatic infection refers to a situation when an individual does not exhibit any clinical signs or symptoms of illness, and their chest imaging is normal. However, they have positive for the SARS-CoV-2 nucleic acid (SARS CoV-2 specific PCR) test or have SARS CoV-2 specific antibodies in their serum.

- 2) Mild manifestations of an acute infection including fever, fatigue, myalgia, coughing, sore throat, runny nose, and sneeze. The physical examination reveals pharyngeal congestion and no abnormalities detected during auscultation. Certain instances may exhibit an absence of fever or just present digestive symptoms, such as nausea, vomiting, stomach discomfort, and diarrhea.
- 3) Moderate clinical presentation is characterized as pneumonia. The individual experiences episodes of fever and cough, which is predominantly dry initially and then becoming productive. Some individuals may also have wheezing, but there are no clear signs of low oxygen levels or difficulty breathing. Lung examination may reveal the presence of rhonchi.
- 4) Severe cases have respiratory symptoms, such as fever and cough, which may be followed by gastrointestinal symptoms, such as diarrhea. The condition often progresses in a week, leading to the onset of dyspnea accompanied by central cyanosis. The oxygen saturation level is $<92\%$, accompanied by additional symptoms of hypoxia.
- 5) Critical disease represents acute respiratory distress syndrome (ARDS) or respiratory failure, and may also have shock, encephalopathy, myocardial injury or heart failure, coagulation dysfunction, and acute kidney injury, including multiple organ dysfunction.

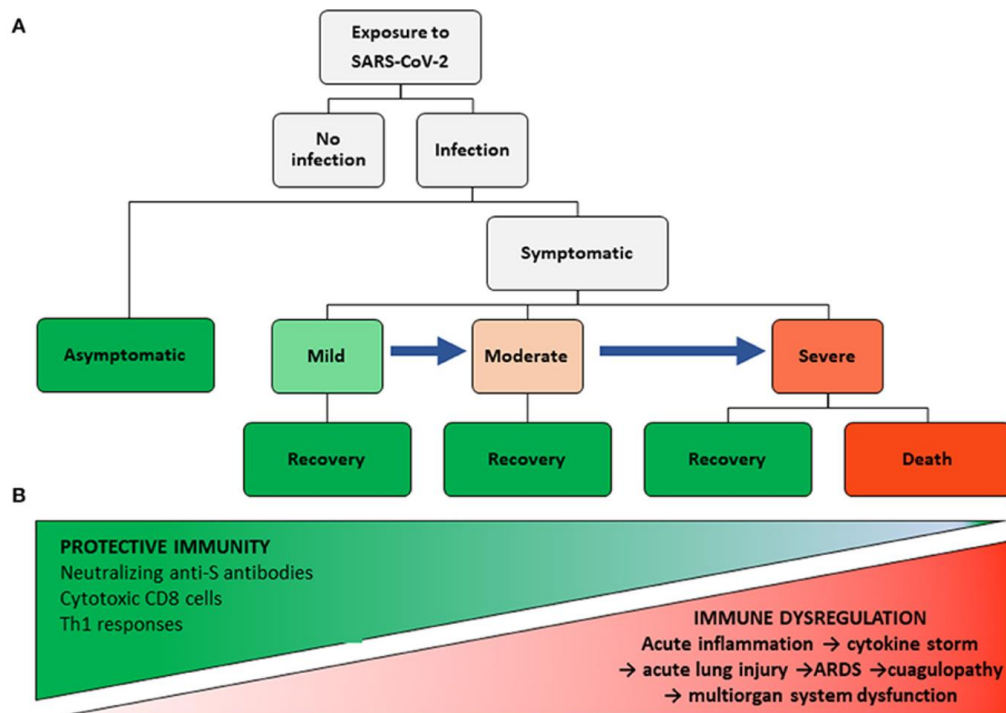


Figure 1.12. Clinical and immunological spectrum of COVID-19, (a) clinical stages of the disease, (b) Protective immunity and immune dysregulation seen in COVID-19 (Adapted from Ref. 188).

1.5.7. Post-infectious Complications

Multisystem Inflammatory Syndrome in Children (MIS-C) and long COVID-19 can also be seen as post COVID-19 complications.

a) Multisystem Inflammatory Syndrome in Children (MIS-C)

Children who get SARS-CoV-2 may experience a hyperinflammatory response known as MIS-C following the infection. In April 2020, first reports from the UK documented cases of children with clinical characteristics of both Kawasaki disease (KD) and toxic shock syndrome (TSS) (189). The highest occurrence usually happens 4 weeks following the highest point of COVID-19 patients (190). There are notable variations in epidemiology when comparing to KD. The prevalence of older children being afflicted is higher, with the highest incidence occurring between the ages of 5 and 14, compared to children under 5 years old who are more commonly impacted by KD. Additionally, there is a slightly higher occurrence of KD in males. In addition, MIS-C predominantly impacts individuals of African or Afro-Caribbean ancestry as well as Hispanic adolescents. KD is more commonly seen in individuals of East Asian ancestry as compared to other populations (191).

MIS-C encompasses a wide range of clinical manifestations. Nevertheless, to be diagnosed with MIS-C, certain criteria must be met, including having a fever for a minimum of 24 hours, laboratory findings indicating inflammation, significant illness requiring hospitalization with involvement of multiple systems, evidence of past or current infection with SARS-CoV-2, and the absence of any other reasonable explanation for the symptoms. Through a comprehensive analysis of 35 research, it was found that the cardiovascular system was the most often impacted organ system, with 82% of patients experiencing an abnormally rapid heart rate and 61% experiencing low blood pressure. Children with MIS-C may exhibit coronary artery anomalies resembling Kawasaki disease (KD) (189). The occurrence rate of

coronary artery anomalies is predicted to range from 13% to 26% (192). Pulmonary involvement was infrequent, occurring in just 9.6% of patients (193). The involvement of other organ systems includes gastrointestinal symptoms such as hepatitis, pancreatitis, abdominal pain, and diarrhea; hematological symptoms such as coagulopathy and thrombus formation; dermatological symptoms such as rash; musculoskeletal symptoms such as myositis, myalgia, arthritis, and arthralgia; neurological symptoms such as headache, confusion, and altered mental status; and renal symptoms such as acute kidney injury (194). Children under the age of 5 had increased frequencies of Kawasaki disease (KD) characteristics and dermatological manifestations, while experiencing reduced frequencies of gastrointestinal symptoms, cardiomyopathy, and neurological symptoms (195).

b) Post-acute sequelae of SARS-CoV-2 infection

Children, like adults, may develop long-lasting symptoms after being infected with COVID-19 that can damage several organ systems, including sensory, neurologic, cardiorespiratory, and mental systems. More than 200 symptoms have been associated with "long COVID," yet many of these symptoms are ambiguous, such as fatigue, sleep disruption, difficulty concentrating, lack of appetite, and muscle/joint discomfort. There is a scarcity of evidence about the condition known as "long COVID" in pediatric population. In a meta-analysis of 5 research, Zimmerman et al. discovered that all the trials exhibited significant constraints or failed to show a distinction between children infected with SARS-CoV-2 and those who were not. Nevertheless, in the majority of adolescents, symptoms did not endure beyond a period of 12 weeks (196).

1.5.8. Immune response to SARS CoV-2

1.5.8.1. Innate Immune Response

Following the infection with SARS-CoV-2, the host cell will initiate a strong innate immune response as shown in **Figure 1.13** (197). Pattern recognition receptors (PRRs) such as RIG-I, MDA-5, and TLRs can detect the presence of SARS-CoV-2 within the

infected cell (198–200). Starting with TLR2, it detects the SARS-CoV-2 envelope protein to trigger an immune response prior to viral entry and replication, resulting in the production of TNF- α and IFN- γ (199). TLR2 also triggers the formation of the NLRP3 inflammasome. TLR1 is anticipated to create a heterodimer with TLR2 and has notably elevated RNA levels in severe and serious cases of COVID-19 (199). Turning our attention to TLR3, this receptor engages with viral pathogen-associated molecular patterns (PAMPs) and double-stranded RNA (dsRNA) (201). The activation of TLR3 triggers the activation of both IRF3 and NF- κ B signaling pathways through the TRIF-dependent pathway. This activation also leads to an upregulation of NLRP3 expression, which allows for the recognition of SARS-CoV-2 and the creation of an inflammasome in conjunction with other proteins. The inflammasome mediates the processing and secretion of IL-1 β and IL-18, which induces pyroptosis (198,202,203). TLR4 responds to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) that arise from SARS-CoV-2 infection. This reaction leads to an increase in the production of IL-6 through the activation of NF- κ B and MAPK pathways (203–205). TLR1, TLR4, and TLR5 have been suggested, and computationally predicted, to recognize the SARS-CoV-2 spike glycoprotein, which triggers the MyD88 and TRIF signaling pathways of the innate immune system (206). Regarding TLR7, TLR8, and TLR9, these receptors located in the endosomes have specific and separate functions. TLR7 and TLR8 detect single-stranded RNA and trigger the secretion of IL-6, TNF α , as well as type I and III IFNs (200,207). TLR9 recognizes viral RNA and DNA with unmethylated CpG motifs, as well as mitochondrial DNA released because of the cellular damage caused by SARS-CoV-2. TLR9 induces the secretion of cytokines, such as IL-1 β , IL-6, IL-10, IL-17, TNF α , and type I IFN (207).

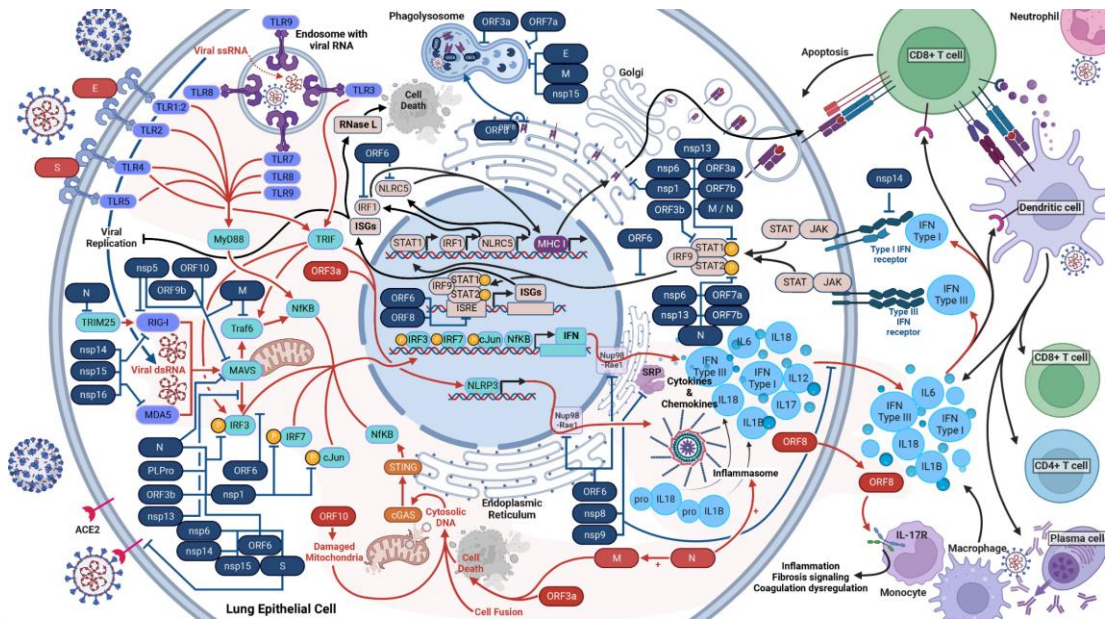


Figure 1.13. Innate immune response to SARS CoV-2 infection. SARS-CoV-2 induces and amplifies an immune response (indicated by red arrows) by causing damage to cellular structures (specifically dysfunctional mitochondria), as well as through the presence of single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), cytosolic DNA, open-reading frame 8 (ORF8), the envelope (E) protein, the spike (S) glycoprotein, and the nucleocapsid (N) protein (highlighted in red). The components of the virus trigger the immune response, leading to the generation of type I interferons (IFN), type III IFN, and inflammasomes (shown by red lines). The cytokine responses will stimulate the IFN receptors (IFNAR, IFNLR) in autocrine and paracrine ways, leading to the activation of interferon-stimulated genes (ISGs), interferon regulatory factor 1 (IRF1), and NOD-like receptor family CARD domain-containing five (NLRC5) (shown by black lines). IRF1 and NLRC5 stimulate the expression of major histocompatibility complex (MHC) class I. The cytokine responses will also attract dendritic cells, CD8⁺ cytotoxic T cells, and trigger the adaptive immune response. The immunological response to SARS-CoV-2 involves the activation of CD8⁺ T cells, CD4⁺ T cells, and B cells (specifically plasma cells) to generate antibodies (Adapted from Ref. 197).

During the development of the immune response, RIG-I and MDA5 engage with MAVS when activated by dsRNA. This interaction triggers the phosphorylation of IRF3 (208,209). Simultaneously, Toll-like receptors (TLRs) trigger the activation of MyD88, which in turn begins the activation of NF-κB (202,210). The activation of these responses triggers the synthesis of several cytokines, such as type I (IFNα and IFNβ)

and type III IFN (IFN λ), through various routes (198,200,202). The IFN antiviral pathway is a crucial innate mechanism in the defense against viral infections (211). The suppressed interferon (IFN) response has a significant role in determining the clinical severity of COVID-19 (212). Simultaneously, the host cell generates cytokines and chemokines, including IL-6, IP-10, and TNF- α , leading to the occurrence of a cytokine storm (198,199,213).

During the subsequent phase of the immune response, type I interferons (IFNs) attach to IFNAR receptors in an autocrine and paracrine manner, whereas type III interferons (IFNs) connect to IFNLR receptors. When IFNs bind to IFNAR and IFNLR, it triggers the activation of the JAK/STAT pathway, which leads to the production of MHC class I and ISG through the ISRE (200,202,210). ISGs inhibit viral replication and induce the activation of RNase L, which breaks down both viral and host single-stranded RNA, resulting in cell death and preventing viral dissemination (208).

In addition, SARS-CoV-2 infection triggers the activation of cGAS-STING signaling by several mechanisms such as cell death, cell fusion, mitochondrial stress, and DNA damage. This activation allows for the identification of the viral genome DNA within fused cells, leading to the activation of NF- κ B and subsequent increase in the production of TNF and IL-6 (214). The cGAS-STING complex also activates the IRF3 signaling pathway. Finally, in the innate response, proteasomes will break down viral proteins and display them on MHC class I proteins to enhance the death of infected cells by cytotoxic T-cells. The instability resulting from the several pathways of cytokine release eventually leads to tissue and organ damage (199). Activated inflammatory cells move to the lungs, resulting in an intensified cytokine response that contributes to the cytokine storm (198).

1.5.8.2. Adaptive Immune Response

The immune cell recruitment and initiation of the adaptive response are triggered by the pro-inflammatory cytokine response. The adaptive immune response to SARS-CoV-2 involves important components such as plasma cells, CD4⁺ T helper cells, and CD8⁺ Killer T cells (215,216) (**Figure 1.14**) (217). Antibodies, CD4⁺ T cells and CD8⁺ T cells cooperate to resist SARS-CoV-2 infection. This occurs when DCs recognize the virus and activated DCs migrate to lymph nodes (218). Neutralizing

antibodies (nAbs) are the primary way by which the immune system prevents the transition of SARS-CoV-2 to COVID-19. Therefore, they are crucial and a key target for vaccine-induced immune responses (215,219,220). nAbs attach to the virus and inhibit its ability to enter cells. Memory B cells play a crucial role in providing enduring immunity to infections by producing antibodies which last up to 6 months after receiving the SARS-CoV-2 mRNA vaccine and up to a year after being infected with SARS-CoV-2 (208,221). B cells undergo differentiation into plasma cells, which promptly generate neutralizing antibodies (IgG, IgA, and IgM) within a few days following infection or immunization (221). Differentiation can take place either in the extrafollicular area during the extrafollicular (EF) phase or in the germinal centers during the germinal center (GC) phase. After the initial EF phase, B cells undergo somatic hypermutation and selection in germinal centers during the GC phase. Subsequently, they localize in the bone marrow (221). Nevertheless, a distinctive observation in cases with acute COVID-19 is the absence of germinal centers inside lymph nodes. Germinal centers play a crucial role in the formation and specialization of memory B cells and plasma cells that produce antibodies with a high level of specificity (222)(223). The absence of germinal centers in acute COVID-19 is associated with the presence of "disease-related" extrafollicular B lymphocytes. The B cells that are associated with diseases are a product of class-switching rather than selection in germinal centers. These B cells do not provide durable immunity. Hence, the maturation of B cells highlights the significance of immunization in producing a potent neutralizing antibody (nAb) reaction and safeguarding against COVID-19 (224).

CD4⁺ T helper cells play a crucial role in antibody responses by interacting with B cells. They are involved in nearly all infections caused by SARS-CoV (215,216). CD4⁺ T cell responses are more dominant than CD8⁺ T cell responses in SARS-CoV-2 infection. These CD4⁺ T cell responses are significantly linked to reduced illness severity. The responses of CD4⁺ T cells are primarily directed against 21 different SARS-CoV-2 proteins, including S, M, N, nsp3, nsp4, nsp12, ORF3a, ORF7a, and ORF8 (216,225,226). Memory CD4⁺ T cells have long-term persistence and retain the ability to mount a response upon subsequent exposure. They become detectable in the circulation about 30 days after the beginning of symptoms and have a lifespan of around 94 days (227). COVID-19 has many cellular roles for CD4⁺ cells (216). Tfh fraction of CD4⁺ T cells play a crucial role in facilitating the production of neutralizing

antibodies (nAbs) and inducing B cells. The presence of Tfh can be detected and remains stable for a period beyond six months after the beginning of symptoms (227). However, the presence of Bcl-6⁺ GC-Tfh cells, which are necessary for the formation of germinal centers, is reduced in individuals with COVID-19. Besides, COVID-19 triggers a strong TH1 immune response (224), and TH1 response is responsible for the release of cytokines, specifically IFN γ , and the recruitment of innate cells. The host's humoral immune response undergoes continual evolution through affinity maturation with CD4⁺ T cells in germinal centers following viral resolution (228). Furthermore, there is a direct correlation between a larger frequency of CD4⁺ T cells and slower decay rates of antibodies, as well as a stronger antibody response with higher affinity (228). Tissue-resident memory (TRM) cells are a specific type of memory T cells that do not circulate throughout the body and instead remain in tissues. Their main function is to prevent re-infection and they play a crucial role in limiting the spread of pathogens (229). The duration of TRM in the respiratory tract is 10 months after infection (229). CD4⁺ T-cells that secrete IL-22 contribute to the process of healing wounds in mucosal tissues (216).

CD8⁺ T lymphocytes are crucial in several viral infections and are accountable for the eradication of contaminated cells (215). CD8⁺ T cell responses have been identified targeting the S protein, M protein, N protein, nsp6, and ORF3a of SARS-CoV-2 (225,226). Memory CD8⁺ T cells begin circulating between 20-50 days after the beginning of symptoms and have a lifespan of 225 days. The majority of these circulating CD8⁺ T cells are effector memory T cells expressing CD45RA (TEMRA), with smaller proportions of effector memory T cells (TEM) and central memory T cells (TCM). TEMRA contributes to the defense against severe illness, as demonstrated in previous viral infections (227).

Following infection with SARS-CoV-2, individuals who possess the ability to produce immunological responses through B cells, CD4⁺ cells, and CD8⁺ cells are capable of reducing the severity of the disease (215). In addition, the development of a strong reaction using these three strategies was found to have a negative correlation with age (215). The comprehensive range of all three reactions characterizes the adaptive immune response to SARS-CoV-2 infection and mitigates the severity of the illness (215). A crucial factor to consider when designing vaccines is the incorporation of M

and N components in order to accurately replicate the natural CD4⁺ T cell and CD8⁺ T cell responses to SARS-CoV-2 (226). Memory CD4⁺ T cells and memory CD8⁺ T cells remain intact and unaffected by emerging variations in vaccinated people, independent of the kind of vaccination used. The detection of spike proteins by memory B cells, as well as the reactivity of antibodies, is noticeably diminished against variations in both vaccinated individuals and those who have been naturally infected (230).

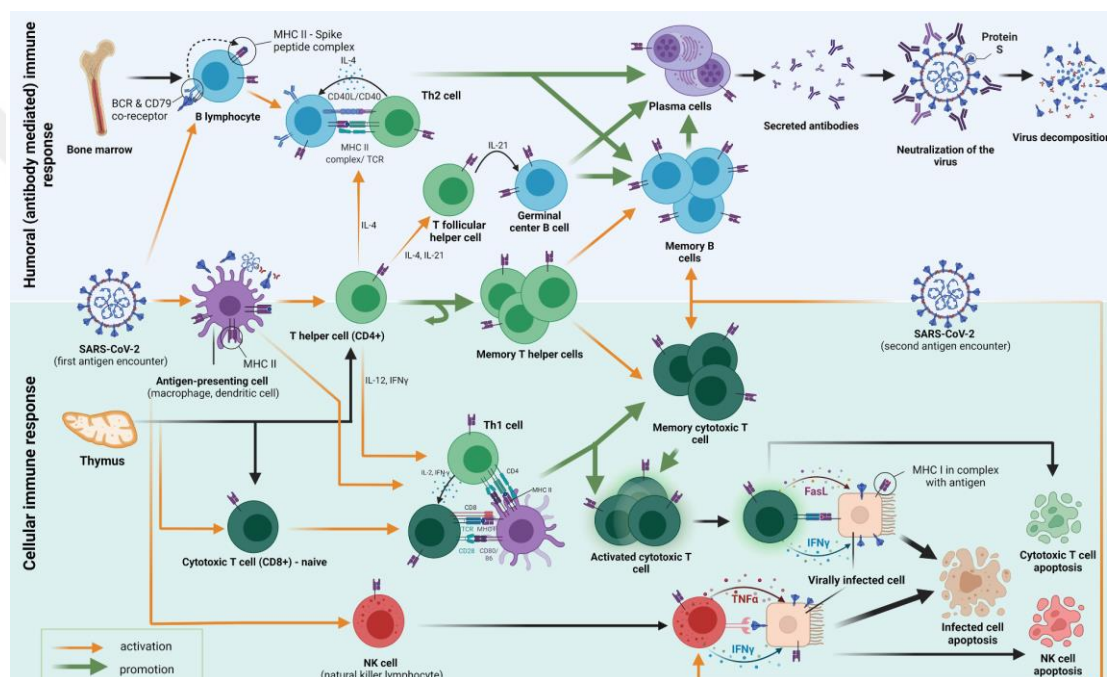


Figure 1.14. Adaptive immune response to SARS CoV-2. After the first interaction, different antigens undergo phagocytosis and subsequent breakdown within the antigen-presenting cells (APCs). APCs degrade the antigen into smaller peptides, which they display on their surface using surface receptors known as major histocompatibility complex class II (MHC-II) molecules. The antigens are then shown to several cell types inside the host, with particular emphasis on CD4⁺ T helper cells (as well as B and CD8⁺ cells). B cells undergo differentiation into plasma cells, which subsequently generate antibodies that impede the viral particle from entering healthy cells. APC-induced activation of T helper cells leads to their differentiation into several subtypes, each with specialized tasks facilitated by cytokine release and intercellular communication. Th2-differentiated T helper cells enhance the maturation of humoral responses by delivering a secondary signal to B cells, mostly through the release of IL-4 and the interaction of CD40/CD40L. CD4⁺ cells

can differentiate into T follicular helper cells (Tfh), which play a crucial role in regulating the interactions that occur in the germinal centers. These interactions are essential for the development of memory B cells and the production of long-lasting high-affinity antibodies by plasma cells. A distinct subgroup of CD4⁺ T cells undergo differentiation into a reservoir of memory T helper cells. The T helper cells are essential in the establishment of cellular immune responses. The MHC-I activation of CD8⁺ cells (CTL, cytotoxic T lymphocytes) is modulated by their simultaneous interaction with APCs. Activated cytotoxic T lymphocytes (CTLs) induce apoptosis in host cells infected with SARS-CoV-2 through the binding of Fas to FasL. Certain cytotoxic T lymphocytes (CTLs) undergo differentiation into memory cytotoxic T cells. These cells are responsible for rapidly restoring the CTL response upon subsequent encounters with the same antigen. An analogous process of devastation takes place when NK cells engage with the cell that has been infected by a virus. Within their cytoplasm, they possess granules that contain IFN γ and TNF α . The secretion of these granules induces programmed cell death. Additionally, the activation of NK cells does not rely on MHC molecules, as the presence of MHC is not consistently observed on cells that are infected by viruses (Adapted from Ref. 217).

1.6. COVID-19 and Asthma Relationship

Asthma is a chronic disease characterized by a past of respiratory symptoms such as wheezing, difficulty breathing, chest constriction, and coughing. A key characteristic of asthma is the fluctuating severity of restriction in the outflow of air from the respiratory airway. Asthma is also a complex disorder due to various phenotypes and endotypes (231).

Patients with asthma frequently experience more severe viral illnesses, as viral respiratory tract infections are the primary cause of severe asthma exacerbations. There has been a great suspicion of susceptibility of individuals with asthma to SARS-CoV-2 infection. At the beginning of the pandemic, many researchers analyzed the connection between being diagnosed with asthma and the occurrence and seriousness of SARS-CoV-2 infection. In a preliminary investigation conducted in China, it was shown that comorbidities such as hypertension and diabetes were more prevalent compared to obstructive lung disease (232). Similarly, a preliminary investigation shown that the occurrence of asthma among patients hospitalized with SARS-CoV-2 was notably lower (2%) compared to the incidence of asthma in the whole community (6%) (233). Skevaki et al, showed considerable variations in the incidence of

SARSCoV-2 infection between individuals with asthma and the general population across different nations (234).

It is crucial to acknowledge that during the initial stages of the pandemic, there was a restricted amount of testing conducted on people who did not need to attend the emergency room or be hospitalized. It is conceivable that variations in the severity of different strains of SARS-CoV-2 prevailing throughout different stages of the pandemic would have had diverse effects on individuals with varied underlying health conditions.

The association between asthma and COVID-19 is a subject of debate. Two comprehensive meta-analyses, each comprising over 100 publications, have determined that there is no significant correlation between asthma and increased severity, hospitalization, death, or worse prognosis in COVID-19 patients as compared to individuals without asthma (214,235). Furthermore, a study has found that asthma patients who take regular inhaled corticosteroids (ICSs) had less frequent and severe SARS-CoV-2 infection, decreased severity of COVID-19, and lower levels of ACE2 in their sputum compared to asthma patients who do not use ICSs (236).

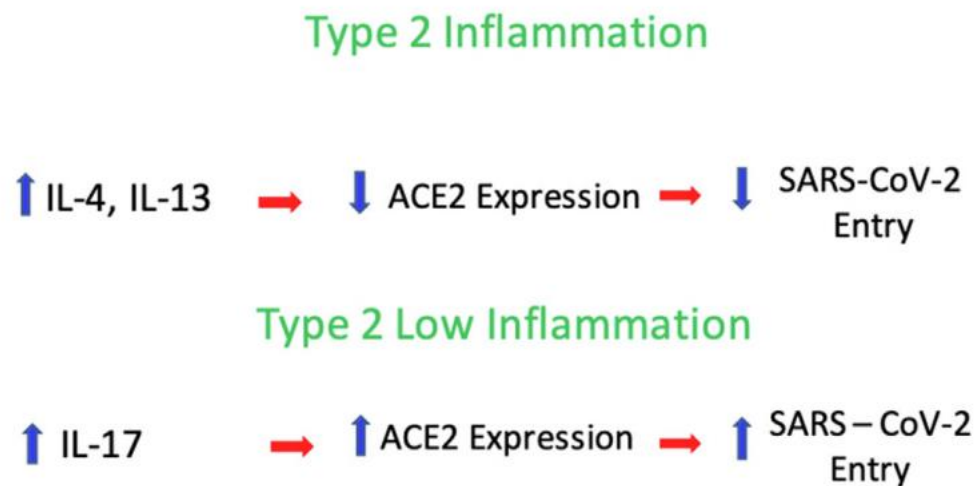
1.6.1. Effect of SARS CoV-2 in Asthma

Respiratory viruses are the primary cause of asthma aggravation. Seasonal coronaviruses have a little impact on asthma exacerbations when compared to other respiratory viruses, and this remained consistent during the SARS-CoV-2 pandemic (237,238). Several hypotheses explain the reduced correlation between asthma and SARS-CoV-2 infection.

SARS-CoV-2 enters host cells by using the angiotensin-converting enzyme 2 (ACE2) receptor and transmembrane protease serine 2 (TMPRSS2). TMPRSS2 enzymatically cleaves the SARS-CoV2 S protein, facilitating its entrance into host cells via ACE2. Both upper and lower airways exhibit co-expression of ACE2 and TMPRSS2, with their expression levels being influenced by various host variables (239). A study was conducted to investigate the expression of ACE2 and TMPRSS2 mRNA in airway epithelial cells following 48 hours of exposure to IL-13 (240). All individuals included in the research had mild to moderate asthma and signs of atopy. Every participant received a bronchoscopy procedure, during which bronchial brushings were collected.

The collected epithelial cells were then subjected to additional analysis after being exposed to IL-13. The study revealed a reduction in ACE2 expression and an increase in TMPRSS2 expression in the airway epithelial cells of both asthmatic and non-asthmatic individuals with atopy. Additional examination of two extensive genetic databases containing nasal and bronchial epithelial cells from both children and adults in this study provided more evidence supporting the observed reduction in ACE2 and elevation in TMPRSS2 mRNA expression patterns with asthma and allergic rhinitis (240). Furthermore, it was also shown that the expression of ACE2 exhibited an inverse correlation with the allergen-specific IgE results, fractional exhaled nitric oxide, total IgE levels, and nasal epithelial production of IL-13 (241). The decreased expression of ACE2 might be the possible reason for the reduced incidence of severe SARS-CoV-2 infection seen in asthmatic patients with type 2 inflammation, which encompasses atopy or the presence of type 2 cytokines like IL-13. The impact of asthma types and treatment of asthma on COVID-19 infection was given in **Figure 1.15** (242).

(a)



(b)

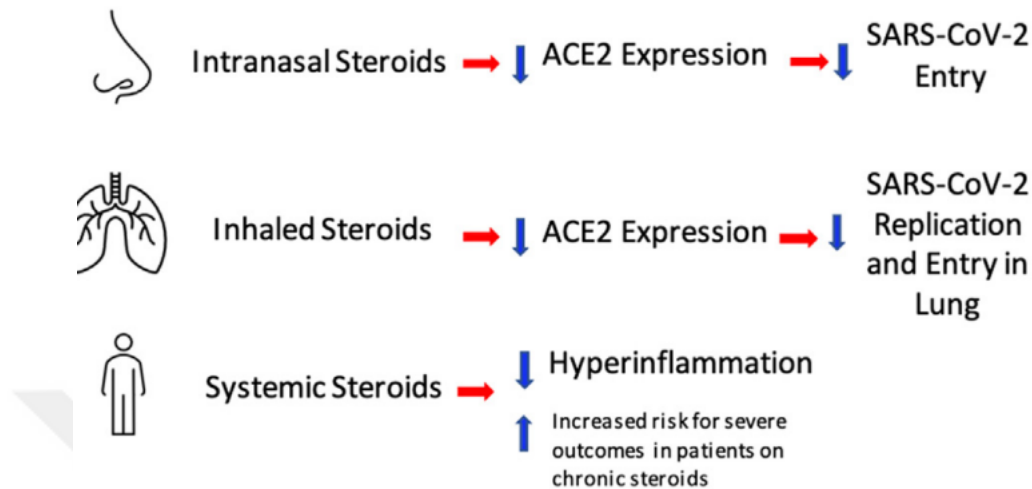


Figure 1.15. Relationship of asthma and asthma treatment on the severity of the COVID-19, (a) Type 2 high and low inflammation types in asthma and their relationship with SARS CoV-2, (b) Effects of asthma treatment on SARS CoV-2 entry and viral load (Adapted from Ref. 242).

1.7. Extracellular Vesicles

1.7.1. Biogenesis and function of extracellular vesicles (EVs)

Previously, EVs have been considered as cellular "junk bags" responsible in the elimination of undesirable substances. Currently, EVs have understood that they have significant functions in several biological systems as transporters of biomolecules, including proteins, lipids, and nucleic acids, and play a vital role in facilitating cell-to-cell communication. Moreover, EVs have been employed as vehicles for medical drugs and used for the diagnosis of many diseases (243). Extracellular vesicles (EVs) are double layered membrane nanoparticles that are released by many cells. They may be detected in multiple biofluids, such as blood, urine, cerebrospinal fluid, saliva, breast milk, and bronchoalveolar lavage fluid (244). EVs provide a challenge in terms of categorization due to their sizes. The Minimal Information for Studies of Extracellular Vesicles (MISEV) has adopted a practical strategy by categorizing extracellular vesicles (EVs) into two groups: 'big EVs' (more than 200 nm) and 'small EVs' (less than

200 nm). However, it would be more appropriate to group them in more detail for academic studies and to better classify them due to biogenesis differences. Therefore, EVs may be categorized into three primary subtypes based on their biogenesis pathways: exosomes (small EVs, 30–100 nm), microvesicles (ectosomes, small to large EVs, 100–1,000 nm), and apoptotic bodies (larger EVs more than 1,000 nm) (245).

1.7.1.1. Exosomes

Exosomes are produced from multivesicular bodies (MVBs) by the inward protrusion of the late endosomal membrane (246). This process results in the formation of intraluminal vesicles (ILVs) that contain biomolecules within the MVBs. Subsequently, multivesicular bodies (MVBs) merge with the plasma membrane and discharge intraluminal vesicles (ILVs) into the extracellular environment as exosomes by exocytosis (247). The "endosomal sorting complexes required for transport" are a group of peripheral membrane protein complexes that play a crucial role in the creation of intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) and the release of microvesicles from the plasma membrane. The ESCRT system has four complexes, namely ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, each with its own set of related proteins (Tsg101, ALIX, and VPS4). Each complex plays a distinct role during biogenesis. To start the production of the EVs, ESCRT-0 creates a protein network on the membranes of endosomes. This network captures cargo proteins that have been marked with ubiquitin and enables their organization according to the MVBs pathway (248). ESCRT-I attaches to ubiquitinated cargo proteins located on the endosomes and triggers the activation of ESCRT-II, which directly participates in the sorting of cargo at multivesicular endosomes (MVEs). ESCRT III, along with the charged multivesicular body proteins, is responsible for modifying the membrane by performing tasks such as fission, dissociation of proteins, and recycling of the endosomal sorting machinery. These processes rely on the involvement of the Vps4 AAA-ATPase (249). In addition to these activities, ESCRT-III and its associated proteins, including as Vps4, Vta1, Vsp60, and ALIX, also participate in viral budding, cytokinesis, and nuclear envelope remodeling (250). An alternative mechanism for the formation of exosomes includes the syndecan/syntenin complex, which relies on the function of phospholipase D 2 (PLD2) and ADP-ribosylation factor 6 (Arf6) (251). In addition to the well-

established ESCRT-dependent pathway, the SMase2/ceramide pathway, which operates independently from the canonical ESCRT pathway, has also been identified (252). RAB GTPases are involved in the transport and release of EVs (253). RAB GTPases control the movement of substances in both the endocytic and secretory pathways. They accomplish this by attracting certain effector proteins to the surfaces of membranes, which helps with the collection of cargo, movement of organelles, or docking of vesicles. Increasing evidence suggests a connection between RAB GTPases and the release of exosomes, particularly RAB5 and RAB7 (254). Additional research has shown that RAB27A and RAB27B are involved in the transportation of MVEs, whereas the proteins RAB11 and RAB35 control the fusion of MVEs with the plasma membrane and the direct or indirect secretion process (255). In addition, it has been discovered that RAB22A coexists with emerging microvesicles, contributing to the organization of cargo in hypoxic breast cancer cells (256).

1.7.1.2. Microvesicles (MVs)

Microvesicles (MVs) are produced by direct plasma membrane budding and fission. These vesicles are also a kind of ectosomes larger than 150 nm (257). Multiple proteins, including Ca^{2+} dependent amino phospholipid translocases, scramblases, and calpains, play a role in the formation of MVs. These proteins facilitate the rearrangement of membrane phospholipids and the rebuilding of the actin cytoskeleton. Ultimately, they determine the curvature of the plasma membrane and the release of MVs into the extracellular space (258). Although exosomes and micro vesicles have separate biogenesis processes, they may have comparable sorting machinery and intracellular mechanisms. This often leads to difficulties in distinguishing between different subpopulations due to their overlapping size and similar composition. There is a necessity to elucidate the precise mechanisms behind the formation of extracellular vesicles (EVs) (255). According to International Society of Extracellular Vesicles (ISEV), it is recommended to use the name "EVs" as a general term because there is currently no specific identifier that can distinguish each subpopulation of EVs (259,260).

1.7.1.3. Apoptotic bodies/vesicles

Apoptotic bodies, also known as apoptosomes, are specifically generated during the process of apoptosis. This process is distinguished by the compaction of nuclear chromatin and involves the sequential occurrence of nuclear chromatin condensation and membrane blebbing (261). In addition to ESCRT proteins, EVs may contain an abundance of tetraspanins (CD81, CD82, CD63, CD9), as well as proteins responsible for cargo transport and membrane fusion (annexins, RABs). Furthermore, EVs may also be enriched with proteins involved in various processes such as cell adhesion (integrins), antigen presentation (MHC-I, MHC-II), stress regulation (HSP70, HSP90), cell migration (actin, myosin, tubulin), and metabolic enzymes (262). EVs can also include several nucleic acids, including messenger RNAs (mRNAs), micro-RNAs (miRNAs), transfer RNAs (tRNA), and long non-coding RNAs (lncRNAs) (263). The capacity of EVs to transport cargo, undergo budding, shedding, and be taken up again by nearby cells, might potentially facilitate the dissemination of infections, advancement of illnesses, and modification of immune-related mechanisms. The involvement of EVs in the development of infectious diseases indicates that EVs might be used as biomarkers to monitor moderate bacterial infections and chronic viral diseases. Additionally, there is potential to utilize EVs to prevent the transmission of infections or to protect against them (264).

1.7.2. Role of EVs in viral infections

Viruses invade the cells and use the cell's structures to reproduce and complete their life cycle. Due to the significance of extracellular vesicles (EVs) in cell-to-cell communication and their involvement in disease processes, the interaction between viruses and EVs has received considerable interest, particularly due to their shared characteristics in terms of structure and formation (265). EVs serve a complex function in viral infections, primarily aiding in viral reproduction, spread, and evasion of the host immune system by influencing immune system activation. Moreover, EVs have the potential to obstruct or activate the antiviral response, leading to increased harm and various negative consequences, including the development of cancer, inflammation, or autoimmune reactions. Many viruses take use of internal membrane

rearrangements to generate unique double-membrane structures called "replicative organelles". These structures aid in the reproduction of the virus and the production of new virions, which are then released from the host cell (266). Coronaviruses are RNA viruses with positive-stranded RNA (+RNA) that replicate their large genetic material in the cytoplasm of the host cell. The viral replication organelle (RO) is a complex network of altered endoplasmic reticulum (ER) membranes generated by the virus, which supports this process (267,268). Double-membrane vesicles (DMVs) are the predominant constituents of the replication organelles (ROs) and serve as the primary sites for viral RNA production (269) (**Figure 1.16**) (270). The inside of the DMV contains double-stranded RNA, which are likely the products of viral genome replication and sub genomic mRNA synthesis (267,269). DMVs could provide an advantageous small-scale environment to produce viral RNA and may protect viral RNA from detection by innate immune sensors that are triggered by double-stranded RNA. Nevertheless, coronaviral DMVs have been identified as compartments that do not have any connections to the cytosol (267,269,271), even though newly-synthesized viral mRNAs require exportation for translation. Furthermore, the cytosolic nucleocapsid (N) protein is responsible for packaging the coronavirus genome before it is directed to virus assembly sites on secretory route membranes (272).

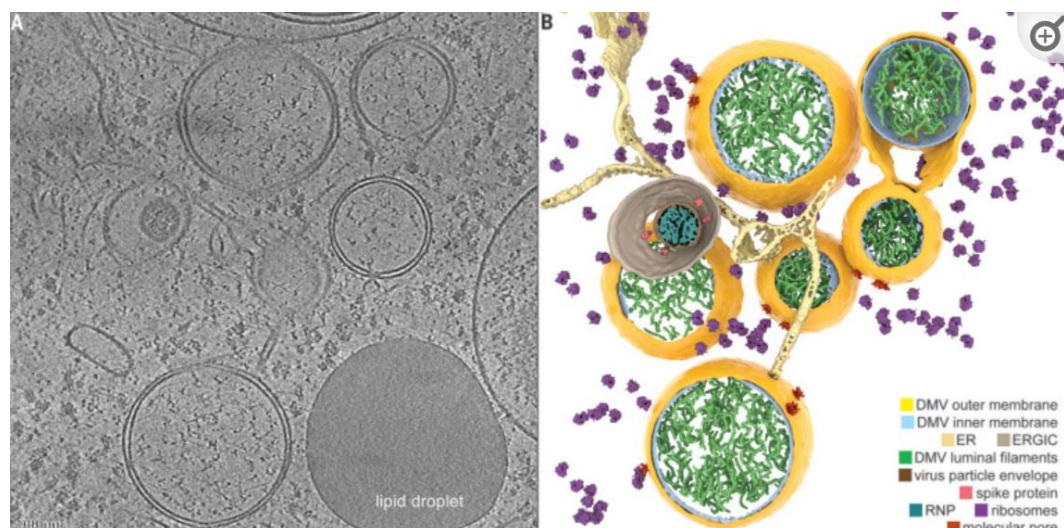


Figure 1.16. Cryo-electron tomography exposes DMVs triggered by the coronavirus. (A) Here, the murine hepatitis coronavirus (MHV) that serves as an extensively researched model for the genus Betacoronavirus, which encompasses severe acute respiratory

syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2 was studied. A 7 nm thick tomographic slice of a cryo-lamella that has been milled through MHV-infected cell at an intermediate stage of infection. **(B)** A 3D representation of the tomogram, displaying the annotated segmented information. *ERGIC*, an intermediary compartment between the endoplasmic reticulum and the Golgi apparatus. (Adapted from Ref. 270).

Furthermore, it also appears that viral infection affects lipid metabolism and processing, leading to the creation of protective structures that are crucial for a successful viral cycle (273). Lipid rafts and myelin and lymphocyte protein (MAL), which is a membrane protein located in cholesterol and glycolipids-enriched membranes, are thought to play a role in the absorption of virions and serve as platforms for the assembly of viral particles (274). In addition to the development of replication organelles, viruses must leave the infected cell and spread, while also evading the host immune system, in order to provide their biological life cycle. Both enveloped and non-enveloped viruses utilize EVs as a vehicle to enhance their ability to infect a wider range of cells, while evading detection by the host's immune cells (275). EVs can facilitate and amplify viral spread by forming "collective infectious units," which are vesicles containing several virions or virions containing multiple viral genomes. Viruses enhance their multiplicity of infection (MOI) by employing this dispersion method (276,277). The debate regarding whether these vesicles are extracellular vesicles (EVs) containing viral molecules or defective viral particles is ongoing. However, due to the similarities in size, density, and composition between EVs and viruses, it is challenging to separate and analyze distinct fractions. Therefore, there is a need for enhanced isolation strategies (278). An intersection between the release of viral particles and the pathway of extracellular vesicle (EV) formation is observed in "quasi-enveloped" viruses. Unlike enveloped viruses, which typically cause cell lysis to be released and are not surrounded by a host membrane, quasi-enveloped viruses have been discovered to be enclosed within vesicles during their spread. During this process, viruses use the host ESCRT machinery to obtain a protective membrane and leave the infected cell without causing its destruction. This allows the viruses to evade detection by the host immune system. The observed phenomenon, initially documented in hepatitis A virus (HAV) (279), has also been observed in hepatitis E virus (HEV)

(280), Coxsackie B virus (CBV) (281), as well as enveloped viruses such as hepatitis C virus (HCV) (282) and others. Moreover, the "Trojan exosome" hypothesis posits that retroviruses, such as the human immunodeficiency virus (HIV), exploit the pathways involved in the formation of extracellular vesicles (EVs) to exit, disseminate, and enter host cells. This process enhances their ability to infect cells and influences the expression of host genes and immune responses (283,284). An alternative theory suggests that viruses might exit infected cells without causing cell death by utilizing a process called "secretory autophagy." This process involves the formation of LC3-positive vesicles derived from autophagosomes, which fuse with the cell's plasma membrane instead of lysosomes. It is shown that LC3-positive organelles, which resemble autophagosomes, preferentially catch numerous viral particles (284). These organelles are rich in phosphatidylserine lipids. The collected viral particles are then released from the infected cells without causing cell lysis. Several diseases have the ability to disrupt the autophagic route through various methods (285,286). Compelling evidence indicates that viral infection significantly modifies the composition of EVs, leading to various effects that depend on the specific virus-host environment. These effects include promoting viral replication, transmission, and stability through the transfer of viral RNAs and proteins, as well as by modulating interactions between membranes and receptors. Consequently, this makes other cells more vulnerable to infection. Nevertheless, the exploitation of EVs trafficking by viruses can also result in varied immune responses that aid the host in combating the infection. This includes the activation of other cell targets, the stimulation of pro-inflammatory molecule production, and the promotion of antiviral response. To summarize, EVs can either worsen or mitigate viral infections, and in certain cases, a single virus can be responsible for both impacts. Given the similarities in biogenesis, dynamics, and mechanisms of action between viruses and EVs, it is crucial to have a comprehensive knowledge of their interaction in order to further treatment strategies (287).

1.7.3. EVs in SARS CoV-2

Extracellular vesicles (EVs) are found in body fluids and excreted by all types of cells. They contain various biomolecules like proteins, lipids, and nucleic acids. These biomolecules are transported to other cells, causing changes in their function and

structure. Consequently, EVs play a crucial role in regulating communication between cells. EVs and viruses exhibit resemblances in terms of their dimensions, organization, and biochemical composition, employing comparable processes of formation and fusion of membranes (288). Similar to other viruses, vesicular structures play a crucial role in the SARS-CoV-2 infection process. These structures are involved in the construction of the virus, its release from infected cells, and its spread to other cells. Both SARS-CoV and SARS-CoV-2 utilize similar processes for replication, virion assembly, and egression. These coronaviruses may use the ERGIC or Golgi apparatus to exit the cell, most likely through the exocytic pathway (269,289,290) as shown in **Figure 1.16** (291).

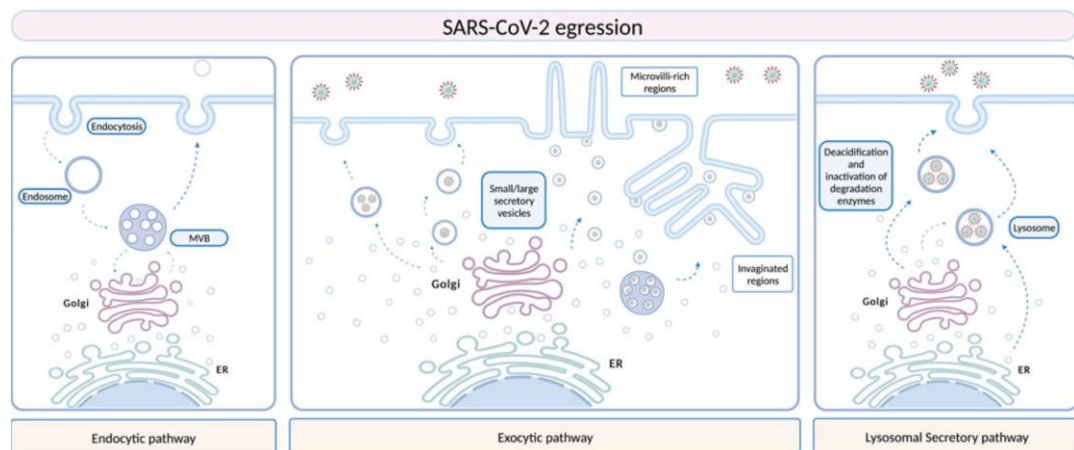


Figure 1.17. SARS CoV-2 egression pathway resembling extracellular vesicles. Multiple morphological studies provide evidence for the involvement of extracellular vesicles (EVs) in the release of SARS-CoV-2, particularly through the exocytic route. This process entails the modification of cell membranes and the creation of distinct structures, such as areas abundant in microvilli or invaginations. An alternative conjecture is the lysosomal route, however the mechanism by which these organelles merge with the plasma membrane remains undisclosed (Adapted from Ref. 291).

It has been proposed that coronaviruses may utilize lysosomes instead of the secretory route. This idea is reinforced by the observation of viral particles within lysosomal compartments using Transmission Electron Microscopy (TEM) (292). Nevertheless, the precise fusion process by which these organelles merge with the plasma membrane, enabling the release of the virus into the extracellular environment, remains uncertain.

Early-stage studies have utilized morphometric and ultrastructure analyses to address the limited understanding of the replication cycle of SARS-CoV-2, with a particular focus on egress events (293–296). The concept of "exit tunnels" has been suggested as a means of connecting intracellular structures containing viral particles to the external environment (296). Conversely, a different group emphasized the existence of morphologically distinct regions within infected cells that allow SARS-CoV-2 to exit. These regions include areas with a significant inward folding of the cell's cytoplasm and small projections on the cell's outer membrane, as well as sections of the membrane that have not undergone noticeable changes, and situations that fall in between. The distinguishing feature of these locations was the presence of either several intracellular vesicles, carrying multiple viral particles, or a single viral particle in smaller vesicles. Based on this study, the most common way that SARS-CoV-2 particles exit cells is via passing through tiny secretory vesicles in locations with a lot of invaginated microvilli (294). However, further research is required to elucidate the mechanism responsible for the release of vesicles harboring viral particles into the extracellular space. Vesicular pathways have a crucial role in facilitating SARS-CoV-2 infection, both in terms of maintaining the viral morphological life cycle and its functional processes. EVs have a significant role in several biological processes, such as maintaining tissue balance, contributing to the development of diseases, and facilitating interactions between the host and pathogens, including viral infections (297). Viruses can utilize EVs to facilitate their entry, packaging, dissemination, and evasion of immune system reactions. EVs can have either a beneficial or detrimental effect on SARS-CoV-2 infection, depending on the specific cargo they carry. This is similar to their involvement in other viral infections, where EVs can either promote or hinder the transmission of the virus, depending on the virus-host interaction. Infected cells exhibit heightened production of EVs, which facilitate the transfer of viral nucleic acids, proteins, and complete virions, along with viral entry receptors, to uninfected cells. This process not only aids in the spread of the virus, but also plays a role in modifying the immune responses of target cells and their vulnerability to infection. CD9 tetraspanin and ACE2 receptor are two components found in EVs that play a role in SARS-CoV-2 infection. CD9 tetraspanin, which is naturally abundant in the membranes of EVs, may be involved in the fusion of coronaviruses. On the other hand, the ACE2 receptor can be packaged into EVs and transferred to cells that lack this receptor, making them more susceptible to viral infection (160,298). Moreover, extracellular vesicles (EVs) released by infected cells

can potentially trigger inflammatory reactions and initiate programmed cell death and cell toxicity, hence causing harm to tissues (299). The capacity of EVs to carry viral and self-antigens can have both positive and negative effects. On one side, it enables the host immune system to identify the virus and regulate immunological responses against it (300). Several studies have observed an elevated presence of extracellular vesicles (EVs) carrying lung-related self-antigens, viral antigens, and 20S proteasome during coronavirus infection. This finding provides additional evidence for the hypothesis that EVs may also play a role in the spread of SARS-CoV-2, which is still being investigated, along with the intracellular vesicular structures that support viral replication (301,302). Since EVs produced from infected cells present a unique molecular signature than healthy cells, defining their cargo, as well as their dynamics, may result important for a better knowledge of COVID-19 etiology, to contribute new insights to diagnostic and therapeutic efforts.

1.8. Aim of the Study

Corona virus disease 2019 (COVID-19), which is caused by SARS CoV-2, affected millions of people, and the course of the disease varies from asymptomatic to multi-organ failure. At the beginning of the pandemic, children were thought to be less affected than other age groups and have a favorable course of COVID-19. However, the rate of infected children rapidly increased from 2% of all patients in 2022, to 17.9% in 2023 (303). Children had also cytokine storm and severe course of disease similar to adults (304).

Some risk factors for severe COVID-19 have been identified. Asthma, which is the most frequent chronic disease of the childhood, was previously considered a risk factor (13,305). However, publications have showed that asthma does not pose risk for severe COVID-19 (305,306). Moreover, people who have asthma had fewer hospitalizations during the pandemic possibly due to the medications given for asthma, more compliance with the face mask, hand washing and social isolation rules due to the fear of asthma exacerbation (306).

The mechanisms underlying COVID-19 are complex, with multiple immunological pathways and profound immune dysregulation appearing to be involved (307). To elucidate the underlying pathophysiology especially in severe and critical patients, numerous studies have been conducted. T lymphocytes, monocytes, large amounts of inflammatory cytokines and chemokines are shown to be responsible for the disease severity (307). Other mechanisms may also have role in COVID-19.

Although human angiotensin-converting enzyme 2 (hACE-2), the receptor to which SARS CoV-2 binds, is accepted very crucial in disease pathogenesis and progression. Although hACE-2 is found at a very low level in organs including lungs and heart, they are the most frequently involved organs in COVID-19, and RNA of SARS CoV-2 is commonly isolated in these tissues. This raises the question of other mechanisms for the disease propagation (308,309).

Coronaviruses form double-membrane vesicles, uses Trans-Golgi network pathway in replication phase within the host cells. By this way, they can escape from the cytosolic innate immune receptors of the host cells and form a protective microenvironment (269,293). This indicates that vesicular trafficking is important in SARS CoV-2 immunity and viral spread. Vesicular trafficking is a way of intercellular communication both in physiological and pathological conditions. Extracellular vesicles (EVs) are the key elements of this cellular trafficking and have significant impact on the immunological responses (310,311). Considering that SARS CoV-2 uses a double membrane vesicular system similar to EVs for replication and immune evasion, EVs are likely to be a pathway for SARS CoV-2 related responses.

EVs are 30 to 5000 nm in size, lipid-bilayer microparticles released by cells. There are three types of EVs defined so far. These are, from small to large, exosomes (20–150 nm), microvesicles (MVs) (100–1000 nm in diameter), and apoptotic blebs (1000–5000 nm in diameter) (312). Cargo contents of these particles represent the cellular origin, physiological and pathological conditions of the cells (313,314). The fact that SARS CoV-2 uses similar pathways as EVs in its intracellular life cycle, reinforcing the significant role of EVs in the pathogenesis of COVID-19. However, the number of studies on EVs in COVID-19 is relatively scant.

In this study, we aimed to understand the effects of EVs in severe and mild COVID-19 pediatric patients with and without asthma. The impact of EVs on CD4+T cells and monocytes, which are important two immune cells involved in SARS CoV-2 were assessed (226,315). EVs were purified by serial ultracentrifugation and characterized by several methods. Peripheral blood mononuclear cells (PBMCs), naïve CD4+T cells and monocytes from healthy donors were stimulated with plasma derived EVs of healthy controls, severe and mild patients at the time of admission and during convalescence period. The cytokine milieu in the culture medium and cellular changes after the stimulation were evaluated by surface markers and chemokine receptors.



2. CHAPTER 2

MATERIALS AND METHODS

2.1. Patients and Healthy Controls

This prospective study involved children (<18 years old) with SARS CoV-2 infection who were admitted to the Hacettepe University School of Medicine, which is a tertiary hospital for treating COVID-19 pediatric patients in Turkey, between November 16, 2021, and May 23, 2022.

Children who were unvaccinated, symptomatic, and confirmed by SARS-CoV-2 nucleic acid real-time reverse transcription-polymerase chain reaction (RT-PCR) testing were enrolled in the study. Patients who did not undergo SARS-CoV-2-specific PCR testing with at least one family member with PCR-confirmed COVID-19 infection and with symptoms occurring in the patient within a few days following the contact were also included if these patients had positive SARS-CoV-2 IgG results. Patients who did not have any contact with a physician-diagnosed COVID-19 patient along with a negative SARS CoV-2 PCR test, who were vaccinated against SARS CoV-2, who had an accompanying chronic illness other than allergic diseases, and who did not want to participate were excluded.

Data including demographic features, clinical presentation, symptoms during COVID-19 infection, hospitalization, duration of hospitalization, specific RT-PCR results, medical history, laboratory results, treatments (medications, non-invasive ventilation, and oxygen therapies), outcomes and presence of asthma were obtained from the medical records. Childhood asthma control test (C-ACT; 4-11 years) (316) and ACT (≥ 12 years old) (317) scores were assessed during outpatient follow-up.

The severity of pediatric COVID-19 patients was defined according to the classification of Dong *et al.*, which is based on the clinical characteristics, laboratory results and radiologic imaging as follows: (a) mild disease, with acute symptoms but without evidence of pneumonia or hypoxia, and (b) severe disease with progressive respiratory disease, dyspnea, and central cyanosis (186). The detailed inclusion and exclusion

criteria and characteristic features of the patients were given in **Figure 2.1** and **Figure 2.2**.

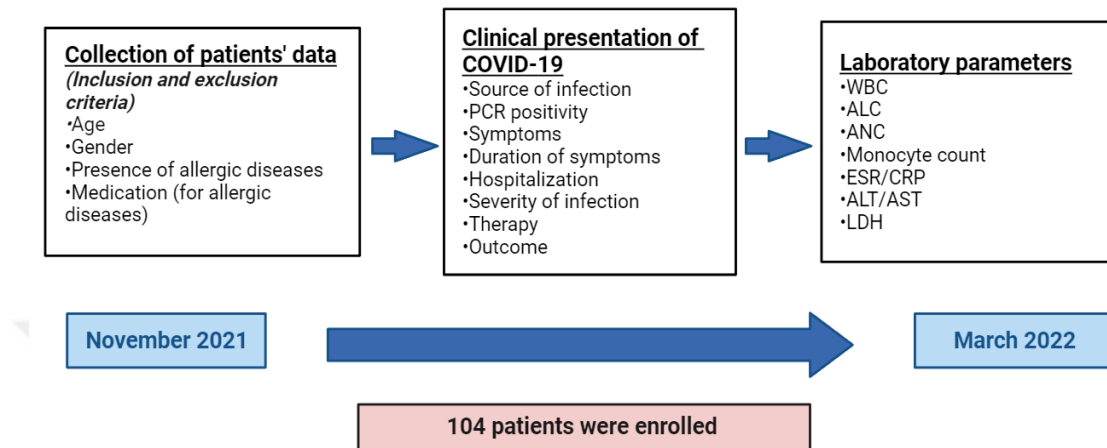


Figure 2.1. Overview of the study design, enrollment process, study period, data collection and collected parameters are shown. BioRender™ was used to illustrate.

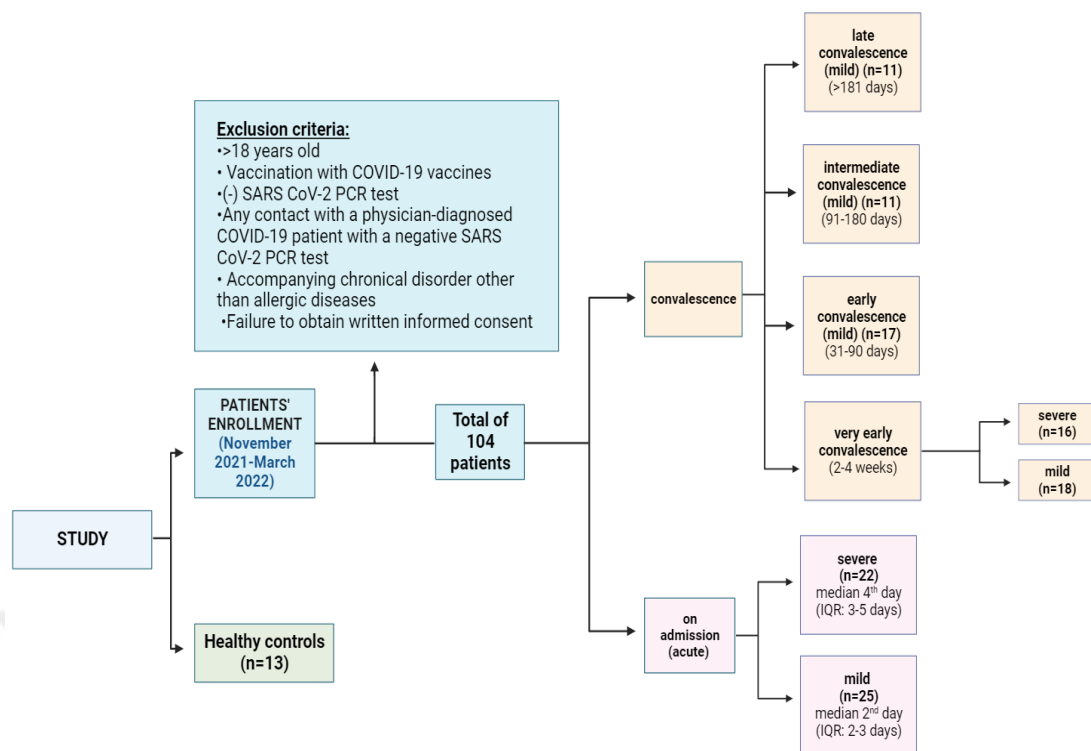


Figure 2.2. Classification of the patients included in the study according to the severity of COVID-19 disease. BioRender™ was used to illustrate.

Blood samples were obtained at the first admission to the pediatric infectious diseases' outpatient and inpatient clinics and between 2 and 4 weeks (very early convalescent phase) after initial symptoms from the patients with acute COVID-19 disease. Samples were also collected from children who were followed up in the pediatric allergy outpatient clinic with the history of COVID-19 infection and no acute infection at the time of admission. These convalescent individuals were neither vaccinated nor reinfected with SARS CoV-2 during the first 12 months of follow-up period after the first COVID-19 infection. Convalescent patients were further categorized as very early convalescent phase (between 2 and 4 weeks), early (between 31 days and 90 days of the diagnosis), intermediate (between 91 days and 180 days of the diagnosis) and late convalescent (>181 days of the diagnosis) (318–320). Long-term follow-up of patients with severe disease could not be performed for various reasons, therefore only 12-month follow-ups of patients with mild disease were recruited. Since the blood samples of severe patients were taken only in the very early convalescence phase, the immunological parameters of both mild and severe convalescence patients could only be compared between 2 and 4 weeks.

Pediatric healthy controls (HCs) ($n=13$) with negative SARS-CoV-2 RT-PCR and SARS-CoV-2 serology were included. These children had no accompanying diseases, no SARS CoV-2 vaccination, and no acute infection at the time of blood sampling. The summary of the patient cohort and experimental design were given in **Figure 2.3**.

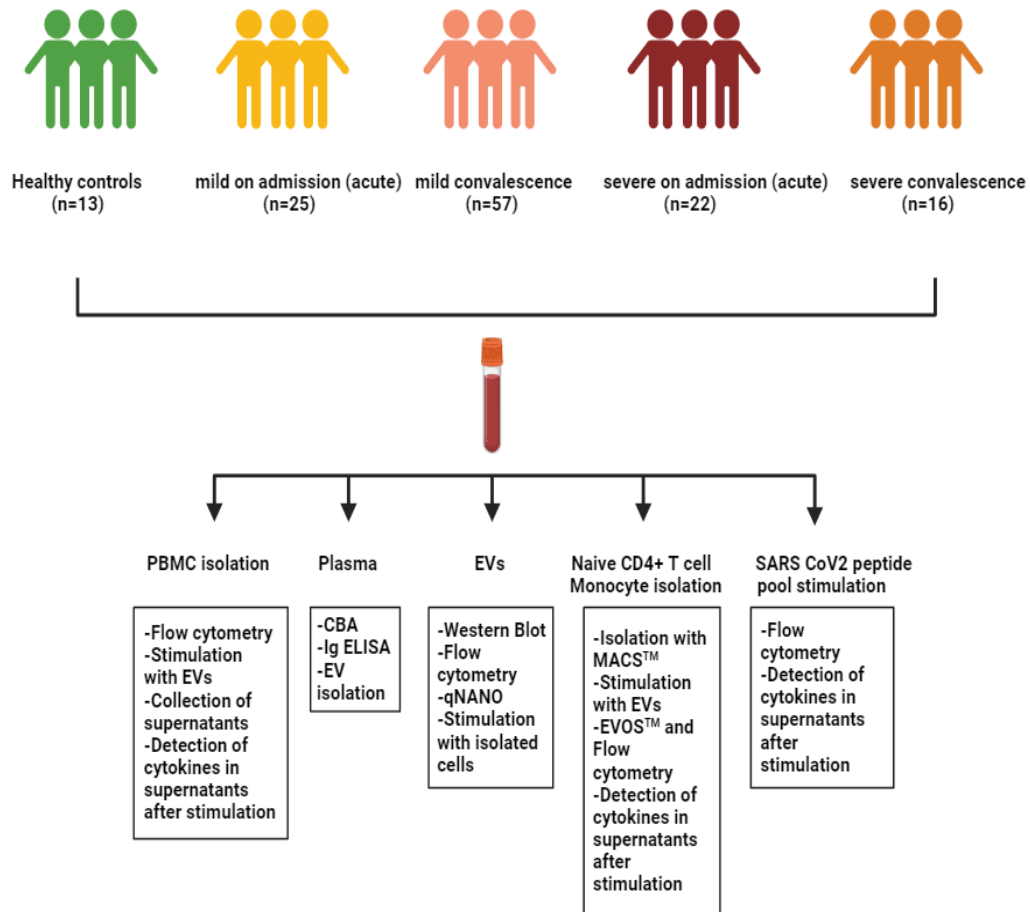


Figure 2.3. Schematic summary of the study design, patient cohort and methods used in the study (BioRender™ was used to illustrate).

Written informed consents were obtained from patients/HCs or their parents. This study obtained ethical approval from the Human Ethics Committee of Hacettepe University School of Medicine and Bilkent University (reference number: GO 22/77).

2.2. Materials

2.2.1. Cell Culture Media Supplements and Buffers

RPMI 1640 (Sigma Aldrich, USA, Cat# R0883) with penicillin (100 IU/ml) and streptomycin (100 µg/ml), 1% L-glutamine 100 mM, 1% sodium pyruvate 1 M, non-essential aminoacids (HyClone, Cat# SH30238.01), HEPES (Biological Industries, Cat# 03-025-1B) and 10% exosome-depleted fetal bovine serum (FBS) (Biological Industries, UK, Cat# 04-127-1A) was used. Cell culture grade water (DNase and RNase-free) was purchase from Biological Industries, USA (Cat# 01-869-1A). EDTA (10 nM final concentration) (Serva, USA, 0.5 M, Cat# 39761.02) in DPBS (Biological Industries, Sartorius, Israel, Cat# BI02-023-1A) was used to harvest the monocytes stimulated with extracellular vesicles. Lymphocyte Separation Medium (density 1.077 g/ml, #LSM-A, Capricorn, Germany) was used to isolate PBMCs. Recipes of homemade buffers were given in **Appendix A**.

2.2.2. Mitogens, Ligands and Cytokines

All stimulants used in the experiments are given in **Table 2.1.** in detail.

Table 2.1. All mitogens, ligands and cytokines used in the experiments during the study

Ligand name	Brand name	Catalog number	Working concentration
Phorbol-12-myristate 13-acetate	Sigma Aldrich, Germany	P1585	50 ng/ml
Ionomycine	Calbiochem, Merckmillipore, Germany	407950	1 µg/ml
LPS (Isolated from E.coli)	Sigma Aldrich, Germany	L-2880-100mg	20 ng/ml
Human recombinant IFN γ	Thermo	RIFNG50	50 ng/ml
Human recombinant IL4	Thermo	RIL4I	20 ng/ml

Human recombinant IL10	BioLegend	575804	20 ng/ml
GM-CSF	Endogen	RGMCSF10	50 ng/ml
M-CSF	TONBO	21-8789-U100	50 ng/ml
SARS CoV-2 specific (SMNO) peptide pool	Mabtech, Sweden	3622-1	2 µg/ml
anti-human CD28	BioLegend	302914	1 µg/ml
anti-human CD3	BioLegend	300314	1 µg/ml

2.2.3. ELISA Reagents and Kits

All antibodies, recombinant standards, Streptavidin-Alkaline Phosphates (SA-ALP) and para-Nitrophenylphosphate (pNPP) used in cytokine ELISA were summarized in **Table 2.2.**

Table 2.2. Antibodies, recombinant proteins and reagents used in cytokine ELISA

Recombinant antibody	Brand name	Catalog number	Clone	Working concentration
IFN γ	Mabtech, Sweden	3420-1A-20	1-D1K	2 µg/ml
Biotin anti-human IFN γ	Mabtech, Sweden	3420-1A-20	7-B6-1	1 µg/ml
Human recombinant IFN γ	Mabtech, Sweden	3420-1A-20	NS*	1000 pg/ml
IL6	Mabtech, Sweden	3460-1A-20	13A5	0.5 µg/ml
Biotin anti-human IL6	Mabtech, Sweden	3460-1A-20	39C3	1 µg/ml
Human recombinant IL6	Mabtech, Sweden	3460-1A-20	NS*	20 pg/ml
TNF α	Mabtech, Sweden	3512-1A-20	MT25C5	2 µg/ml
Biotin anti-human TNF α	Mabtech, Sweden	3512-1A-20	MT20D	1 µg/ml

Human recombinant TNF α	Mabtech, Sweden	3512-1A-20	NS*	1000 pg/ml
IL4	Mabtech, Sweden	3410-1A-20	IL4-I	1 μ g/ml
Biotin anti-human IL4	Mabtech, Sweden	3410-1A-20	IL4-II	1 μ g/ml
Human recombinant IL4	Mabtech, Sweden	3410-1A-20	NS*	500 pg/ml
IL5	Mabtech, Sweden	3490-1A-20	TRFK5	2 μ g/ml
Biotin anti-human IL5	Mabtech, Sweden	3490-1A-20	5A10	1 μ g/ml
Human recombinant IL5	Mabtech, Sweden	3490-1A-20	NS*	1000 pg/ml
IL13	Mabtech, Sweden	3471-1A-20	25K2	2 μ g/ml
Biotin anti-human IL13	Mabtech, Sweden	3471-1A-20	MT1318	0.5 μ g/ml
Human recombinant IL13	Mabtech, Sweden	3471-1A-20	NS*	1000 pg/ml
IL10	Mabtech, Sweden	3430-1A-20	9D7	2 μ g/ml
Biotin anti-human IL10	Mabtech, Sweden	3430-1A-20	12G8	1 μ g/ml
Human recombinant IL10	Mabtech, Sweden	3430-1A-20	NS*	500 pg/ml
IL17A	Mabtech, Sweden	3520-1A-20	MT44.6	2 μ g/ml
Biotin anti-human IL17A	Mabtech, Sweden	3520-1A-20	MT504	0.5 μ g/ml
Human recombinant IL17A	Mabtech, Sweden	3520-1A-20	NS*	1000 pg/ml
Goat Anti-human IgA	Southern Biotech	2050-04	NS*	1:1000 diluted
Goat Anti-human IgG	Southern Biotech	2040-04		
Goat Anti-human IgM	Southern Biotech	2020-04		
SA-ALP	Mabtech, Sweden	NS*	NS*	1:1000

pNPP	Thermo Scientific, USA	NS*	NS*	NS*
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*NS; Not Specified

2.2.4. Antibodies used in Flow Cytometry

All anti-human antibodies used in experiments with flow cytometry are shown in **Table 2.3.**

Table 2.3. Anti-human antibodies that were utilized in the experiments

Anti-human Antibody	Brand name	Catalog number	Clone
Zombie Aqua™ dye Fixable Viability Kit	BioLegend, USA	423101	NS*
Human TruStain FcX™ Fc Receptor Blocking Solution	BioLegend, USA	422302	NS*
CD45 PerCP	BioLegend, USA	368506	2D1
CD3 APC-Cy7	BioLegend, USA	344818	SK7
CD3 PE	BioLegend, USA	300308	HIT3a
CD4 BV570	BioLegend, USA	300534	RPA-T4
CD4 APC	Elabscience, USA	E-ABF1109E	RPA-T4
CD8 PE	Immunostep	8PE1-100T	143-44
CD8 APC-Cy7	BioLegend, USA	344714	SK1
CD19 FITC	BioLegend, USA	302256	HIB19
CD14 BV421	BioLegend, USA	301830	63D3
CD14 APC-Cy7	BioLegend, USA	325620	M5E2
CXCR3 FITC	BioLegend, USA	353704	G025H7
CCR4 APC	BioLegend, USA	131212	2G12
CCR6 PE-Cy7	BioLegend, USA	353418	G034E3
CD25 PerCP-Cy5.5	BioLegend, USA	302626	BC96
CD127 PE	BioLegend, USA	351340	A019D5
CD303 PerCP-Cy5.5	BioLegend, USA	354210	201A
CD123 APC	BioLegend, USA	306012	6H6
CCR7 (CD197) BV421	BioLegend, USA	353208	G043H7
CD45RO PE-Dazzle 594	BioLegend, USA	304248	UCHL1

CD69 FITC	BioLegend, USA	310904	FN50
PD-L1 PE-Cy7	BioLegend, USA	329718	29E.2A3
CD14 APC-Cy7	BioLegend, USA	325620	HCD14
CD68 APC	BioLegend, USA	333810	Y1/82A
CD80 PE-Cy7	BioLegend, USA	305218	2D10
CD80 PerCP-Cy5.5	BioLegend, USA	305232	2D10
CD86 Alexa Fluor® 488	BioLegend, USA	305414	IT2.2
CD163 PerCP-Cy5.5	BioLegend, USA	333608	GHI/61
CD206 BV 421	BioLegend, USA	321126	15-2
IFN γ FITC	BD Biosciences, USA	552887	B27
IL4 PE-Cy7	BioLegend, USA	500824	MP4-25D2
IL10 PerCP-Cy5.5	BioLegend, USA	501418	JES3-9D7

2.2.5. Antibodies used in Western Blotting

All the solutions and buffers used for Western Blotting were home-made and prepared as described in Appendix A. The technical equipment for the experiments were purchased from BioRad (USA). All Western Blotting antibody components were summarized in **Table 2.4**.

Table 2.4. Antibodies used in Western Blot experiments

Antibody	Brand	Catalog number	Clone	Dilution factor
TSG101	Abcam (UK)	ab83	4A10	1:1000
Flotinin-1	Cell Signaling Technology (Germany)	3253S	NS*	1:1000
Grp94 Rabbit	Cell Signaling Technology (Germany)	20292S	D6X2Q	1:1000
HRP-conjugated anti-mouse IgG	Cell Signaling Technology (Germany)	7076S	NS*	1:10000

HRP-conjugated anti-rabbit IgG	Cell Signaling Technology (Germany)	7074P2	NS*	1:10000
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*NS; Not Specified

2.3. Methods

2.3.1. Experiments related to peripheral human blood cells

2.3.1.1. Isolation of peripheral blood mononuclear cells (PBMCs) and plasma

Peripheral blood of the patients and healthy controls were collected in the EDTA tubes and studied within the first four hours immediately after the withdrawal of the blood samples. Blood samples were transferred into the 50 ml conical centrifuge tubes (Sarstedt, Germany, Cat# 62.547.254-300), diluted with 1:1 (v/v) ratio of 1xDPBS (Biological Industries, Sartorius) at room temperature (RT) and mixed gently with 25 ml (Genaxy, India, Cat# GEN-SER-25ML-S) serological pipette. Then, two-thirds of the blood volume of lymphocyte separation medium (Capricorn Scientific, Germany, Cat# LSM-A) was placed in 15 ml conical centrifuge tubes (Grenier, Bio-One, Ireland, Cat# 188 271). One-to-one 1xDPBS diluted blood samples were gently layered onto lymphocyte separation medium in 15 ml conical centrifuge tubes. This layered sample was centrifuged at 540xg for 30 min with off brake setting. The plasma portion with a yellowish color and the interface with a cloudy appearance, which contains peripheral blood mononuclear cells (PBMCs), were collected by 3 ml plastic sterile Pasteur pipette (Nest, Biotechnology, China, Cat# 318212), washed with RPMI-1640 medium (Sigma Aldrich, Merck, Germany, Cat# R0883) supplemented with 2% Fetal Bovine Serum (FBS) (Biological Industries, UK, Cat# 04-127-1A) by centrifugation at 540xg for 10 minutes. Washed cells were resuspended in 1 ml of complete RPMI-1640 medium supplemented with 5% FBS and counted in flow cytometry as explained below. PBMCs were used fresh or stored in liquid nitrogen for future use. Plasma was further

centrifuged at 2,000xg for 15 min to discard cellular debris and was stored at -80°C for the isolation of extracellular vesicles (EVs).

2.3.1.2. Isolation of naïve CD4⁺T cells and monocytes

Healthy human naïve CD4⁺T cells were negatively selected by magnetic cell sorting (MACS[®]) using naïve CD4⁺T cell isolation kit II (Miltenyi Biotec, Cat#130-094-131) according to manufacturer's instructions. Chilly cold running and rinsing buffer for MACS (explained in Appendix A as MACS Buffer) was used throughout the procedure. Sorted cells were washed and stained with anti-human CD4 APC, anti-human CD45RA FITC and anti-human CD45RO PE-Dazzle antibodies. The isolated cells were assessed by flow cytometry.

Healthy human derived monocytes were positively selected by using anti-human CD14 MicroBeads (Miltenyi Biotec, Cat#130-050-201), as per manufacturer's protocol. Isolated cells were stained with anti-human CD14 FITC monoclonal antibody and analyzed by flow cytometry.

2.3.1.3. SARS CoV-2 specific peptide pool stimulation of patient and healthy PBMCs

For stimulation assay, cryopreserved PBMCs were thawed at 37°C and washed twice with 2% FBS containing RPMI. 8×10^5 PBMCs were plated per well in 96 well U-bottom plate in 100µl 10% FBS supplemented RPMI. A stimulation solution was prepared with 2 µg/ml of SARS CoV-2 specific (SMNO) peptide pool (Mabtech, PepPool: SARS-CoV-2 (SNMO), human) for each peptide and 1 µg/ml anti-human CD28 in 50µl 10% FBS containing RPMI. For positive control, anti-human CD28 and anti-human CD3 were added at 1 µg/ml each in 50µl medium. Some wells were left unstimulated and added only 50µl of medium. Total volume was completed to 250µl with 10% FBS containing RPMI. All detailed information about the stimulants were given in **Table 2.1**. SARS CoV-2 specific (SMNO) peptide pool was lyophilized and prepared according to manufacturer's instructions. Plates were incubated at 37°C and 5% CO₂ for 48 hours. At the end of the incubation, supernatants were collected and stored at -80°C for analyzing of IFN γ , IL13, IL10 and IL17A cytokines by ELISA (**Table 2.2.**), and PBMCs were stained with anti-human CD69, HLADR, CD25 and PDL1 surface markers (**Table 2.3.**).

2.3.2. Experiments related to Plasma Derived Extracellular Vesicles (EVs)

2.3.2.1. Isolation of Extracellular Vesicles (EVs) from Plasma

Frozen or fresh plasma was used for the isolation of extracellular vesicles (EVs). The night before the isolation, frozen plasma stored at -80°C was taken to thaw slowly at $+4^{\circ}\text{C}$. Samples were centrifuged at $2000\times g$ for 20 min to discard the debris. Then, upper part of the plasma was moved to 15 ml ultracentrifuge tubes (Beckmann Coulter, Cat# 326823) and completed to 14 ml with 1x cold DPBS in case it is necessary. Tubes were centrifuged at $10,000\times g$ for 20 min at $+4^{\circ}\text{C}$ to discard debris and large vesicles by Beckmann Coulter ultracentrifuge. At the end of 20 min, the supernatant was transferred to a new 15 ml ultracentrifuge tube and was centrifuged at $100,000\times g$, at $+4^{\circ}\text{C}$ during 2 hours for two times. After ultracentrifugation, the supernatant was totally discarded, and EV pellet was dissolved within $150\text{ }\mu\text{l}$ 1xDPBS and collected into 1.5 ml conical microcentrifuge tubes (Greiner Bio-One, Ireland, Cat# 616201). EVs in 1xDPBS was snap frozen in liquid nitrogen and stored at -80°C . Multiple freeze-thaw cycles of EVs (>3 times) were avoided.

2.3.2.2. Determination of protein content, particle size and concentration of plasma derived EVs

Protein amount of EVs was determined with BCA Protein Assay Kit (PierceTM, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, $25\text{ }\mu\text{L}$ /well of 1/2 serially diluted standards ranging from $2000\text{ }\mu\text{g/ml}$ to $7.81\text{ }\mu\text{g/ml}$ and $5\text{ }\mu\text{L}$ /well of samples (also by completing the samples' volumes up to $25\text{ }\mu\text{L}$) were transferred into 96-well flat bottom plates by mixing with $200\text{ }\mu\text{L}$ of working reagent. Working reagent was prepared by adding 1 volume of Solution A and 50 volume of Solution B and mixing thoroughly. Prepared mixtures in the 96-well plate was placed into the incubator (37°C , 5% CO_2) for 30 minutes. The samples were incubated for 30 minutes, and then the absorbance at 562 nm was measured using a microplate reader (BioTek®, Synergy, HT). The protein amount was adjusted to the plasma volume (ml) in order to facilitate the comparison of the protein levels of EVs.

2.3.2.3. Plasma Derived EV Characterization

Characterization of isolated patients' and healthy donor EVs, Tunable-resistive pulse sensing (TRPS), flow cytometry and Western blotting were used.

2.3.2.3.1. EV analysis by using Tunable-resistive pulse sensing (TRPS)

The particle-based quantification and size profiling of EVs were measured with TRPS technology by using qNano gold (IZON, Biosciences) system and analyzed with IZON Control Suite 3.4.2.48 software (IZON Science LTD). All sample readings were repeated three times.

2.3.2.3.2. Detection of surface markers by flow cytometry analysis

The surface markers attributed to EVs were analyzed by flow cytometry as explained in the previous study (321). For this experiment, carboxyl latex beads (Invitrogen™, Thermo Fisher Scientific) were used, since EVs are too small to be determined by flow cytometry. First, beads were washed twice with 1xDPBS, then 1 µl of latex beads were incubated with 1 µg anti-human purified CD9 antibody (Biolegend, Cat#312102) in 50 µl 1xDPBS for 30 min at RT. The solution was prepared by adding 500µl of 1xDPBS and incubated overnight at room temperature on a tube rotator set at a low speed.

Next day, latex beads/anti-CD9 complex was centrifuged at 10,000Xg for 10 min at RT (Eppendorf™, Model 5415 R) and was blocked with 5% BSA (Capricorn Scientific, Germany, Cat# BSA 1T) prepared in 1xDPBS for 4 hours at RT on tube rotator. Then the mixture was centrifuged at 10,000Xg for 10 min at RT and resuspended in 1% BSA. For each staining, 2 µg of EVs were combined with 1 µL of the final solution containing coated beads. The volume was completed to 50 µL 1xDPBS, and EV-bead-antibody mixture was left to incubate at room temperature for 30 minutes. After the incubation period, the volume was adjusted to 500 µL by adding 1xDPBS. The EV-bead-antibody mixture was subjected to overnight rotation at room temperature. Following an overnight conjugation, the EV-bead conjugates were subjected to an incubation process with PE-conjugated fluorochrome antibodies specific for human CD63 (Biolegend,

Cat#353004), human CD81 (Biolegend, Cat#349505) and the corresponding isotype control (PE Mouse IgG1, κ Cat#400114). The incubation was carried out at a concentration of 1 $\mu\text{g/mL}$ in a volume of 100 μL for a duration of 1 hour at room temperature in the darkness. After the incubation period, the samples were rinsed with 1xDPBS, spined at 10,000xg for 5 minutes, suspended in 150 μL of 1xDPBS, and acquired using the Novocyte[®] flow cytometer (ACEA Biosciences). At least 30,000 or more latex beads were analyzed in each experiment. A forward scatter area versus forward scatter height density plot (singlet) was used to exclude latex bead doublets.

To measure the specific markers on the surface of plasma EV samples, we used bead conjugates coated with EV anti-CD9. These bead conjugates were stained with an antibody (diluted 1:100) against one of the following cell surface markers: anti-human CD3 PE, anti-human CD14 PE (monocytes), and anti-human CD19 PE (B cells). The same method was used for all these markers. The particle count was divided by the total bead-EV conjugate counts to determine the proportion of antibody-positive beads. To further determine whether T cell-derived EVs carry activation or inhibition markers, EV anti-CD3-coated bead conjugates were stained with anti-human CD69 PE and PDL1-PE.

2.3.2.3.3. SDS-PAGE and Western Blotting

To extract the protein contents, cells (RAW 264.7) (5×10^6 cells) and EV samples (at least 100 μg EV per patient's, used 4 different patients' and 1 healthy EVs) were treated with RIPA buffer (prepared as explained in Appendix A) for SDS-PAGE. They were incubated on ice during 30 minutes by vortexing for 15 seconds at every 5 minutes. After the incubation period, the mixtures were subjected to centrifugation at 13,000xg for 20 minutes. The supernatant was collected. The protein content was determined using the BCA Protein Assay Kit (Pierce[™], Thermo Scientific). Then cell lysates and EV samples were mixed with reducing 4xLaemmli Buffer and denatured at 95°C for 5 min, then they were left on ice until use. Stacking and separating gels were prepared at 5% and 10% concentration, respectively. After solidification, gels were transferred into Tank (Bio-Rad[®], Germany) that was filled with 1xRunning buffer (as explained in Appendix A). Denatured samples were loaded as 10 μg for cell line and 30 μg for each EV sample in 40 μL per well. After loading, 100V power was applied to separate the

samples. After running, SDS gel was transferred into a cassette between 4 Whatman papers (GE Healthcare, USA) and one 0.45µm PDVF membrane (ThermoFischer Scientific, USA). Transfer cassette was put into tank and filled with cold 1xTransfer buffer (as explained in Appendix A). This step was carried out at 110V for 2 hours on ice.

PDVF membranes were washed with PBS-T (as explained in Appendix A) and blocked with blocking buffer (as explained in Appendix A) for 2 hours at RT. Then, membranes were incubated with 1:1000 diluted primary antibodies against anti-tumor susceptibility gene 101 (TSG-101) antibody (Abcam, Cambridge, UK, clone: EPR7130(B)), anti-flotillin (Cell Signaling Technology, Main, Frankfurt, Germany, Cat#3252) and anti-glucose regulated protein 94 (GRP94) (Cell Signaling Technology, clone: D6X2Q) for 16 hours at +4°C. After primary incubation, membranes were incubated with corresponding HRP-conjugated 1:5000 diluted anti-mouse or anti-rabbit IgG secondary antibodies for 2 hours at RT. The HRP activity was detected with ECL™ Western Blotting Reagent (GE Healthcare, USA) and imaged by an Amersham Imager 600 (GE Healthcare, USA).

2.3.3. Experiments related to the stimulation of cells with patients' and healthy donors' EVs

2.3.3.1. Determination of EV Binding and Uptake of PBMC, naïve CD4⁺T lymphocytes and monocytes

The EV uptake and binding capacity of human PBMCs, naïve CD4⁺T lymphocytes and monocytes was analyzed prior to stimulate of the cells with EVs as described in a previous study (322). In this context, EV surface was stained with the lipophilic dye SP-DiOC₁₈(3) (Invitrogen™, Cat#D7778) at a ratio of 1 µg:10 µg (SP-DiOC₁₈(3) to EV) at 37°C for 1 hour at the dark. After the incubation, the mixture was completed to 14 ml with 1xDPBS and ultracentrifuged at 100,000xg for 90 min to remove the excess dye. Following the ultracentrifugation step, EVs were passed through size exclusion columns (IZON qEV column, 70 nm) to further purify and separate from other particles. Human healthy donor PBMCs (400.000/well), isolated naïve CD4⁺T lymphocytes (75.000/well) and monocytes (150.000/well) were seeded into 96 U-bottom well plates. Cells were incubated with 20 µg/mL of SP-DiOC₁₈(3)-stained patient (mild (n=1) and

severe (n=1) SARS CoV-2 patients) and healthy donor (n=1) EVs for 2, 4, 8, 16, or 24 h. Fluorescence signals of SP-DiOC₁₈(3) stained EVs in cells were visualized with auto cell imaging system (EVOS[®] FL, Thermo Fisher Scientific) at specified time points. After fluorescence imaging, cells were washed twice with 1xDPBS. Then cells were stained with anti-human CD3-APC-Cy7, anti-human CD4 APC, anti-human CD8 PE anti-human CD14-BV421 for 30 min at +4°C. After the staining period, cells washed twice with 1xDPBS to remove excess dyes, resuspended in 500µl 1xDPBS and analyzed with flow cytometry (Novocyte[®], ACEA Biosciences). SP-DiOC₁₈(3) is analyzed at FITC channel in flow cytometry. FITC-positive cells were accepted as both bound to and internalized (uptake) EVs. To reveal only EVs internalized cells and exclude only EV-bound cells, trypan blue was added to tube at 0.1% concentration to quench the cell membrane bound EV fraction. After trypan quenching, FITC channel was used to reveal the internalized EV partition by PBMCs, naïve CD4⁺T lymphocytes and monocytes at each specified time points.

2.3.3.2. Co-culture of plasma derived EVs with PBMCs, naïve CD4⁺T lymphocytes and monocytes

Both healthy PBMCs, isolated naïve CD4⁺T lymphocytes and monocytes were stimulated with plasma derived patient and healthy EVs. Therefore, PBMCs, isolated naïve CD4⁺T lymphocytes and monocytes were plated in 96 well plates (Sarstedt, Germany) at a concentration of 4x10⁵ cells, 15x10⁴ cells and 75x10³ cells per well in RPMI 1640 (Sigma Aldrich) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml), 1% L-glutamine 100 mM, 1% sodium pyruvate 1 M, and 10% exosome-depleted FBS, respectively. Cells were either stimulated with various EVs (15µg/well) or left unstimulated (w/o EV, non-EV wells) and completed to 250 µl with 10% exo-free FBS supplemented RPMI.

As positive control in experiments with monocytes, some of wells were stimulated with either M-CSF (50ng/ml) (to trigger M2 type macrophage polarization) or GM-CSF (50ng/ml) (for M2 type macrophage polarization) on day 0 without applying any EVs. For the last 24 h of this culture, IL4 (20ng/ml) and IL10 (20ng/ml) were added to M-CSF, IFNγ (20ng/ml) and LPS (20ng/ml) were added to GM-CSF stimulated wells. Supernatants of these positive control wells were replaced with fresh M-CSF and GM-CSF supplemented 10% exosome free FBS containing RPMI medium every

48 hours at the same concentration on day 0. PBMCs and EVs were incubated for 3 days, naïve CD4⁺T lymphocytes and isolated CD14⁺monocytes were incubated with EVs at 37°C and 5% CO₂ for 5 days.

To harvest the monocytes, cells were treated with 150 µl/well of chilly cold 1xDPBS containing 2mM EDTA (Serva, USA, 0.5 M, Cat#39761.02), left at +4°C for 5 min and collected by gentle pipetting. Monocytes were totally detached by repeating this step for two to three times.

As the next step of this experiment, we cocultured isolated CD14⁺monocytes (15x10⁴/well) and naïve CD4⁺T lymphocytes (75x10³/well) with patient and healthy EVs (15 µg/well) in 96 flat bottom well (Sarstedt, Germany) plate at 37°C and 5% CO₂ for 5 days. Supernatants were stored at -80°C for the measurement of various cytokines with ELISA, and cells were stained and analyzed in flow cytometry for surface markers and intracellular cytokines. Each experiment was repeated two times and obtained similar results. Flow cytometry data in experiments with EVs were plotted as “**Fold Induction**”, the ratio of CD4⁺T lymphocytes gated in healthy PBMCs stimulated by EVs to healthy CD4⁺T lymphocytes not stimulated by EVs (non-EV wells).

2.3.4. Flow Cytometry Experiments

All experiments related to flow cytometry was performed with Novocyte[®] (ACEA Biosciences) flow cytometer. Data acquired from flow cytometry was analyzed by Novoexpress software (version 1.6.1).

For the viability, cells were stained with Zombie Aqua[™] dye at a concentration of 1:500. AmCyan channel was used, and negatively stained cells with Zombie Aqua[™] were accepted live cells.

2.3.4.1. Cell Counting in Flow Cytometry

Washed samples were resuspended in 1 ml of complete RPMI-1640 medium containing 5% FBS, and 20 µL of suspension was mixed well with 5 ml of isotonic solution (Beckman Coulter, USA, Cat#25300-07). 1 ml of this suspension was transferred into 1.5 ml conical microcentrifuge tubes (Greiner Bio-One, Ireland, Cat# 616201), and 100

μL was analyzed with flow cytometer. To eliminate the cellular debris, FSC-SSC gating was used. To quantify the number of cells obtained from the analyzed patient/healthy control blood sample, the number of cells counted in 100 μL suspension was multiplied by the calculated dilution factor $((5000 \mu\text{L}/20 \mu\text{L}) \times (1000 \mu\text{L}/100 \mu\text{L}) = 2500)$.

2.3.4.2. Cell surface staining of PBMCs

For the surface stain, 15×10^4 PBMCs were resuspended in 100 μl FACS buffer (as given in Appendix A) and stained with anti-human CD45 PerCP, CD3 PE, anti-human CD4 APC, anti-human CD8 APC-Cy7, anti-human CD19 FITC, CD14 APC-Cy7, CD303 PerCP-Cy5.5 and CD123 APC antibodies for 30 min at +4°C in dark. Following the surface staining, cells were washed twice with FACS buffer at 300xg for 5 min, resuspended in 150 μl of FACS buffer and analyzed on flow cytometer. At least 30,000 cells with median 94.82% (IQR, 92.69%-96.82%) viability were analyzed for each experiment. List of antibodies used in flow cytometer was given in **Table 2.3**.

2.3.4.3. Cell surface marker expression on PBMCs, naïve CD4⁺T lymphocytes and monocytes after stimulation with plasma derived EVs

At the end of the specified incubation times for PBMCs (3 days), naïve CD4⁺T lymphocytes (5 days) and monocytes (5 days), cells were stained with 5 μl of Human Fc block per million cells in 100 μl 1xDPBS. Without any washing step, cells were stained with 50 μl/well Zombie AquaTM dye per million cells diluted 1:500 in 1xDPBS for 15 min at RT in dark and washed twice with FACS buffer at 300xg for 5 min at RT. Then, PBMCs were stained with anti-human CD4 BV570, CD8 APC-Cy7, HLA-DR PE, CD69 FITC, PD-L1 PE-Cy7, CD25 PerCP-Cy5.5, CXCR3 FITC, CCR4 APC, CCR6 PE-Cy7, CD127 PE, naïve CD4⁺T cells were stained with anti-human CD4 APC, CXCR3 FITC, CCR4 PE, CCR6 PE-Cy7, CD25 PerCP-Cy5.5 and CD127 PE, monocytes were stained with anti-human CD68 APC, CD80 PE-Cy7, CD86 Alexa Fluor[®]488, CD163 PerCP-Cy5.5, CD206 BV421, monocytes in monocyte-naïve CD4⁺T lymphocyte co-culture were stained with anti-human CD14 APC-Cy7, HLA-DR PE, CD69 FITC, CD80 PerCP-Cy5.5 and PD-L1 PE-Cy7 monoclonal antibodies

prepared in FACS buffer and left at +4°C for 30 min. After washing twice at +4°C and 300xg for 5 minutes, the cells were kept on ice in dark until analyzing in the flow cytometer. At least 30,000 cells with >90% viability were analyzed for each experiment.

2.3.4.4. Intracellular staining

To determine the intracellular profiles of IFN γ , IL4 and IL10 cytokines in the EV stimulated healthy PBMCs, isolated healthy naïve CD4⁺T lymphocytes and SARS CoV2 peptide pool treated PBMCs were stimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml) for the last 6 hours of the incubation, and after 2 hours, Brefeldin-A (Sigma Aldrich, Cat#B6542) (1 μ g/ml) was added to stop the reaction. After incubations, cells were stained with anti-human CD4 APC and anti-human CD8 APC-Cy7. Then, cells were fixed with Fixation Buffer (BD Biosciences CytoFix™, Cat#554655) for 15 min at RT and washed twice with 1xPerm/Wash buffer (BD Biosciences Perm/Wash™, Cat#51-2091KZ) (diluted in Nuclease-Free water, Thermo Fischer Scientific) at 300xg for 5 min. Permeabilization was obtained by using 100 μ l cold 1xPerm/Wash buffer for 15 min at RT. Following fixation/permeabilization step, cells were washed twice with 1xPerm/Wash buffer at 300xg for 5 min and stained with anti-human IFN γ FITC, anti-human IFN γ PE-Cy7, anti-human IL4 PE-Cy7 and anti-human IL10 PerCP-Cy5.5 antibodies, which were prepared in 1xPerm/Wash buffer at 1 μ g/ml concentration, for 1 hour at RT in the dark. Samples were washed twice with 1x Perm/Wash buffer following staining. After the final wash, cells were resuspended in 150 μ l 1x Perm/Wash buffer and were acquired on flow cytometer. A forward scatter area versus forward scatter height density plot (singlet) was used to exclude doublets. At least 30,000 or more cells in singlet gate were analyzed in each experiment.

2.3.4.5. Cell surface marker expression on PBMCs after SARS CoV-2 specific peptide pool stimulation

To analyze the surface markers on patients' and healthy donors' PBMCs after peptide pool stimulation for 48 hours, cells were centrifuged at 250xg for 5 min at RT and

washed with 1xDPBS. Then, cells were stained with 50 μ l/well Zombie Aqua™ dye per million cells diluted 1:500 in 1xDPBS for 15 min at RT in dark and were washed twice with FACS buffer at 300xg for 5 min at RT. Washed cells were stained with anti-human CD4 APC, CD8 APC-Cy7, HLA-DR PE, CD69 FITC, PD-L1 PE-Cy7, CD25 PerCP-Cy5.5, CCR7 (CD197) BV421 and CD45RO PE-Dazzle 594 monoclonal antibodies prepared in FACS buffer and left at +4°C for 30 min. After washing twice at +4°C and 300xg for 5 minutes, the cells were kept on ice in the dark until analyzing by flow cytometer. At least 100,000 cells in singlet gate with >90% viability were analyzed for this experiment. Surface markers expressed on CD4⁺ and CD8⁺T lymphocytes were calculated and analyzed as percent of PBMCs in the gate.

2.3.4.6. Cytokine bead array measurement of CD4⁺ helper T responses

CD4⁺T helper cell cytokine levels were assessed with the LEGENDplex™ HU Th Cytokine Panel (12-plex) w/ VbP V02 kit (BioLegend, Cat#741044) to quantify 12 human cytokines, including IL-2, 4, 5, 6, 9, 10, 13, 17A, 17F, 22, IFN- γ and TNF- α according to the manufacturer's instructions from the plasma of patients and healthy donors and performed as described previously (323). Briefly, thawed plasma samples were centrifuged at 2000 $\times$ g for 10 min at RT. 25 μ L of plasma samples were incubated with monoclonal capture antibody-coated microbeads for 2 h at RT on shaker with medium speed. After incubation, samples were washed twice with 200 μ l/well of wash buffer at 250xg for 5 min. After washing step, biotin-labeled detection antibodies were introduced subsequently to the tests and incubated for 1 hour at RT on shaker with medium speed. Streptavidin-PE was added to the reaction at the end of the incubation step without washing, and samples were further incubated for 30 min at RT on shaker. After staining, samples were wash once again with washing buffer at 250xg for 5 minutes. Pellets were resuspended in 150 μ l FACS buffer and analyzed in flow cytometry. The data was analyzed with LEGENDplex™ V8.0 software (BioLegend) and CD4⁺T cell-secreted cytokine levels at plasma were presented as pg/ml.

2.3.5. Enzyme Linked Immunosorbent Assay (ELISA)

2.3.5.1. Immunoglobulin ELISA

SARS CoV-2 specific RBD, S and N Immunoglobulin analyses were performed by ELISA as described previously (323). Briefly, Maxi-binding 96-well enzyme linked immunosorbent assay (ELISA) plates (SPL[®] Life Sciences, South Korea, Cat#32296) were coated with 50 µl/well in-house (made in collaboration with Mayda Gürsel's lab METU) Recombinant Spike (5 µg/ml), nucleocapsid (20 µg/ml) and recombinant RBD (3 µg/ml) in 1xDPBS at 4°C overnight. Next day, plates were blocked at RT for 2 hours with 200 µl/well blocking buffer. After blocking step, plates were washed by hand for three times with wash buffer, then two times with dH₂O. Plates were inverted and tapped on a paper towel to remove excess liquid. Meanwhile, using another 96-well plate, serially four-fold dilutions were made starting from the first row from top to bottom with patient and healthy donor plasmas diluted at 1:25 concentration with blocking buffer. 50µl diluted plasmas of individuals at the bottom row were transferred to the coated and blocked ELISA plates and incubated overnight at 4°C. After washing step, plates were incubated with 50µl/well of 1:1000 diluted ALP-conjugated anti-human IgA, anti-human IgG and anti-human IgM for 2 hours at RT. Following the last washing step, p-nitrophenyl phosphate (PNPP) substrate solution was prepared according to the manufacturer's instructions and added as 50µl/well. In certain time points, optical density (OD) values at 405nm of each well were recorded with spectrophotometer (SpectraMax[®] Molecular Devices, USA). Log10 titers were calculated by using GraphPad Prism 9 software.

2.3.5.2. Cytokine measurement at the supernatants after stimulation of cells with EVs and SARS CoV-2 specific peptide pool

Maxi-binding 96-well ELISA plates (SPL[®] Life Sciences, South Korea, Cat#32296) were coated with 50µl of coating antibodies (Mabtech, Sweden) of human IFN γ , IL6, TNF α , IL4, IL5, IL13, IL10 and IL17A which were prepared in 1xDPBS, for overnight at +4°C. Plates were blocked with 200 µl/well 5% BSA-1XPBS-Tween[®] 20 blocking buffer for 2 hours at RT. After plates were washed by hand for three times with PBS plus 0.05% Tween[®] 20 with 2 min intervals, then two times with dH₂O, they were incubated with either 50µl of supernatants or two-fold serially diluted 50µl of corresponding recombinant cytokines (Mabtech, Sweden) prepared according to

manufacturer's instructions for overnight at +4°C. Next day, incubated plates were washed as described previously, then were added 50µl of 1:500 or 1:1000 diluted (in blocking buffer) corresponding biotinylated antibodies (Mabtech, Sweden) for 2 hours at RT. Following washing step, plates were incubated with 50µl Streptavidin-ALP (Mabtech, Sweden) (1:1000 diluted in blocking buffer) for 1 hour at RT. Following washing steps, p-nitrophenyl phosphate (PNPP) substrate solution (Thermo Scientific, USA) was added 50µl/well. Optical density (OD) values at 405nm of each well were recorded with spectrophotometer (SpectraMax® Molecular Devices, USA).

2.3.6. Statistical analyses

Normality tests for continuous variables were performed and if all the continuous variables were distributed non-normally, the results were given as median and inter-quartile range (IQR). Categorical variables were presented as frequencies and percentages (%). Two-tailed Mann-Whitney U and Kruskal-Wallis following Dunn's multiple comparison tests were used, as appropriate, to compare the data from two groups and more than two groups, respectively. One-way Anova test with Dunnet's multiple comparisons was used to compare the means of the groups. Spearman's correlation test was applied for calculation of the correlation coefficients between different variables. A test with p value of less than 0.05 was considered statistically significant. All statistical analyzes were performed by GraphPad Prism 9 (GraphPad Software Company) and SPSS version 25.0 statistical software package (IBM SPSS Statistics, 2017, USA).

3. CHAPTER 3

RESULTS

3.1. Clinical characteristics of the patients

A total of 104 patients (M/F:53/51) and 13 age ($p=0.579$) and gender ($p=0.721$) matched healthy controls (M/F:6/7) were included in the study, and the demographics and clinical features are summarized in **Table 3.1**. The median age of the patients was 11.5 years (IQR, 7.5-14.3 years). Eighty-one children had the history of allergic diseases, including asthma (74%) and/or allergic rhinitis (15.5%). Of these patients, 54 (51.9%) were under the regular treatment of inhaler corticosteroid, 5 (4.8%) were given LTRA and 23 (22.1%) were taking short acting β -agonist when required.

Table 3.1. Demographics and clinical features of the study population according to the presence of allergic disorders

	Whole study population (n=104)	Patients without any accompanying diseases (n=24)	Patients with allergic disorders (n=80)	p value
Gender (n; M/F) Φ	53/51	6/18	47/33	0.004
Age (year) $\#$	11.5 (7.5-14.3)	12.0 (9.6-14.7)	11.0 (7.1-14.4)	0.605
Covid-19 disease severity Φ	24 (23.1%)	15 (14.4%)	9 (8.6%)	<0.001
Severe	80 (76.9%)	9 (8.6%)	71 (68.2%)	0.827
Mild				
Source of infection Φ	85 (81.7%)	19 (18.3%)	66 (63.8%)	0.712
Family	19 (18.3%)	5 (4.8%)	14 (13.5%)	0.764
Undefined				
Hospitalization Φ	24 (23.1%)	15 (14.4%)	9 (8.6%)	<0.001
Duration of hospitalization (days) $\#$	7.0 (5.0-8.0)	7.0 (5.0-8.0)	7.0 (5.0-8.5)	0.926

Fever ^Φ	85 (81.7%)	17 (16.3%)	68 (65.4%)	0.117
Duration of fever [#]	2.0 (2.0-4.0)	4.0 (2.5-5.5)	2.0 (1.0-3.0)	0.002
Cough ^Φ	62 (59.6%)	18 (17.3%)	44 (42.3%)	0.089
Sore throat ^Φ	25 (24%)	2 (1.9%)	23 (22.1%)	0.041
Rhinorrhea ^Φ	14 (13.5%)	3 (2.8%)	11 (10.6%)	0.876
Shortness of breath ^Φ	31 (29.8%)	13 (12.4%)	18 (17.3%)	0.003
Malaise ^Φ	58 (55.7%)	15 (14.3%)	43 (41.3%)	0.451
Myalgia ^Φ	34 (32.7%)	7 (6.7%)	27 (25.9%)	0.676
Loss of taste and smell ^Φ	6 (5.7%)	4 (3.8%)	2 (1.9%)	0.009
Abdominal pain ^Φ	26 (25%)	6 (5.7%)	20 (19.2%)	0.976
Vomiting ^Φ	18 (17.3%)	6 (5.7%)	12 (11.5%)	0.258
Diarrhea ^Φ	10 (9.6%)	4 (3.8%)	6 (5.7%)	0.184
Exacerbation of asthma ^Φ	15 (14.4%)	0	15 (14.4%)	<0.001
Pneumonia ^Φ	24 (23.1%)	16 (15.4%)	8 (7.7%)	<0.001
Antipyretic ^Φ	91 (87.5%)	21 (20.2%)	70 (67.3%)	0.982
Antibiotic ^Φ	33 (31.7%)	17 (16.3%)	16 (15.3%)	<0.001
Antifungal ^Φ	2 (1.9%)	1 (0.9%)	1 (0.9%)	0.369
Favipiravir ^Φ	20 (19.2%)	12 (11.5%)	8 (7.7%)	<0.001
Systemic glucocorticoid ^Φ	25 (24%)	15 (14.3%)	10 (9.6%)	<0.001
Nebulized corticosteroid ^Φ	3 (2.8%)	1 (0.9%)	2 (1.9%)	0.678
Short acting β2-agonist ^Φ	31 (30.5%)	3 (2.8%)	28 (26.9%)	0.033
Mechanical ventilation ^Φ	0	0	0	-
Non-invasive ventilation ^Φ	9 (8.6%)	3 (2.8%)	6 (5.7%)	0.447
Supplemental oxygen ^Φ	24 (23.1%)	18 (17.3%)	6 (5.7%)	0.005

[#]; Median with Interquartile Range (IQR), ^Φ; number/percentage (n/%)

Children who were not vaccinated against COVID-19 were enrolled to the study. Of the patients, 88.5% (n=92) had positive PCR test. The rest of the patients (11.5%, n=12) were not tested with PCR for SARS CoV-2. One of the family members of these pediatric patients was symptomatic and positive for SARS CoV-2 PCR. The patient's symptoms began within few days following the family member's symptoms. Therefore, those patients were accepted as infected with SARS CoV-2 virus and in the group of convalescent patients who had COVID-19 infection more than one month after the symptoms.

Of the patients, 23.1% had severe, and the rest of the study population had mild COVID-19 infection. The blood samples were collected from severe COVID-19 patients in a median of 4.0 days (IQR, 3.0-5.0 days) in the acute phase (n=22), and in 2 days (IQR, 2.0-3.0 days) from the patients with acute mild COVID-19 infection (n=25) (p=0.001). This period was median 28.5 days (IQR, 28.0-30.0 days) and 25.0 days (IQR, 21.5-28.5 days) in patients with severe (n=16) and mild (n=18) COVID-19 infection in very early convalescent period, respectively (p=0.019). Convalescent patients at least one month after COVID-19 diagnosis had the infection with a median time of 5.25 months (IQR, 3.0-7.5 months). All these patients in the latter group had mild COVID-19 disease.

Common symptoms in pediatric COVID-19 patients were fever (81.7%, n=85), cough (59.6%), malaise (55.8%), myalgia (32.7%), shortness of breath (29.8%), abdominal pain (25%), sore throat (24%), vomiting (17.3%), rhinorrhea (13.5%), diarrhea (9.6%), and loss of taste and smell (5.7%) respectively. The median duration of the fever was 2 days (IQR, 2-4 days) in the whole study population, whereas this was median 5 days and 2 days in severe and mild groups, respectively (p<0.001). Of the patients with asthma (n=77), 19.5% had asthma exacerbation during COVID-19 infection. Twenty-four severe patients (23.1%) were hospitalized for a median of 7 days (IQR, 5.0-8.0 days), and all these patients recovered.

The most common treatment that were given to patients with COVID-19 infection was antipyretics (n=91, 87.5%). Other treatments were antibiotics such as sulbactam ampicillin, ceftriaxone, amikacin, vancomycin, teicoplanin, clarithromycin, amoxicillin-clavulanate (31.7%), short acting β 2-agonist (29.8%), systemic glucocorticoids (24%), systemic antiviral agent (favipiravir) (19.2%), nebulized

corticosteroid treatment (2.9%) and antifungal treatment (1.9%). All the treatments except antipyretics and short acting β 2-agonist were given significantly to the patients with severe disease (**Table 3.2**). None of the patients were treated with mechanical ventilation. Supplemental oxygen (23.1%) and non-invasive ventilation treatment (8.7%) were also applied to severe patients.

Table 3.2. Demographics and clinical features of the patients with acute COVID-19 disease according to the presence of allergic disorders vs the severity of COVID-19 infection

	Patients with acute COVID-19 (n=47)	Patients without any accompanying diseases (n=23)	Patients with allergic disorders (n=24)	p value	Mild acute COVID-19 (n=25)	Severe acute COVID-19 (n=22)	p value
Gender (n; M/F) ^Φ	20/27	6/17	14/10	0.027	9/16	11/11	0.338
Age (year) [#]	11.5 (7.5-14.3)	12.0 (9.5-14.8)	11.5 (6.0-15.2)	0.873	11.0 (7.0-14.2)	12.2 (6.8-15.3)	0.693
COVID-19 disease severity ^Φ				0.061			-
Severe	22	14 (29.8%)	8 (17%)		0	22	
Mild	(46.8%) 25 (53.2%)	9 (19.1%)	16 (34%)		25 (53.2%)	(46.8%) 0	
Source of infection ^Φ				0.940			0.822
Family	37	18 (38.3%)	19 (40.4%)		20	17	
Undefined	(78.7%) 10 (21.3%)	5 (10.6%)	5 (10.6%)		(42.6%) 5	(36.2%) 5	
Hospitalization ^Φ	22	14 (29.8%)	8 (17%)	0.061	0	22	<0.001
	(46.8%)					(46.8%)	
Duration of hospitalization (days) [#]	7.0 (5.0-8.0)	7.0 (5.0-8.0)	6.5 (5.0-7.8)	0.794	-	7.0 (5.0-8.0)	-
Fever ^Φ	37	16 (34%)	21 (44.6%)	0.137	18	19	0.235
	(78.7%)				(38.3%)	(40.4%)	
Duration of fever [#]	2.0 (2.0-4.0)	4.0 (2.3-5.8)	3.0 (2.0-5.0)	0.446	2.0 (1.7-3.0)	5.0 (4.0-6.0)	<0.001
Cough ^Φ	34	17 (36.2%)	17 (36.2%)	0.815	17	17	0.483
	(72.3%)				(36.2%)	(36.2%)	
Sore throat ^Φ	7 (14.9%)	2 (4.3%)	5 (10.6%)	0.248	5 (10.6%)	2 (4.3%)	0.300
Rhinorrhea ^Φ	5 (10.6%)	3 (6.4%)	2 (4.3%)	0.605	2 (4.3%)	3 (6.4%)	0.536

Shortness of breath ^Φ	23 (48.9%)	12 (25.5%)	11 (23.4%)	0.667	7 (14.9%)	16 (34%)	0.002
Malaise ^Φ	27 (57.4%)	14 (29.8%)	13 (27.6%)	0.646	10 (21.3%)	17 (36.2%)	0.011
Myalgia ^Φ	14 (29.8%)	7 (14.9%)	7 (14.9%)	0.925	6 (12.7%)	8 (17%)	0.360
Loss of taste and smell ^Φ	5 (10.6%)	4 (8.5%)	1 (2.1%)	0.146	3 (6.4%)	2 (4.3%)	0.749
Abdominal pain ^Φ	13 (27.6%)	5 (10.6%)	8 (12.5%)	0.925	3 (6.4%)	10	0.011
Vomiting ^Φ	13 (27.6%)	5 (10.6%)	8 (12.5%)	0.380	4 (8.5%)	9 (19.1%)	0.059
Diarrhea ^Φ	7 (14.9%)	4 (8.5%)	3 (6.4%)	0.641	1 (2.1%)	6 (12.7%)	0.027
Exacerbation of asthma ^Φ	9 (19.1%)	0	9 (19.1%)	<0.001	4 (8.5%)	5 (10.6%)	0.006
Pneumonia ^Φ	22 (46.8%)	14 (29.8%)	8 (17%)	0.317	0	22 (46.8%)	<0.001
Antipyretic ^Φ	43 (91.5%)	20 (42.5%)	23 (48.9%)	0.281	23 (48.9%)	20 (42.5%)	0.895
Antibiotic ^Φ	27 (57.4%)	16 (34%)	11 (23.4%)	0.104	5 (10.6%)	22 (46.8%)	<0.001
Antifungal ^Φ	0	0	0	-	0	0	-
Favipiravir ^Φ	18 (38.3%)	11 (23.4%)	7 (14.9%)	0.193	0	18 (38.3%)	<0.001
Systemic glucocorticoid ^Φ	22 (46.8%)	14 (29.8%)	8 (17%)	0.061	0	22 (46.8%)	<0.001
Nebulized corticosteroid ^Φ	2 (4.3%)	1 (2.1%)	1 (2.1%)	0.976	0	2 (4.3%)	0.127
Short acting β2-agonist ^Φ	18 (38.3%)	2 (4.3%)	16 (34%)	<0.001	11 (23.4%)	7 (14.9%)	0.396
Mechanical ventilation ^Φ	0	0	0	-	0	0	-
Non-invasive ventilation ^Φ	8 (17%)	2 (4.3%)	6 (12.7%)	0.141	0	8 (17%)	0.001
Supplemental oxygen ^Φ	22 (46.8%)	16 (34%)	6 (12.7%)	0.933	0	22 (46.8%)	<0.001

#, Median with Interquartile Range (IQR), ^Φ; number/percentage (n/%)

When we grouped the whole study population including convalescent patients into those with (n=80) and without allergic diseases (n=24), the children without any disease had significantly more severe COVID-19 infection (p<0.001). Since there were more severely ill patients in this group, hospitalization (p<0.001), the duration of fever (p=0.002), different treatment modalities (p<0.001), shortness of breath (p=0.003) and

covid pneumonia ($p<0.001$) were apparently higher. Only short acting β_2 -agonist treatment application was higher in allergic group ($p=0.033$) (**Table 3.1**). However, when we compare only the patients in the acute phase ($n=47$), it is seen that the statistical differences are only related to the severity of the disease, not whether they have an allergic disease or not (**Table 3.2**).

3.2. Laboratory findings of the study population

The laboratory findings of the patients were given in **Table 3.3**. In the comparison of the parameters in acute phase, no statistical difference was found between groups except for increased eosinophil count in patients with asthma. However, parameters differed according to the severity of COVID-19 rather than the presence of asthma. In severe acute patients, ESR ($p=0.017$), CRP ($p=0.008$), LDH ($p=0.003$) and AST ($p=0.028$) on admission were elevated, absolute monocyte ($p=0.020$) and lymphocyte counts ($p=0.017$) were decreased compared to mild group (**Table 3.3**).

Table 3.3. Laboratory findings of the patients infected with SARS CoV-2 virus on admission to hospital based on the presence of allergic disorder vs the severity of COVID-19 infection

	Patients with acute COVID-19 (n=47)	Patients without any accompanying diseases (n=23)	Patients with allergic disorders (n=24)	p value	Mild acute COVID-19 (n=25)	Severe acute COVID-19 (n=22)	p value
Duration of the symptoms before admission (days) [#]	3.0 (2.0-4.0)	3.0 (2.5-4.0)	3.0 (2.0-4.0)	0.202	2.0 (2.0-3.0)	4.0 (3.0-5.0)	<0.001
Duration of fever [#]	4.0 (2.0-5.0)	4.0 (2.7-5.2)	3.0 (2.0-5.0)	0.280	2.0 (2.0-3.0)	5.0 (4.0-6.0)	<0.001
Hb (mg/dl) [#]	12.4 (11.8-13.8)	12.3 (11.9-13.2)	12.6 (10.9-13.8)	0.853	12.2 (10.9-14.4)	12.4 (11.9-13.8)	0.998
WBC count ($\times 10^9/L$) [#]	7.5 (5.0-11.9)	7.4 (5.7-14.20)	6.85 (4.77-10.28)	0.411	5.65 (5.07-10.42)	7.8 (4.8-12.1)	0.525

ALC (x10 ⁹ /L) #	1.2 (0.95-1.85)	1.2 (0.56-1.48)	1.25 (0.98-2.1)	0.284	2.00 (1.57-2.9)	1.2 (0.72-1.3)	0.017
ANC (x10 ⁹ /L) #	5.8 (2.9-10.4)	6.2 (4.64-13.28)	5.65 (2.67-8.0)	0.325	5.25 (2.95-8.0)	5.8 (2.9-12.02)	0.685
Eosinophil count (x10 ⁹ /L) #	0.30 (0.20-0.40)	0.20 (0.20-0.25)	0.30 (0.25-0.35)	0.002	0.30 (0.24-0.35)	0.25 (0.20-0.30)	0.089
Monocyte count (x10 ⁹ /L) #	0.50 (0.43-0.55)	0.50 (0.40-0.55)	0.50 (0.44-0.55)	0.803	0.55 (0.50-0.60)	0.46 (0.40-0.52)	0.020
Platelet count (x10 ⁹ /L) #	167.0 (116.0-232.0)	167.0 (109.0-232.0)	177.0 (116.5-227.3)	0.975	221.5 (194.2-237.5)	165.0 (112.0-216.0)	0.114
ESR (mm/h) #	27.5 (12.25-43.25)	27.5 (12.75-39.75)	30.5 (14.25-46.25)	0.509	12.25 (8.6-14.75)	12.75 (7.95-14.99)	0.017
CRP (mg/dl) #	26.9 (15.6-38.0)	27.79 (17.6-36.3)	24.27 (13.79-39.65)	0.931	12.75 (7.95-14.99)	30.25 (20.55-39.65)	0.008
ALT (U/L) #	40.0 (22.0-49.5)	43.0 (25.5-48.5)	29.5 (21.5-54.0)	0.644	23.0 (21.5-29.75)	43.0 (22.5-53.5)	0.128
AST (U/L) #	32.0 (24.5-50.2)	35.0 (27.5-50.2)	27.5 (54.0-28.5)	0.374	24.5 (21.75-25.0)	33.0 (27.5-55.5)	0.028
LDH (U/L) #	342 (226-455)	354 (314-483)	284 (175-415)	0.145	166 (148.5-198.5)	357 (314-480)	0.003

Hb hemoglobin, *WBC* white blood cell, *ALC* absolute lymphocyte count, *ANC* absolute neutrophil count, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *LDH* lactate dehydrogenase, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, #Median with Interquartile Range (IQR). Two-sided Mann-Whitney U test was used to determine the significance of differences between groups.

3.3. SARS CoV-2 specific Immunoglobulins

ELISA was performed to assess specific immunoglobulin (Ig) responses against RBD (receptor binding domain), N (nucleocapsid) and S (spike) proteins of SARS CoV-2 in the plasma of patients and HCs. There was not any difference between patients with or without asthma regarding anti-RBD, anti-S and anti-N Ig levels (**Table 3.4**).

Table 3.4. Comparison of the cytokine, Ig levels and cellular percentages between children with and without asthma at acute and very early convalescence (between 2nd and 4th weeks of the infection) phase pf COVID-19

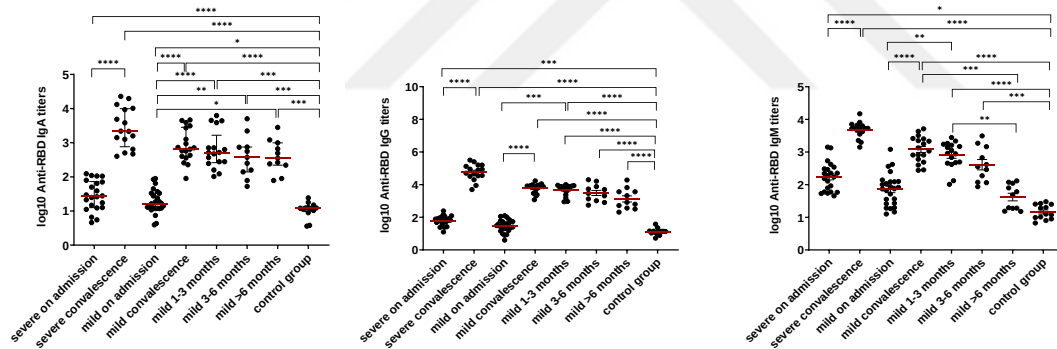
	Patients at acute and very early convalescence phase of COVID-19 (n=65)	Patients with asthma (n=33)	Patients without asthma (n=32)	p value
Protein amount of EVs (µg/mL) [#]	78.0 (68.5-121.5)	72.0 (59.0-105.0)	84.0 (72.3-114.3)	0.443
IFN γ (pg/mL) [#]	98.4 (52.6-155.8)	103.2 (50.3-153.4)	98.5 (58.7-157.1)	0.694
IL2 (pg/mL) [#]	7.4 (5.0-10.4)	7.0 (4.2-10.9)	7.5 (5.9-10.4)	0.839
TNF α (pg/mL) [#]	126.8 (43.1-196.8)	126.8 (40.9-171.6)	130.4 (41.7-246.8)	0.512
IL4 (pg/mL) [#]	24.4 (10.3-36.7)	23.4 (9.3-49.0)	26.7 (10.5-33.7)	0.783
IL5 (pg/mL) [#]	14.4 (7.1-27.9)	13.8 (6.7-26.1)	17.5 (6.9-32.6)	0.503
IL13 (pg/mL) [#]	47.2 (23.1-75.6)	59.2 (17.6-77.4)	41.3 (28.0-65.5)	0.573
IL6 (pg/mL) [#]	103.4 (30.4-198.6)	111.4 (23.7-182.3)	100.1 (37.2-230.0)	0.699
IL10 (pg/mL) [#]	11.6 (4.2-22.6)	7.0 (2.7-24.0)	13.2 (5.9-20.9)	0.325
IL9 (pg/mL) [#]	23.0 (13.5-31.9)	21.4 (12.5-31.3)	24.4 (13.9-34.1)	0.466
IL17A (pg/mL) [#]	6.0 (3.4-10.6)	5.4 (3.0-10.6)	7.4 (3.5-15.4)	0.243
IL17F (pg/mL) [#]	27.8 (16.6-48.0)	27.6 (16.0-41.9)	28.2 (16.8-62.1)	0.443
IL22 (pg/mL) [#]	22.0 (12.3-32.2)	22.0 (11.5-30.0)	22.1 (12.0-43.7)	0.665
panCD3 (%) ^Φ	70.6 (66.7-76.1)	70.3 (64.8-75.4)	71.4 (64.8-75.4)	0.246
CD4 (%) ^Φ	56.3 (49.7-62.0)	56.5 (49.4-70.0)	55.4 (50.8-62.1)	0.927
CD8 (%) ^Φ	32.8 (28.0-39.5)	33.2 (28.2-40.0)	32.6 (27.8-38.0)	0.600
B cells (%) ^Φ	10.9 (8.3-15.3)	11.6 (7.8-17.7)	10.6 (9.6-14.5)	0.948
Monocytes (%) ^Φ	4.6 (2.0-8.0)	4.9 (2.2-9.4)	3.2 (1.4-6.6)	0.307
pDC (%) ^Φ	0.1 (0.05-0.3)	0.2 (0.1-0.3)	0.1 (0.05-0.3)	0.287
Th1 (%) ^Φ	10.7 (6.8-13.8)	10.2 (7.2-12.6)	10.8 (5.8-15.9)	0.767
Th2 (%) ^Φ	5.1 (3.5-8.3)	5.3 (4.3-7.7)	4.5 (2.9-9.8)	0.749
Th17 (%) ^Φ	6.6 (3.3-11.8)	6.6 (3.8-9.5)	7.6 (3.2-13.6)	0.396
Treg (%) ^Φ	1.4 (0.9-1.8)	1.4 (0.9-1.7)	1.4 (0.9-1.8)	0.888
Anti-RBD IgA (log10 titer) ^Φ	2.2 (1.5-2.9)	2.4 (1.2-2.9)	2.2 (1.5-2.8)	0.708
Anti-RBD IgG (log10 titer) ^Φ	3.3 (1.8-3.8)	2.3 (1.8-3.9)	3.4 (2.1-3.9)	0.768
Anti-RBD IgM (log10 titer) ^Φ	2.4 (1.7-3.1)	2.1 (1.6-3.6)	2.5 (2.0-2.9)	0.969
Anti-N IgA (log10 titer) ^Φ	1.7 (1.4-2.4)	1.6 (1.2-2.7)	1.8 (1.6-2.0)	0.376
Anti-N IgG (log10 titer) ^Φ	2.4 (1.8-3.2)	2.5 (1.6-3.3)	2.4 (1.9-2.9)	0.773
Anti-N IgM (log10 titer) ^Φ	2.0 (1.6-2.3)	2.1 (1.6-2.4)	1.9 (1.6-2.2)	0.520
Anti-S IgA (log10 titer) ^Φ	1.6 (1.2-2.3)	1.6 (1.2-2.5)	1.6 (0.9-2.4)	0.984
Anti-S IgG (log10 titer) ^Φ	2.9 (1.7-3.7)	2.8 (1.6-3.5)	3.2 (1.7-3.9)	0.401
Anti-S IgM (log10 titer) ^Φ	2.2 (1.3-2.7)	2.0 (1.4-2.7)	2.3 (1.2-2.8)	0.854

[#]; Median with Interquartile Range (IQR), ^Φ; number/percentage (n/%)

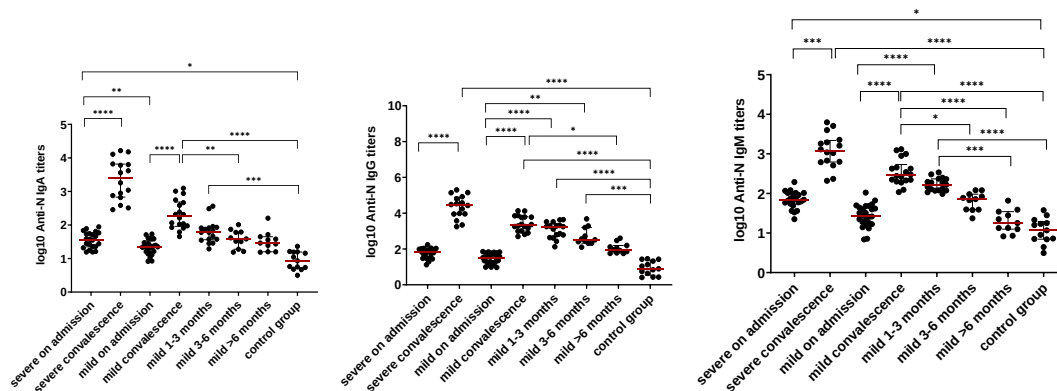
Thereupon, patients were analyzed according to the severity of COVID-19 rather than the presence of asthma (**Figure 3.1a, Figure 3.1b, Figure 3.1c**). All Ig responses were abruptly elevated in acute period, and this was prominent in severe cases. Since we had

early, middle, and late convalescence plasma samples only of mild patients, Ig trends for 12 months could only be observed in mild patients. After increasing in the first 3 months of the infection, IgG titers decreased slowly until 12th month. However, Anti-S IgG levels stayed at a relatively constant level during the first 6 months. IgM rose during the very early convalescent period (between 2nd and 4th week of infection), then declined rapidly throughout the first year of the infection. On the other hand, Anti-S IgA and Anti-RBD IgA showed a similar trend and declined slowly compared to Anti-N IgA (**Figure 3.1d**). Due to the possible relationship between plasma IgA levels and mucosal involvement, we further classified the patients according to the presence of gastrointestinal (GI) symptoms (nausea, vomiting, abdominal pain, diarrhea). Anti-RBD ($p=0.037$) and Anti-N IgA ($p=0.019$) titers were found to be significantly higher in those with GI system manifestations. Unlike IgA, IgG values were not associated with the presence of GI symptoms (**Figure 3.1e**).

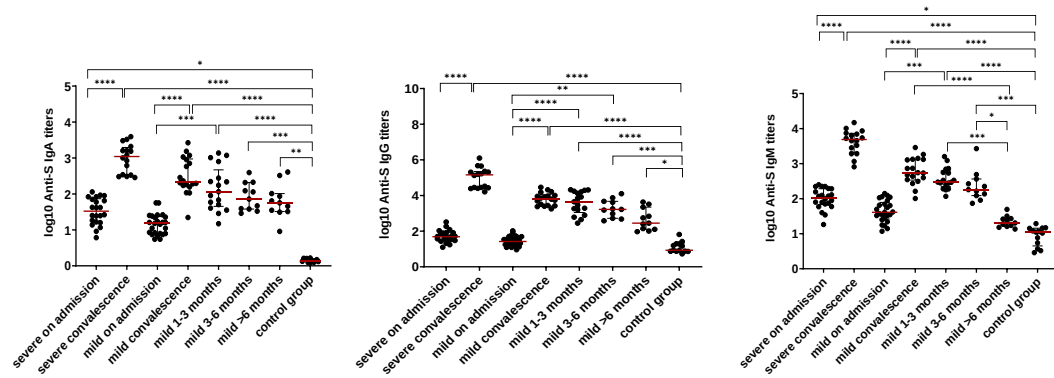
(a)



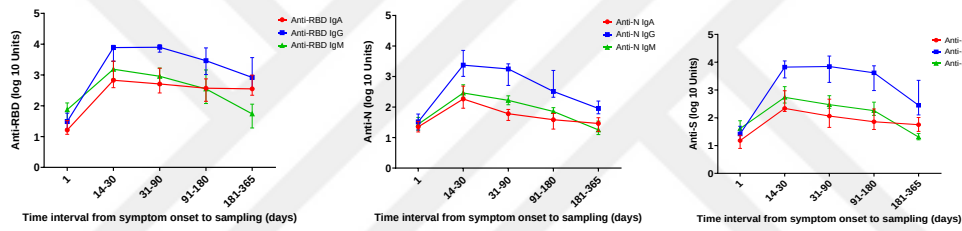
(b)



(c)



(d)



(e)

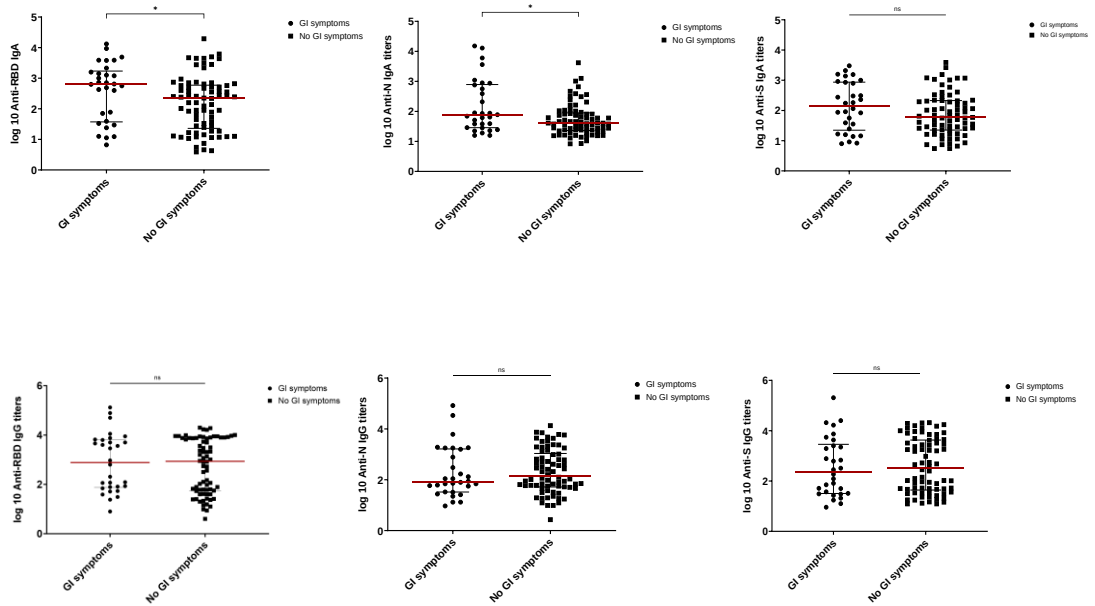
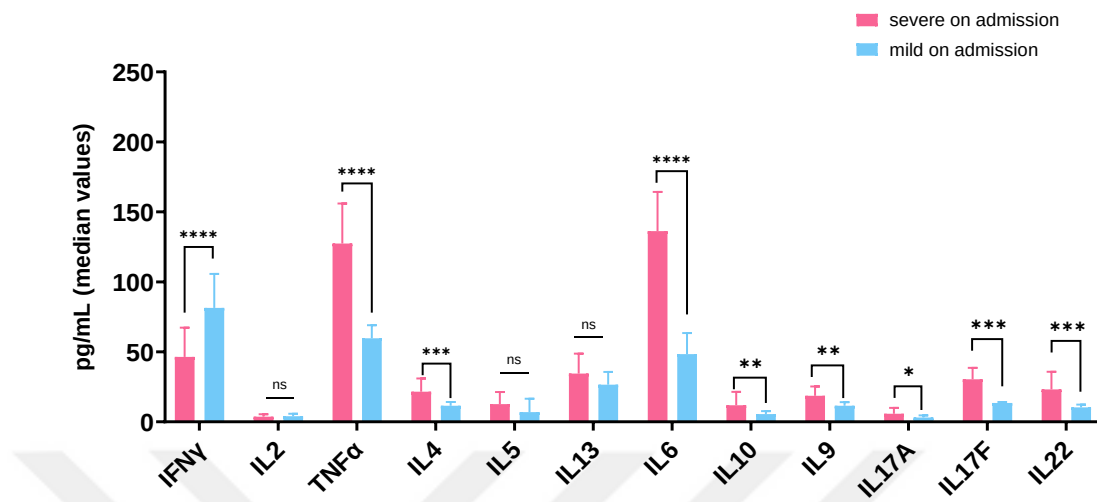


Figure 3.1. SARS CoV-2 specific anti-RBD, anti-S and anti-N IgA, G, M responses in patients with severe COVID-19 disease. Responses on admission (n=22), at convalescence period (n=16) and mild COVID-19 disease on admission (n=23), at convalescence period (n=18), at >1 to ≤3 months (n=17), at >3 to ≤6 months (n=11) and >6 to ≤12 months (n=11) after infection in mild patients and healthy controls (n=13) were measured by ELISA. Plasmas were assessed for **(a)** SARS CoV-2 RBD-specific IgA, IgG, and IgM, **(b)** SARS CoV-2 N-specific IgA, IgG, and IgM, **(c)** SARS CoV-2 S-specific IgA, IgG, and IgM, **(d)** longitudinal titers of IgA (red), IgG (blue) and IgM (green) recognizing different SARS-CoV-2 proteins during time course following the onset of symptoms up to one year in mild patients. In summary, humoral immune responses to SARS-CoV-2 declined over time in mild convalescent patients. IgM antibody titers declined rapidly, while IgG titers showed a slower rate of decline. Anti-RBD IgA titers plateaued after the 3rd month, whereas Anti-S IgA and Anti-N IgA antibodies started to decrease between 1st and 3rd months of the infection, then showed a relatively stable course. Each dot represents one individual. Graphs show median levels (red horizontal lines) with interquartile ranges. One-way Annova with Dunnet's multiple comparisons test was used to determine the significance of differences between groups (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$), **(e)** longitudinal titers of IgA and IgG against RBD, S and N protein domains of SARS CoV-2 based on the presence of the gastrointestinal (GI) symptoms (nausea, vomiting, abdominal pain, diarrhea) (patients with GI symptoms n=30 vs without GI symptoms n=74) regardless of disease severity.

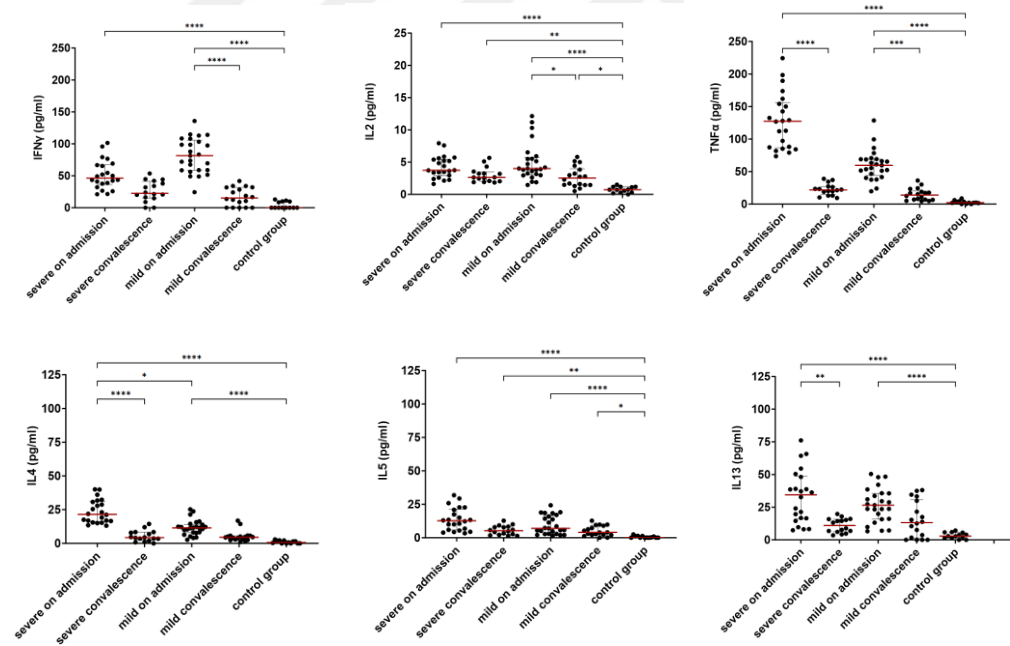
3.4. Cytokine responses to SARS CoV-2 infection

Cytokine levels were assessed by bead-assisted multiplex cytokine profiling in flow cytometry. Our analysis revealed that all Th cytokines, except for IFN γ and IL2, were higher in severe patients compared to mild acute ones (**Figure 3.2a**). Almost all cytokines had significantly declined but remained at relatively high levels compared to HCs at the convalescence period (**Figure 3.2b**). In addition, higher levels of Th2 cytokines and IL-6 were observed in severe cases compared to mild ones (**Figure 3.2c**). Detailed analysis of cytokines showed that TNF α , Th2 related cytokines, IL10, Th9- and Th17-related cytokines were higher in severe acute patients; conversely, IFN γ levels were found to be lower compared to mild acute ones (**Figure 3.2b**). Overall, the decrease in IFN γ , increase in IL6, TNF α , Th2, Treg and Th17 related cytokines in severe cases were similar to previous studies (232,324). However, cytokine patterns were totally opposite in mild acute patients (**Figure 3.2c**).

(a)



(b)



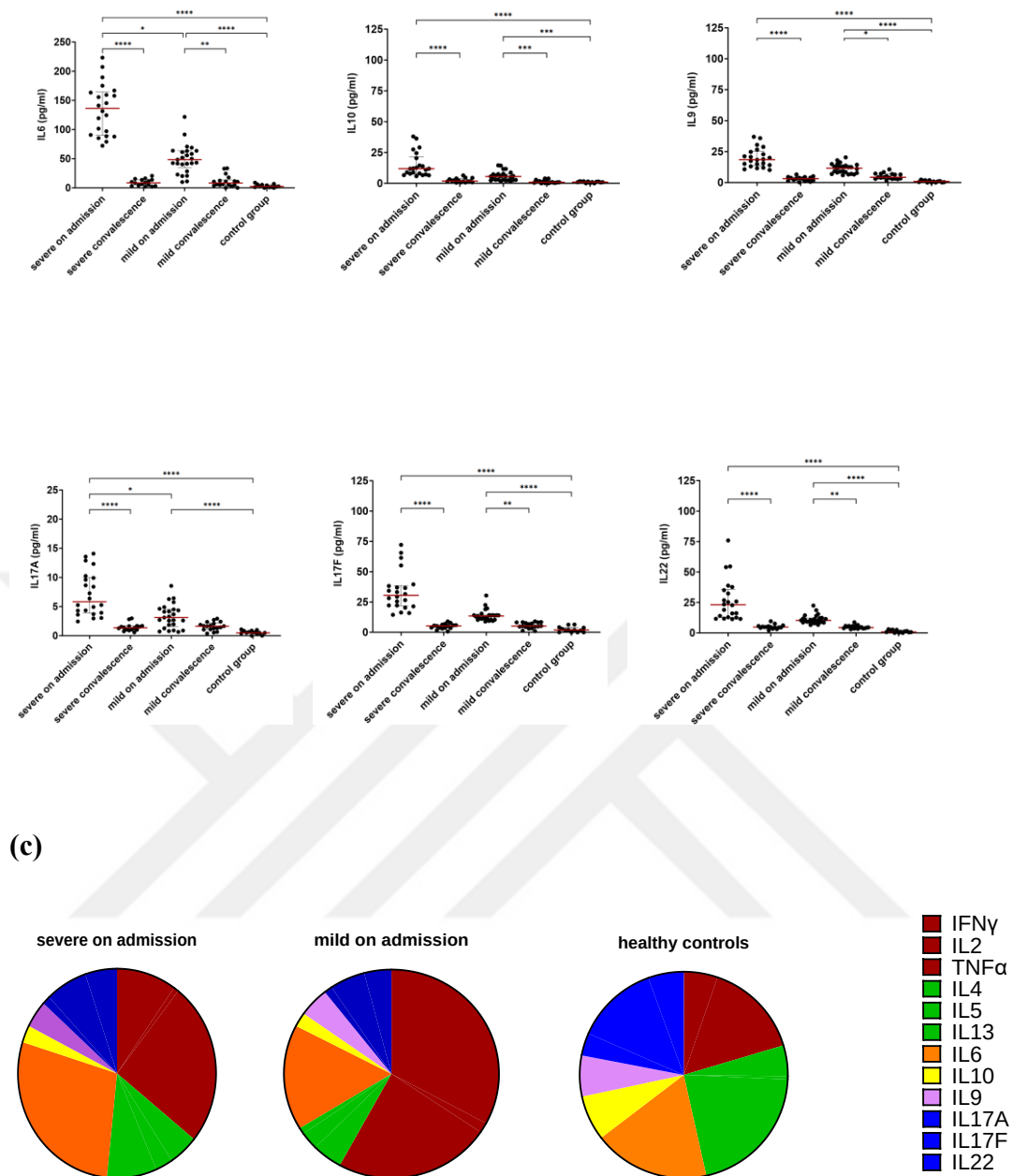


Figure 3.2. T helper (Th) cell plasma cytokine levels were assessed by using LEGENDplex™ HU Th Cytokine Panel (12-plex). (a) Severe (n=22, pink columns) and mild (n=25, blue columns) COVID-19 patients on admission were analyzed. Most of the cytokines were increased in severe patients rather than mild ones, except for IFN γ . Groups were compared in pairs by Mann-Whitney U test. Data are presented as median with interquartile ranges, (b) severe COVID-19 patients on admission (n=22) and at convalescence period (n=16), mild COVID-19 patients on admission (n=25) and at convalescence period (n=18) and HCs (n=13) were depicted. All cytokines, except for IL2 and IFN γ were higher during acute phase in severe cases compared to mild patients. During the convalescence phase, cytokine levels declined, but

did not reach the level of HCs, (c) pie charts representing the proportions of Th cytokines are presented for severe acute and mild acute patients and HCs. Each dot represents one individual. Graphs show median levels (red horizontal lines) with interquartile ranges. The Kruskal-Wallis test with Dunn's multiple comparisons was used to determine the significance of differences between groups. (p values <0.05 were shown, $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$), HCs; Healthy Controls.

3.5. SARS CoV-2 specific T lymphocyte responses of the patients with severe and mild disease during acute and convalescence period

We next investigated the expression of various markers on the patient lymphocytes with SARS CoV-2 infection and lymphocytes of healthy controls upon the stimulation with SMNO peptide pool. Following 48 h stimulation, a significant increase both in CD69⁺, CD25⁺ and PDL1⁺ CD4⁺T cells, and non-significant increase in HLADR marker on CD4⁺T cells of patients after SMNO stimulation were seen. This elevation was prominent in severe acute patients compared to mild acute cases (**Figure 3.3a**).

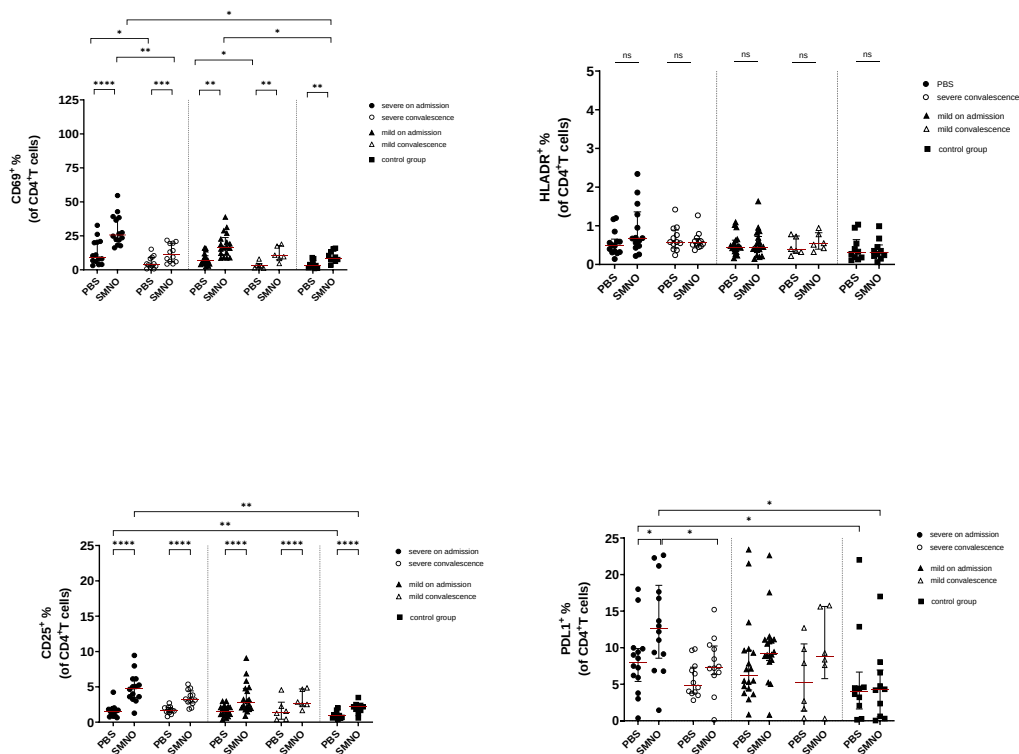
We observed similar trend of CD69⁺, CD25⁺ and PDL1 on CD8⁺T cells like those seen on CD4⁺T cells (**Figure 3.3b**). However, the percentages of CD25 and PDL1 positivity on CD8⁺T cells were lower than those in CD4⁺T cells. Elevation in almost all markers was observed in patient groups after SMNO peptide stimulation compared to both baseline and control groups. Interestingly, we observed elevations in activation and inhibitory markers even in healthy control groups upon the peptide pool stimulation. This may be due to cross reaction among the other coronaviruses (325).

Then we analyzed the differences regarding CD4⁺T cell naïve and memory compartments between groups upon the SMNO peptide pool stimulation (**Figure 3.3c**). Naïve CD4⁺T cells were at the low levels in disease groups compared to healthy subjects, and upon SMNO stimulation, insignificant declines were observed in those groups. The percentage of central and effector memory, also CD4⁺TEMRA cells were higher in all disease groups than those in healthy controls. After stimulation of cells with SMNO peptide pool, a slightly non-significant increase was observed in disease groups (**Figure 3.3c**). Gating strategy was given in **Appendix B (Appendix Figure 1, Figure 2 and Figure 3)**.

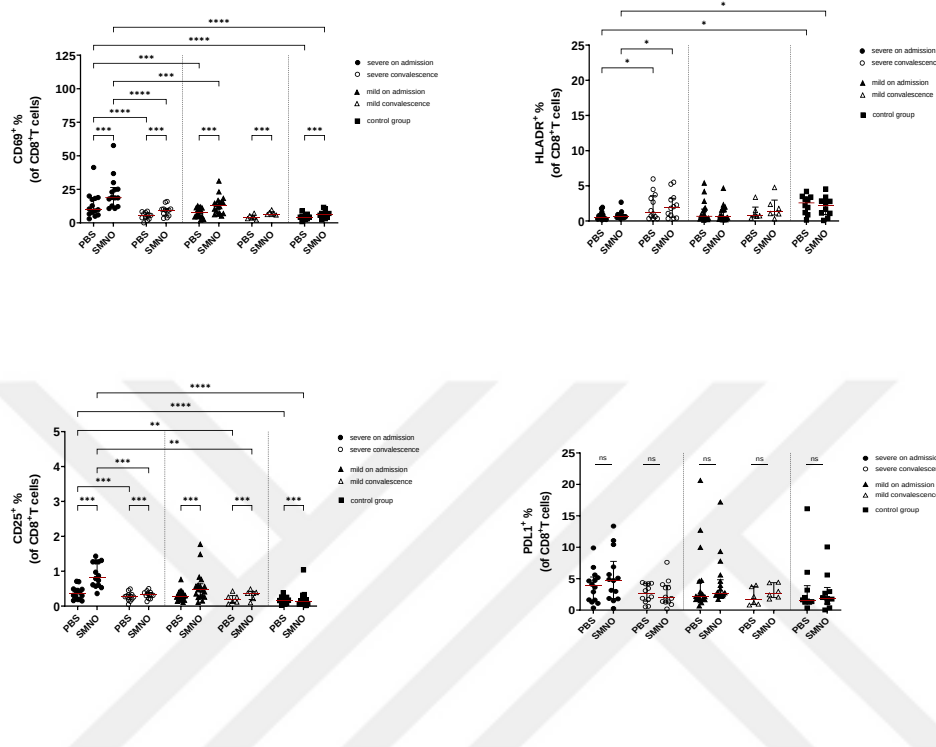
After 48 hours of SMNO peptide pool stimulation, cytokine levels were checked in the supernatants by ELISA method. Th2 (IL13), Treg (IL10), and Th17 (IL17A)-related cytokines were measured elevated in the supernatants of SARS CoV-2 patients upon peptide pool stimulation (**Figure 3.3d**). This increase was more pronounced in severe patients. Although patient PBMCs also produced increased IFN γ levels following the SMNO peptide pool stimulation, this increase was more prominent in those with mild COVID-19 disease. IFN γ secretion was also measured by intracellular staining after peptide pool stimulation. After stimulation with SMNO peptide pool, the IFN γ response was more pronounced in CD4 $^{+}$ T cells than in CD8 $^{+}$ T cells. Interestingly, the IFN γ response was higher in mild patients than in severe patients (**Figure 3.3e**). Gating strategy was given in **Appendix B (Appendix Figure 4)**.

To define any relationship between cytokines and surface markers after peptide pool stimulation, correlation analysis was performed. Moderate positive correlation between CD25 $^{+}$ and PDL1 $^{+}$ CD4 $^{+}$ T cells ($p=0.039$, $r=0.437$), and CD25 $^{+}$ CD4 $^{+}$ T cells and IL10 ($p<0.001$, $r=0.487$) were determined (**Figure 3.3f**).

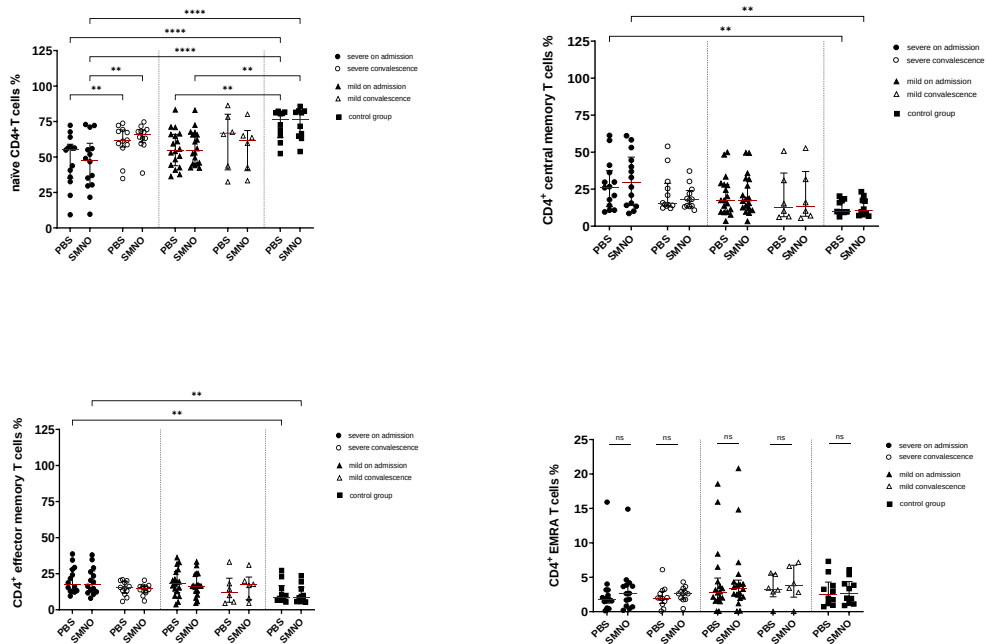
(a)



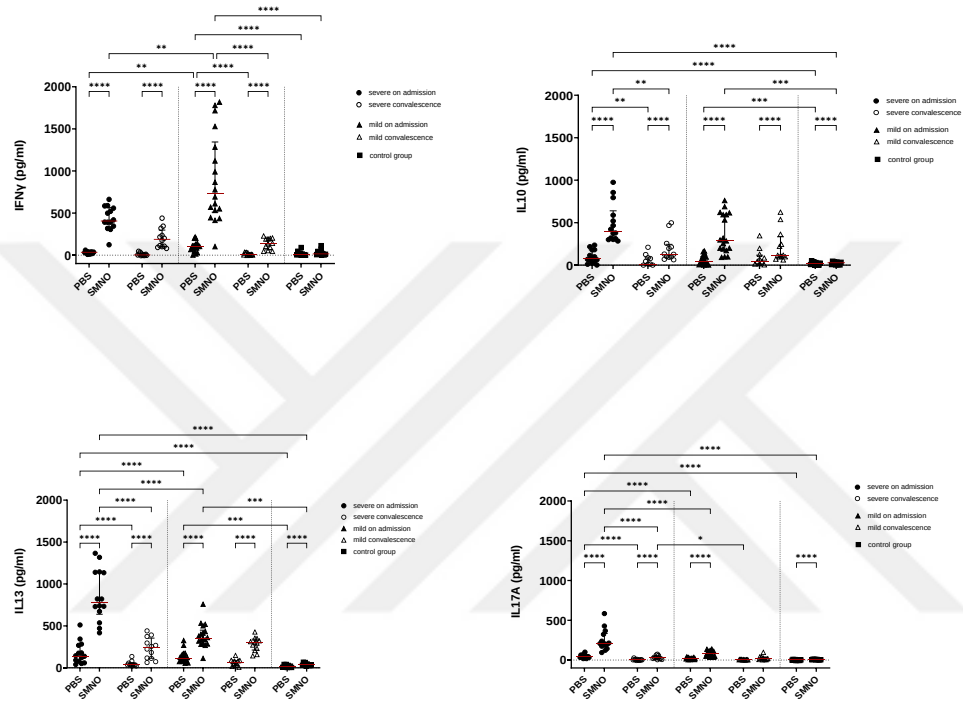
(b)



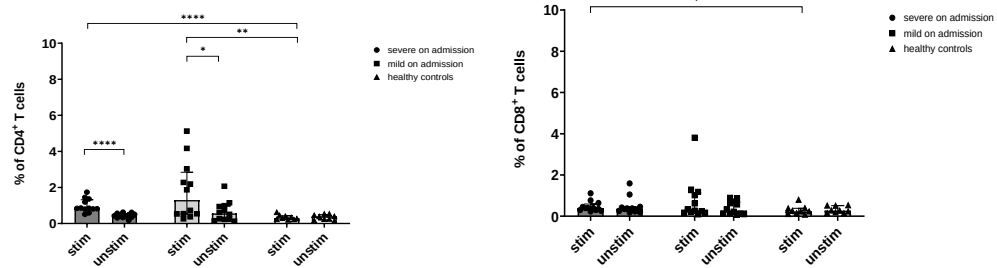
(c)



(d)



(e)



(f)

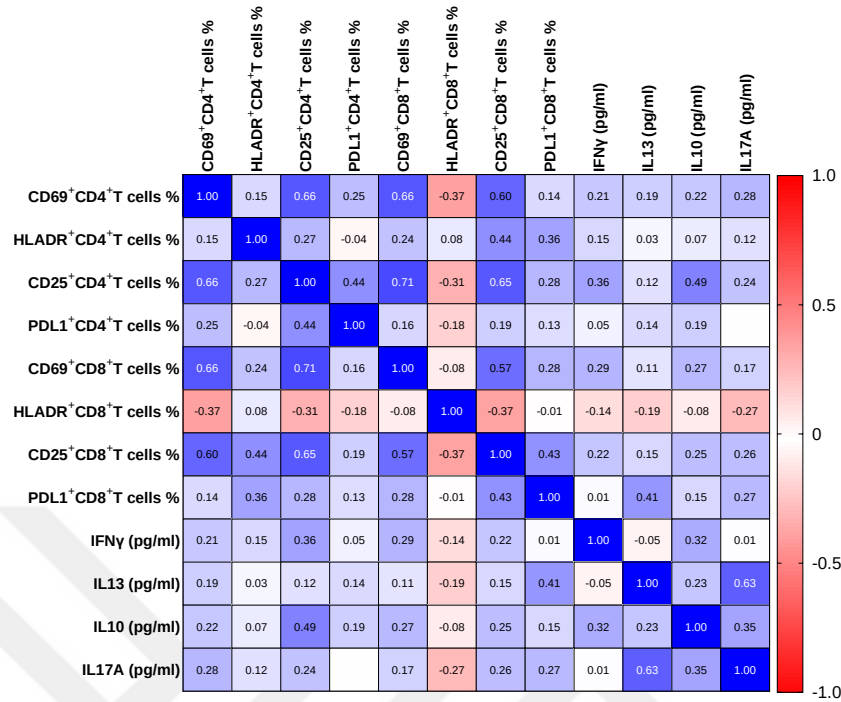


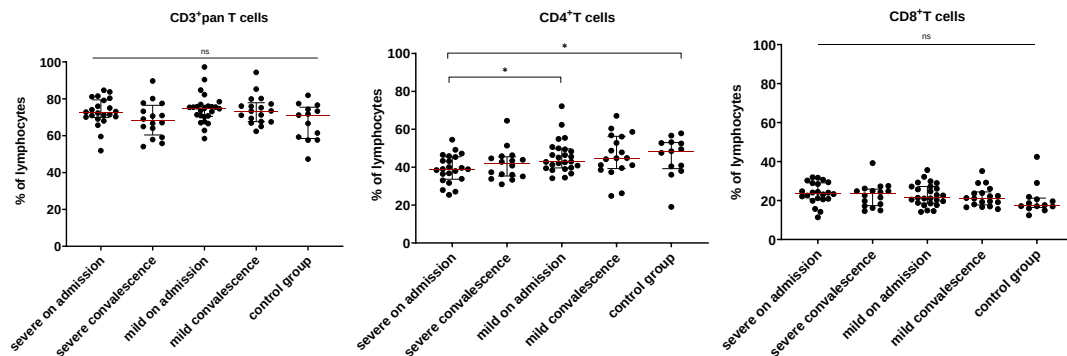
Figure 3.3. Stimulation of patient and healthy PBMCs with SMNO peptide pool to reveal CD4⁺ and CD8⁺T cell responses via surface marker upregulation. 8x10⁵/well PBMCs (severe on admission (n=14), mild on admission (n=18), severe convalescence (n=12), mild convalescence (n=6), healthy controls (n=10)) were plated in 96 well U-bottom plate and stimulated with 2 µg/ml (for each peptide) SARS CoV2 specific SMNO peptide pool plus 1 µg/ml anti-human CD28, and positive control wells were additionally stimulated with 1 µg/ml anti-human CD3. Unstimulated wells were designed as negative, (a) changes in CD4⁺T cell surface markers upon SMNO peptide pool stimulation in various patient and healthy control groups in culture for 48hrs, (b) the distribution of naïve T cells (CD45RO⁻CCR7⁺), central memory T cells (CM, CD45RO⁺ CCR7⁺), effector memory T cells (EM, CD45RO⁺ CCR7⁻), EMRA (CD45RO⁻CCR7⁻) gated from CD4⁺T cells with and without SMNO peptide pool, (c) CD8⁺T cell phenotype change upon SMNO peptide pool stimulation in the groups, (d) intracellular cytokine secretion from PBMCs of patients (severe acute (n=14), mild acute (n=18), severe convalescence (n=12), mild convalescence (n=12)) and healthy controls (n=10) in the supernatants after SMNO peptide pool stimulation for 48 h was determined by ELISA. Each dot corresponds to a donor. Data are given as median and

interquartile ranges (red horizontal lines). Two-sided Mann Whitney-U analysis was performed to determine the significance of differences between two groups. ($*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$), (e) intracellular IFN γ secretion upon SARS CoV2 peptide pool stimulation, Mann Whitney U test was used to compare the significance between the groups, (f) correlation matrix heatmap of IFN γ , IL13, IL10 and IL17A cytokines and surface markers. The numbers in the squares indicate correlation coefficients (Spearman r-values). The color scale symbolizes positive (blue) and negative correlations (red). Spearman's correlation analysis was used to compute the relevance and redundancy of the variables.

3.6. Lymphocyte subsets in peripheral blood during SARS CoV-2 infection

CD4⁺T cells and B cells were significantly decreased in severe acute patients compared to HCs ($p=0.014$) (**Figure 3.4a**). CD4⁺T cells in severe acute cases were also significantly diminished compared to mild patients ($p=0.019$). In addition, plasmacytoid dendritic cells (pDC) and monocytes were decreased both in patients with severe and mild acute disease than those in HCs. Furthermore, Th1 ($p=0.047$) and Treg ($p=0.046$) populations were significantly lower in severe acute patients. However, there was no different in Th2 and Treg subgroups between groups (**Figure 3.4b**). Gating strategy is given in **Figure 3.5**. Gating strategy was given in **Appendix B (Appendix Figure 5, Figure 6 and Figure 7)**.

(a)



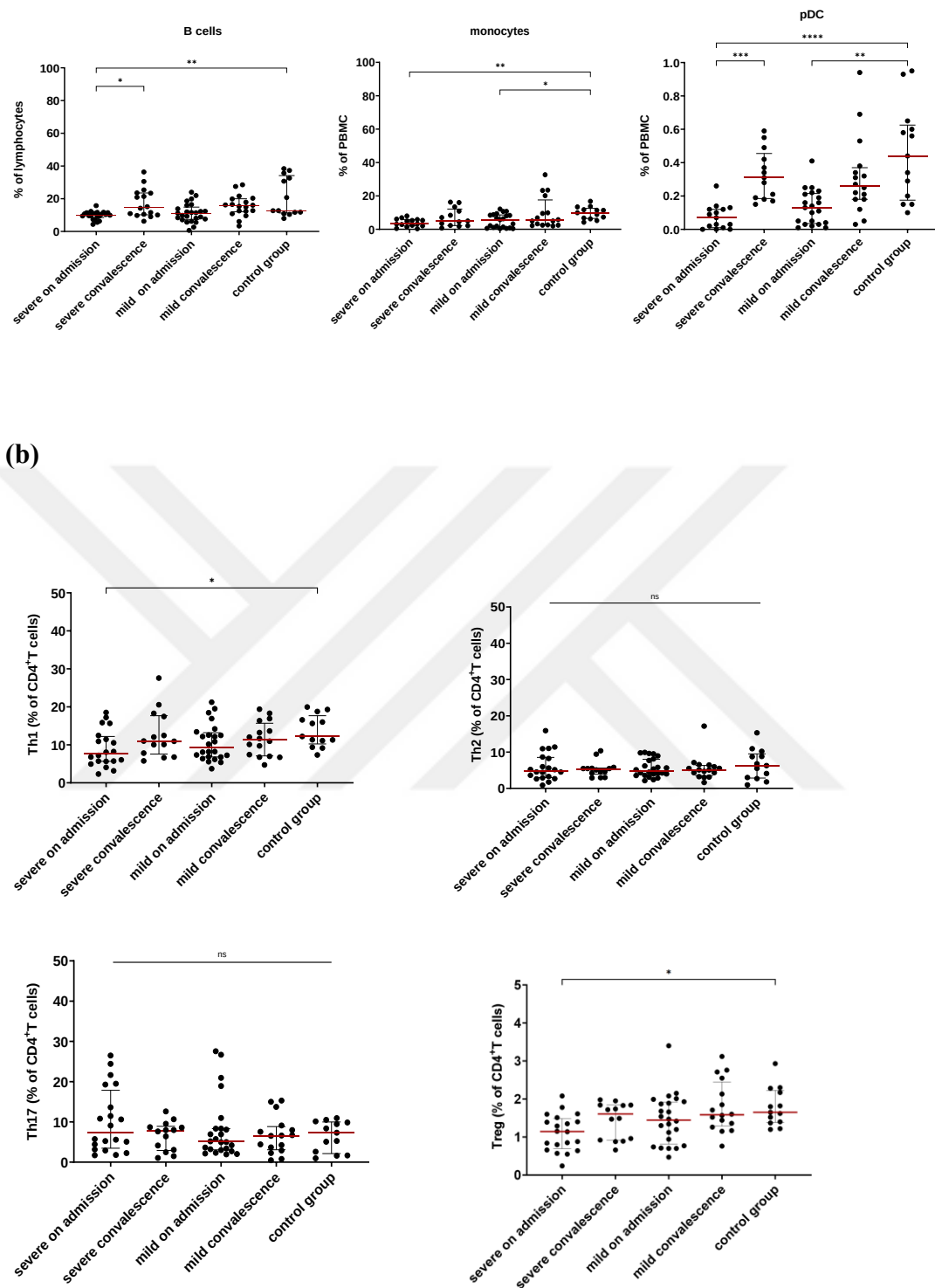
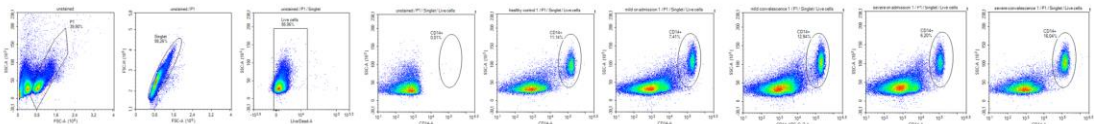


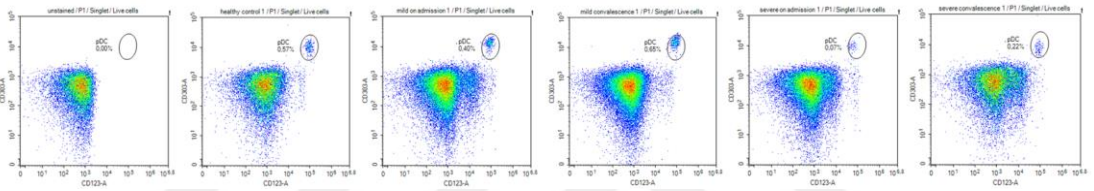
Figure 3.4. Distribution of PBMCs (a) in COVID-19 patients (severe on admission=22, severe very early convalescent=16, mild on admission=25, mild very early convalescent=18 for lymphocytes; severe on admission=15, severe very early convalescent=13, mild on admission=21, mild very early convalescent=16 for pDC and monocytes) and in HCs (n=13).

(b) Characterization of CD4⁺T lymphocyte subgroups in COVID-19. PBMCs were stained for Th1 (CXCR3⁺CCR4⁻CCR6⁻), Th2 (CXCR3⁻CCR4⁺CCR6⁻), Treg (CD25⁺CD127^{low/-}), and Th17 (summation of CXCR3⁻CCR4⁺CCR6⁺ and CXCR3⁺CCR4⁻CCR6⁺ (Th1Th17) cells). The Kruskal-Wallis test with Dunn's multiple comparisons was used to determine the significance of differences between groups.

(a)



(b)



(c)

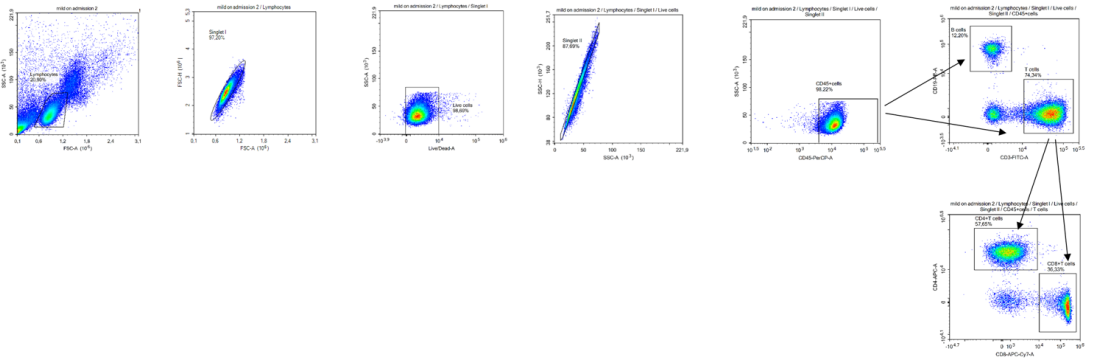
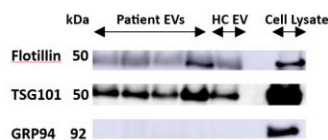


Figure 3.5. Gating strategy of monocytes, pDC and lymphocytes of the COVID-19 patients, (a) CD14⁺ monocytes were gated on HCs and patient groups. Zombie AquaTM was used to determine live and dead cells in patient PBMCs. Suppressed levels of monocytes can be seen in acute patients, especially in severe acute ones, **(b)** pDC were gated by using CD123 and CD303 in the whole PBMC population after live/dead staining. The frequency of pDC were depressed in acute COVID-19 patients and markedly suppressed in severe ones, **(c)** the gating of CD4⁺ and CD8⁺T cells and B cells was depicted.

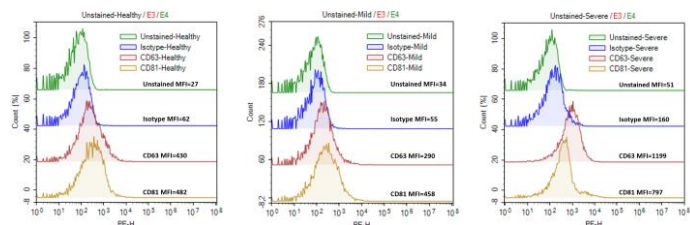
3.7. Characterization of isolated EVs and determination of the kinetics of EV Binding and Uptake by cells

Isolated EVs were validated by Western immunoblot analysis by using TSG101 and flotillin (as positive controls) and glucose-regulated protein 94 (GRP94), which is expected to be positive in cell lysates but absent in EVs, as negative control (**Figure 3.6a**). To further validation of EVs, tetraspanin protein CD63 was detected on EVs by flow cytometry (**Figure 3.6b**). Tunable resistive pulse sensing by qNano (Izon, Biosciences) was used to determine the individual particle sizes and concentration of EVs (**Figure 3.6c**). Total protein content of the EVs and particle size were measured by BCA and qNano (Izon, Biosciences) (**Figure 3.6d**) and were significantly higher in acute patients, specifically in severe ones, than those in HCs ($p < 0.001$ vs. $p < 0.01$). Plasma EVs consist of particles from different cell types. Since T cells and monocytes have important role in SARS CoV-2 infection (307), we studied those two cell groups and their EVs. To clarify the EVs originated from white blood cells (WBCs), bead captured EVs were stained with cell surface molecules of T cells (CD3), B cells (CD19), monocytes (CD14), and activation (CD69) and inhibitory (PDL1) markers on T cells. EVs from T cells ($p = 0.031$), B cells ($p = 0.007$), monocytes ($p = 0.008$), and T cells bearing inhibitory markers ($p = 0.047$) were significantly high in severe acute patients. On the other hand, T cell derived EVs with CD69 was similar between groups ($p = 0.690$) (**Figure 3.6e**). Gating strategy of EV origins were given in **Appendix B (Appendix Figure 8)**.

(a)



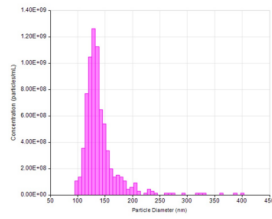
(b)



(c)

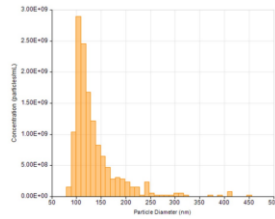
healthy control

Mean size: 141 ±35.7 nm
Mode: 102 nm
Conc: 92x10⁹/ml



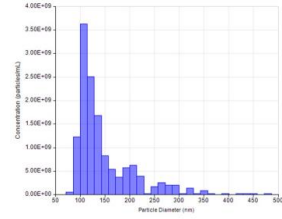
mild on admission

Mean size: 140±59.5 nm
Mode: 105 nm
Conc: 122x10⁹/ml

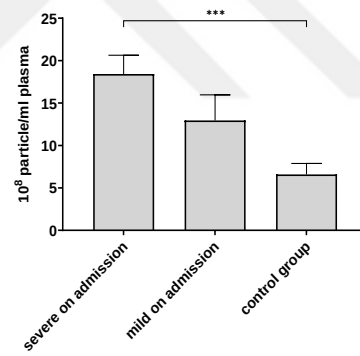
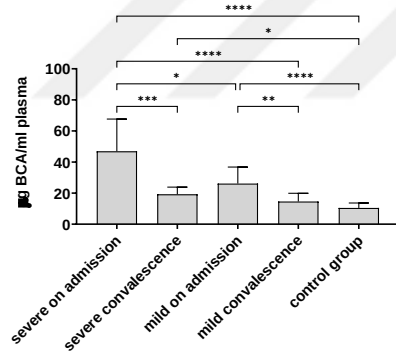


severe on admission

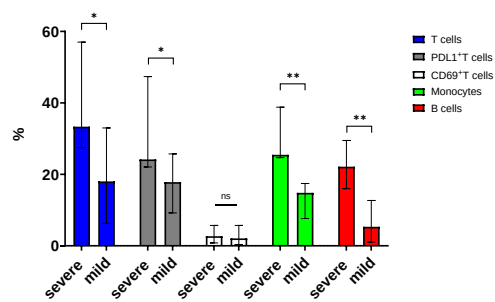
Mean size: 127±35.4 nm
Mode: 98 nm
Conc: 135x10⁹/ml



(d)



(e)



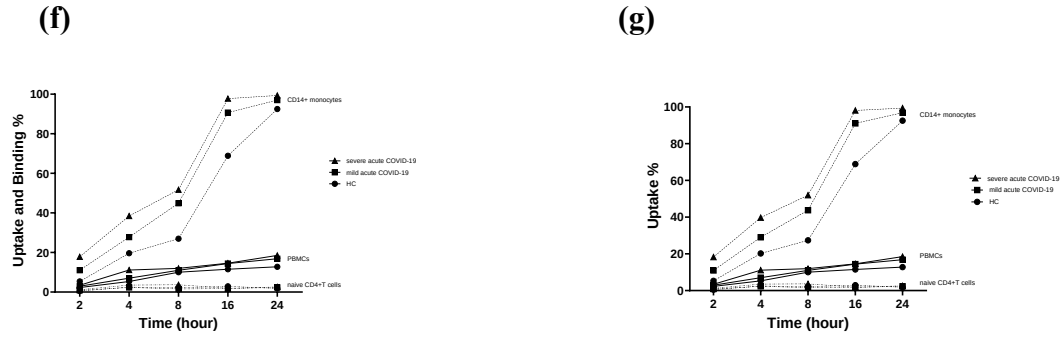


Figure 3.6. Isolation and characterization of extracellular vesicles (EVs). (a) Expression of Tumor susceptibility gene 101 (TSG101) and flotillin proteins on EVs (n=2 severe disease on admission, n=2 mild disease on admission, n=1 healthy control (HC)) and RAW 264.7 cell line lysate. Glucose-regulated protein 94 (GRP94) was used as a negative EV marker, (b) comparison of mean fluorescence intensity (MFI) values of unstained, PE- labelled anti-CD63 stained EVs of patients and healthy control and isotype control antibodies, (c) representative images of particle size distribution and concentration of EVs from healthy control, patients with mild and severe disease during acute phases measured by tunable resistive pulse sensing (qNano, Izon Biosciences), (d) protein and particle concentration of EVs per milliliter of plasma is higher in severe and mild covid cases than those in healthy controls. Protein (n=22 severe cases on admission, n=25 mild cases on admission, n=16 severe convalescence between days 25 to 30, n=18 mild convalescence between days 25 to 30) and particle concentration (n=6 severe cases on admission, n=6 mild cases on admission) of patient EVs normalized to plasma volume were higher compared to healthy EVs (n=13 HC for protein, n=5 HC for particle concentration assay) ($p < 0.001$). The particle number to protein (μg) ratio was calculated, and it was similar between the groups ($p = 0.168$) (median = 0.87×10^8 particles, IQR; $0.78\text{--}0.98 \times 10^8$ particles/ μg protein). A total of $15 \mu\text{g}$ EV protein was used for each EV stimulation assay, (e) circulating WBCs-derived EVs of severe patients on admission (n=5) and mild patients on admission (n=5) were stained with the corresponding cell type-specific markers for T cells (CD3), monocytes (CD14) and B cells (CD19) as well as CD69 activation marker and PDL1 inhibitory marker on the EVs from T cells. Data are presented as median $\pm$ IQR (in %). Two-sided Mann Whitney-U test was used to determine the significance between groups. Comparison of the (f) internalization (uptake) and binding kinetics, and (g) only internalization kinetics after performing 0.1% of trypan blue to quench the cell membrane bound EV fraction on PBMCs, CD14⁺ monocytes and naïve CD4⁺T cells. SPDIOC₁₈(3)-labeled EVs from one healthy control, one patient with severe acute and one patient with mild acute disease were incubated with healthy PBMCs, healthy CD14⁺ monocytes and healthy naïve CD4⁺T cells. Time course uptake/binding and only uptake (internalization) kinetics were determined by

FITC-positive cells by flow cytometry. Nearly all the monocytes had internalized of EVs at 24th hour, while the uptake of EVs by T cells was almost unchanged and remained at a very low level. HC; Healthy Control

To determine the kinetics of EV binding and uptake by PBMCs, isolated human healthy naïve CD4⁺T cells and CD14⁺ monocytes, EVs were co-cultured with SP-DiOC18(3) (Invitrogen™) stained EVs for 24 hours. The EV binding and internalization kinetics of PBMC, naïve CD4⁺T cells, and CD14⁺ monocytes were analyzed by flow cytometry at 0, 2, 4, 8, 16, and 24 hours (**Figure 3.7**). Binding and uptake of EVs were gradually increased by CD14⁺ monocytes over time, and EVs were internalized (uptake) by almost all monocytes at the end of the 24th hour. Similar to previous results, only a certain internalization and almost no uptake were observed by PBMCs and naïve CD4⁺T cells, respectively (**Figure 3.6f and 3.6g**) (326,327).

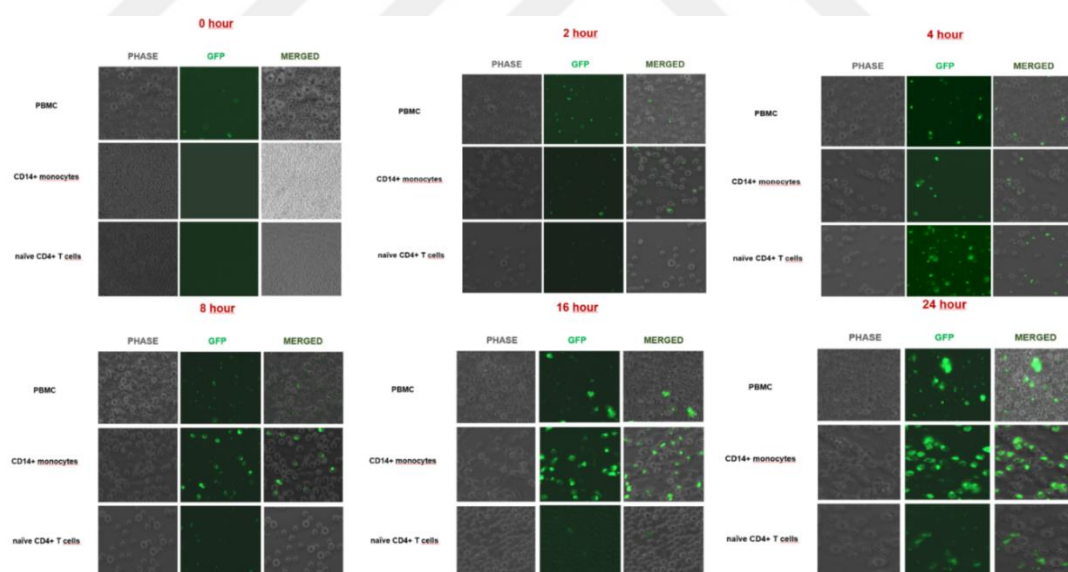


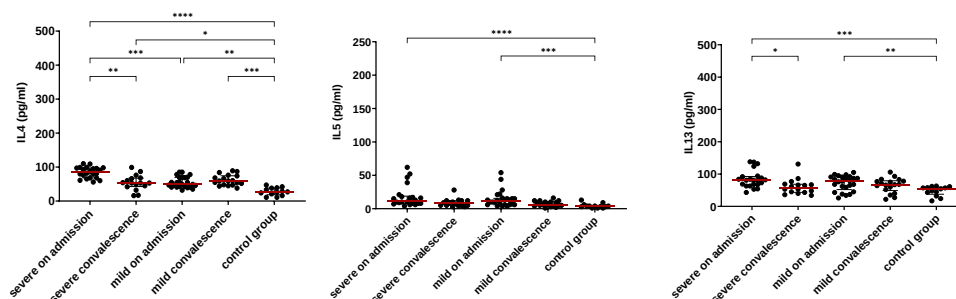
Figure 3.7. Representative images of EV uptake by PBMCs, CD14⁺ monocytes and naïve CD4⁺T cells. Time course (0-, 2-, 4-, 8-, 16- and 24-h EV incubation) representative images of EV uptake by staining with the green lipophilic dye SP-DiOC and was visualized at the green fluorescent protein (GFP) channel at live cell imager (EVOS®). Uptake of EVs by monocytes gradually increased by time. However, EV uptake by T cells did not change significantly.

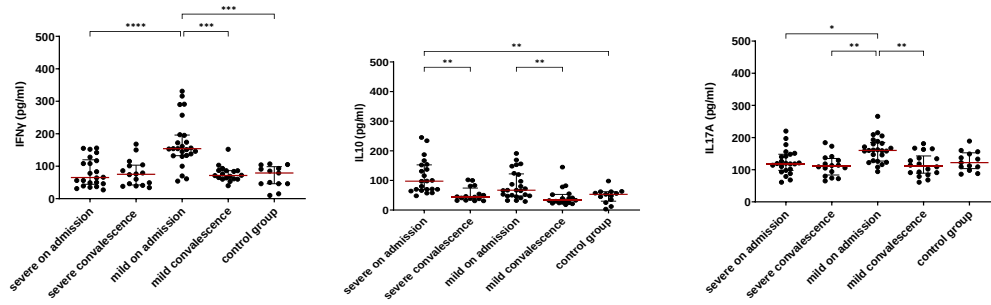
3.8. Extracellular vesicles of severe COVID-19 patients suppress Th1 and induce Th2 responses

Isolated PBMCs were incubated with patient and healthy EVs for 3 days. Supernatants were collected at the end of the 3th day, and cytokine concentrations were evaluated by ELISA (**Figure 3.8a**). Th2 related cytokines and IL10 were higher with severe acute patient EVs compared to mild ones. In contrast, IFN γ and IL17A were quite similar to the level of HCs and significantly lower compared to mild ones. The ratios of IFN γ to the other cytokines were markedly lower in severe acute group than those in all mild and HCs (**Figure 3.8b**).

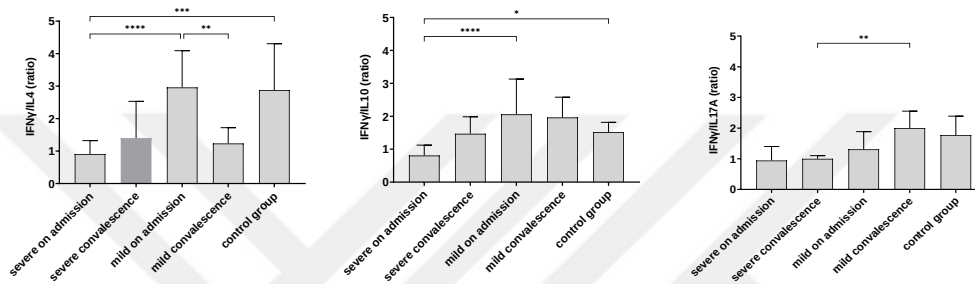
Then we further investigated the frequency of Th1, Th2, Treg and Th17 subsets of PBMCs stimulated with plasma derived EVs. The proportion of Th1 ($p<0.0001$) and Th17 ($p<0.0001$) cells were significantly lower with acute patient EVs than those in HCs. Th2 ($p=0.0003$) frequency was significantly elevated with severe acute EVs compared to EVs of HCs. However, there was no significant differences in Tregs between the groups. Interestingly, CD4⁺T cells in PBMCs showed a slight non-significant decrease with severe acute EVs compared to HCs (**Figure 3.8c**). Then we assessed the expression of the cell surface markers after EV stimulation. The expression of CD69 and CD25 activation and PDL1 inhibitory markers on CD4⁺T cells were upregulated with severe acute EVs (**Figure 3.8d**). Intracellular IL4 and IL10 secretion from CD4⁺T cells were found to be increased with severe acute EVs. In contrast, IFN γ was high in cells stimulated with mild acute EVs (**Figure 3.8e**). Gating strategy was given in **Appendix B (Appendix Figure 9 and Figure 10)**.

(a)

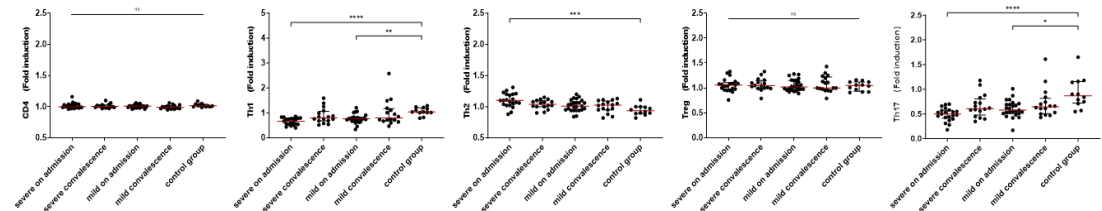




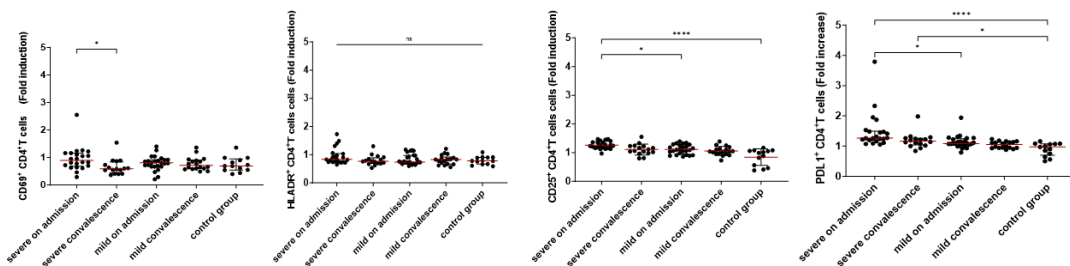
(b)



(c)



(d)



(e)

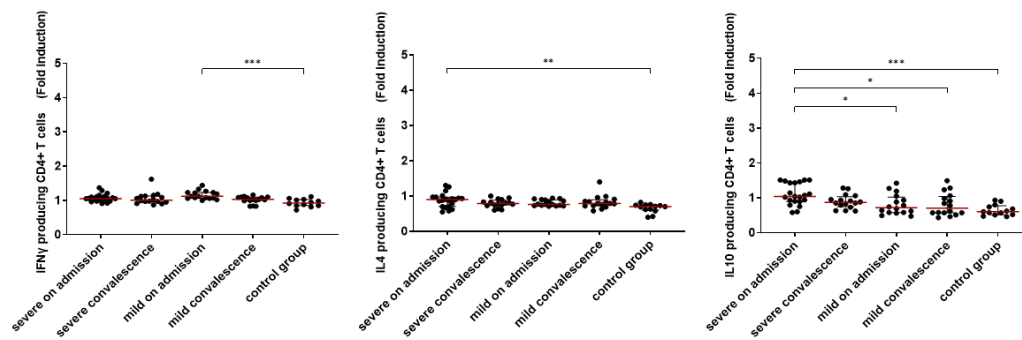


Figure 3.8. Stimulation of healthy PBMCs with plasma derived EVs of patients and healthy controls. 4×10^5 healthy PBMCs were seeded in 96 well plate in 100 μ L of 10% RPMI with exosome free FBS. PBMCs were treated with 15 μ g/well purified patient (severe acute=22, severe convalescence=16, mild acute=25, mild convalescence=18) and HC EVs (n=13) for 72 h. **(a)** IL4, IL5, IL13, IFN γ , IL10 and IL17A levels were plotted. IL4 was significantly increased, whereas IFN γ was decreased in samples treated with severe acute EVs. **(b)** IFN γ /IL4, IFN γ /IL13, IFN γ /IL10, and IFN γ /IL17A ratios were plotted. Th1 cytokine ratio was suppressed in severe acute cases compared to mild patients. For the comparison of the variables, Kruskal-Wallis test with Dunn's multiple comparisons was performed. Data was shown as bar graphic with median values and interquartile ranges, **(c)** Th subsets in EV stimulated healthy PBMCs. Stimulated PBMCs were stained for Th1 (CXCR3⁺CCR4⁻CCR6⁻), Th2 (CXCR3⁻CCR4⁺CCR6⁻), Treg (CD25⁺CD127^{low/-}), and Th17 (summation of Th17 (CXCR3⁻CCR4⁺CCR6⁺) and Th1Th17 (CXCR3⁺CCR4⁻CCR6⁺) cells) surface markers. Results in **Figure c** are expressed as fold induction; the ratio of CD4⁺T cells gated in PBMCs treated with EVs to EV-untreated wells. Red lines indicate median with interquartile ranges (severe acute cases=21, mild acute cases=25, severe convalescence=16, mild convalescence=16, healthy controls=12). Each dot represents an individual. The Kruskal-Wallis test with Dunn's multiple comparisons was used to determine the significance of differences between groups. **(d)** Activation (CD69, HLADR, CD25) and inhibition (PDL1) markers on CD4⁺T cells were presented as fold induction. **(e)** Intracellular IFN γ , IL10 and IL4 levels in CD4⁺T cells (severe acute=22, severe convalescence=16, mild acute=25, mild convalescence=16, healthy control=13). Data are given as fold induction and median with interquartile ranges.

3.9. The severity of COVID-19 infection effects the immune responses of undifferentiated monocytes via Extracellular vesicles of the patients

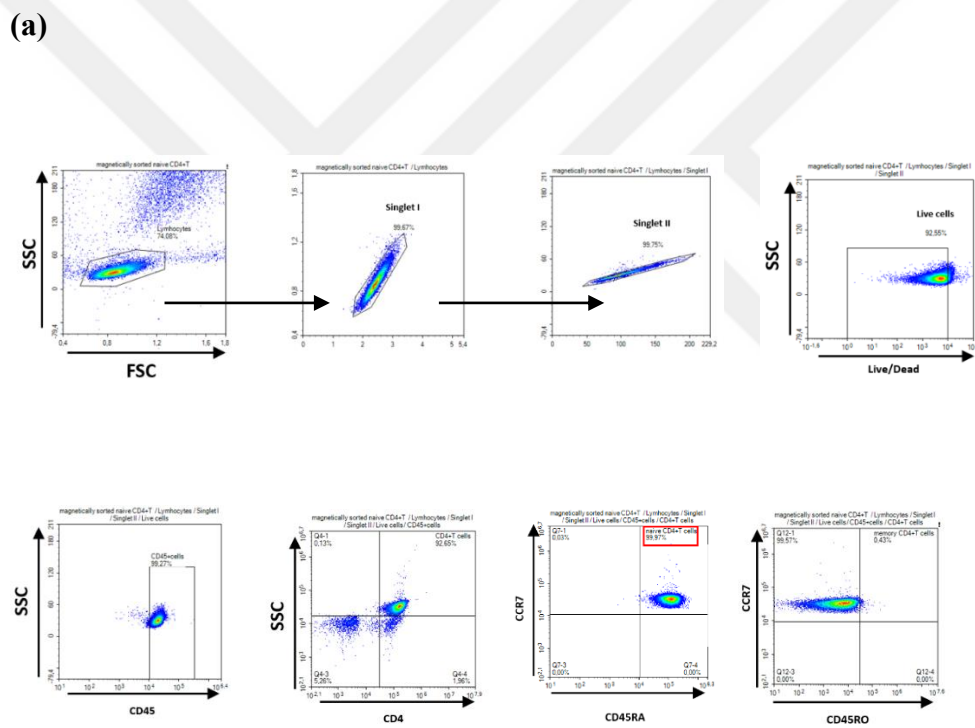
To further evaluate the effects of EVs of the patients infected with SARS CoV-2 on cellular immunity, monocytes and naïve CD4⁺T cells were isolated with magnetic activated cell sorting (MACS[®]) from healthy PBMCs and treated with both healthy and patient EVs. CD14⁺ cells through positive selection (human, CD14 microbeads, Miltenyi Biotec, Germany) and naïve CD4⁺T cells via negative selection (human, CD14 microbeads, Miltenyi Biotec, Germany) were isolated. Purity and viability of the sorted cells was shown in **Figure 3.9a and 9b**. After isolation, monocytes and CD4⁺T naïve T cells were seeded in 96 well-U bottom plate and stimulated for 5 days with EVs of severe patients on admission (n=18), mild patients on admission (n=18) and healthy controls (n=9). At the end of the 5th day, supernatants were collected for ELISA to measure the cytokines released by EV treated isolated healthy cells, and the cells were harvested for flow cytometry analysis (**Figure 3.9c**).

Morphologically, undifferentiated freshly isolated monocytes were round form after isolation on day 0 (**Figure 3.9d**). At the end of the 5th day of the coculture, monocytes treated with M-CSF were round, whereas monocytes stimulated with GM-CSF changed their round form and began to elongate (328). Monocytes that were cultured with EVs from severe patients on admission had more rounded shape, while monocytes that were applied with EVs of mild patients on admission appeared more elongated (**Figure 3.9d**). To investigate the effect of EVs on monocytes, supernatants were collected at the end of the 5th day and cytokine profile was studied (**Figure 3.9e**). While the anti-inflammatory cytokine IL10 and IL4 were higher in cultures of severe patients' EVs than those in mild patients' EVs, IL6 and IFN γ , which are proinflammatory cytokines, were found to be lower. In **Figure 3.9f**, we presented the ratio of proinflammatory cytokine IFN γ to anti-inflammatory cytokines IL10 and IL4. The ratio of IFN γ was significantly lower at the samples treated with EVs of severe cases.

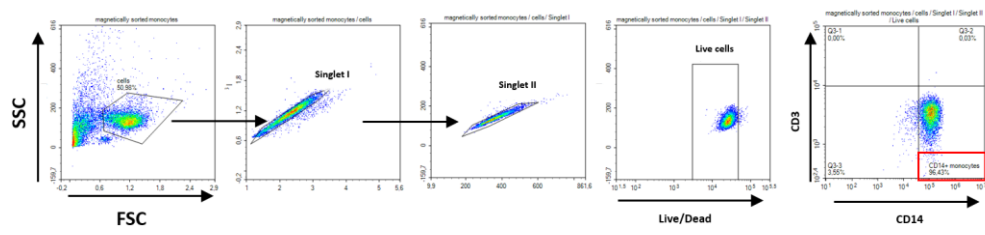
Then, the monocyte to macrophage differentiation and polarization surface markers were analyzed in flow cytometry. We used CD68 as pan macrophage marker, CD80 and CD86 for the discrimination of M1 and CD206 and CD163 for M2 macrophages (**Figure 3.9g**). Gating strategy used for this experiment is shown in **Figure 3.9h**. M1 markers were significantly suppressed on monocytes which were stimulated with EVs

with severe cases compared to mild and healthy donors, whereas M2 markers were not significantly different among the groups. Therefore, it can be concluded that monocytes treated with severe patients' EVs tended to suppress M1 markers compared to mild cases and healthy controls (**Figure 3.9g**).

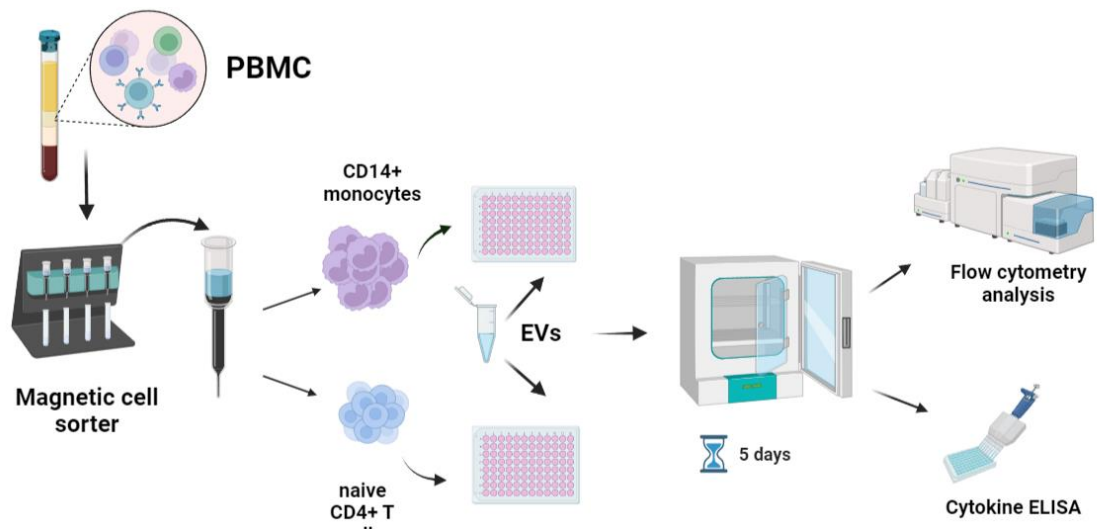
To further evaluate the effect of EVs on monocytes, we also analyzed the monocytes in PBMCs stimulated with EVs for 72 h. Similarly, suppression of M1 surface markers in those stimulated with EVs of severe acute disease compared to other groups was observed (**Figure 3.9i**). Gating strategy was given in **Appendix B (Appendix Figure 11 and Figure 12)**.



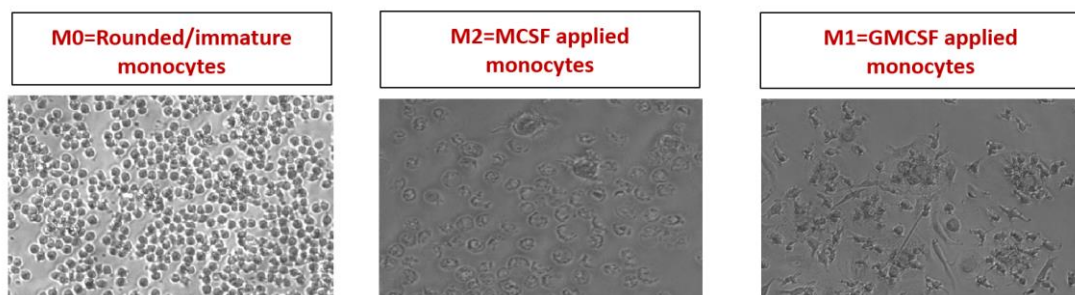
(b)

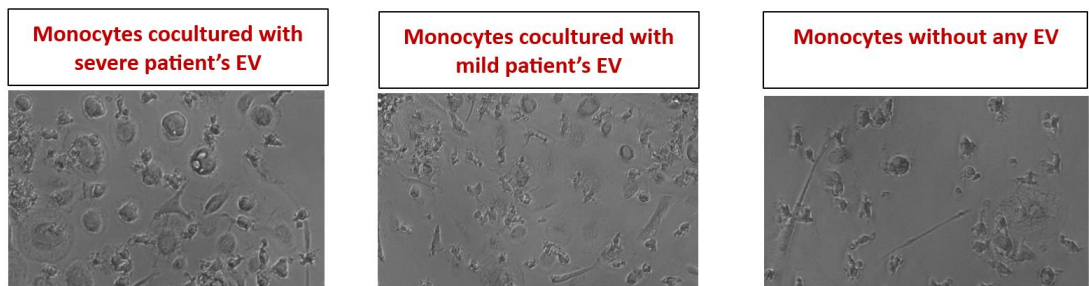


(c)

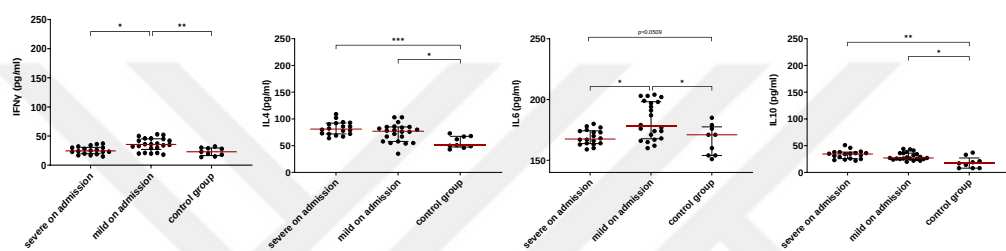


(d)

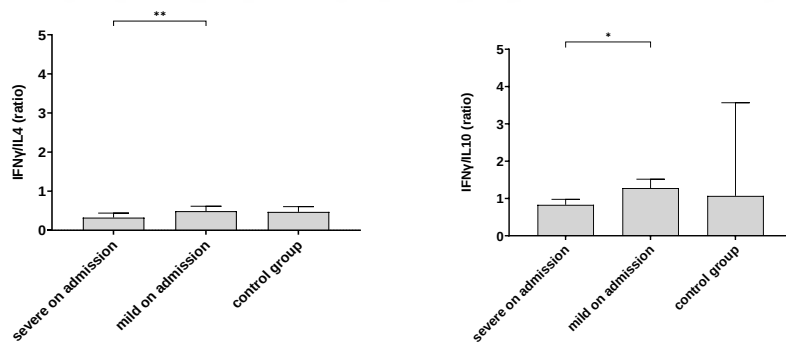




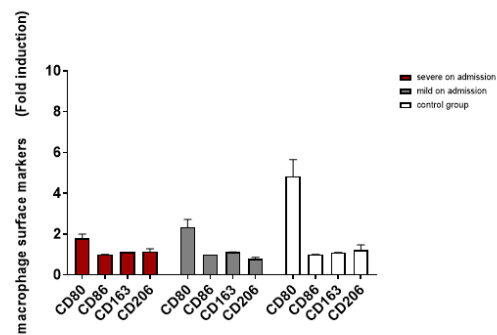
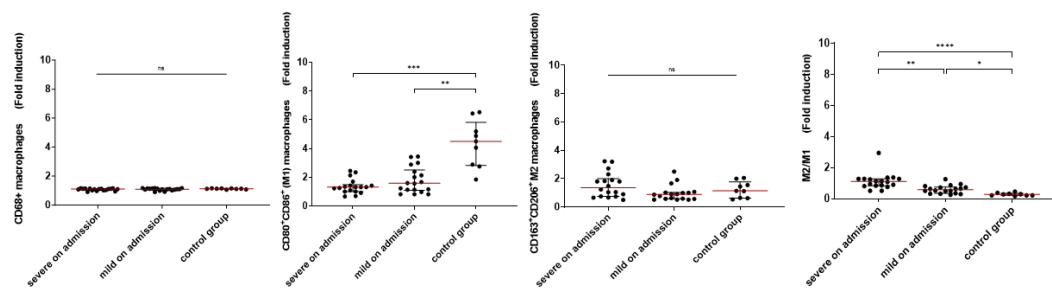
(e)



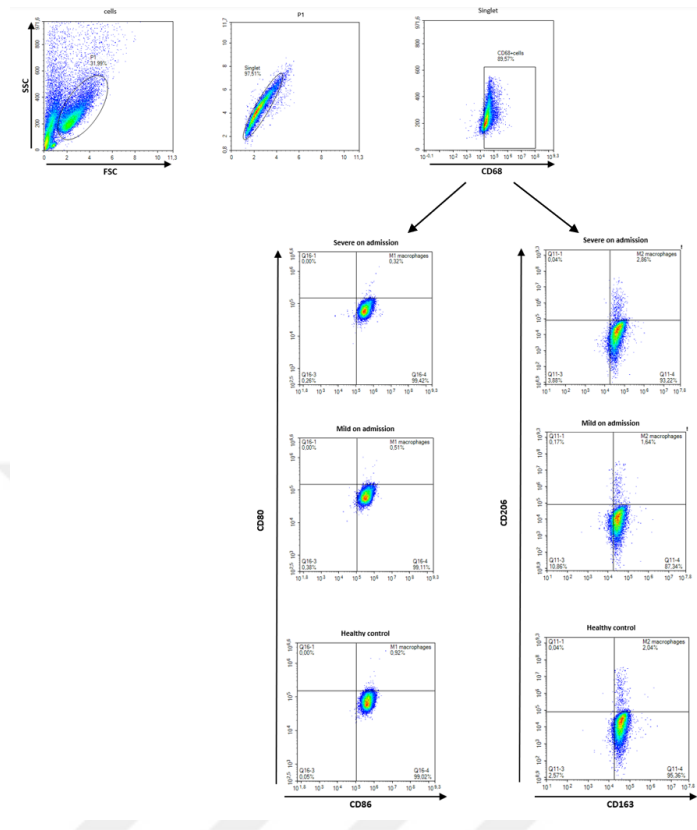
(f)



(g)



(h)



(i)

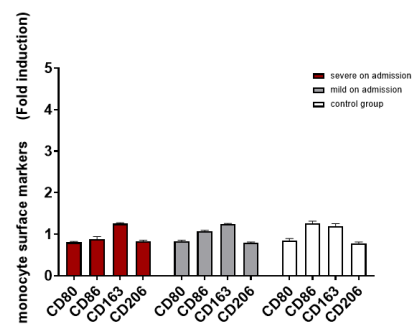
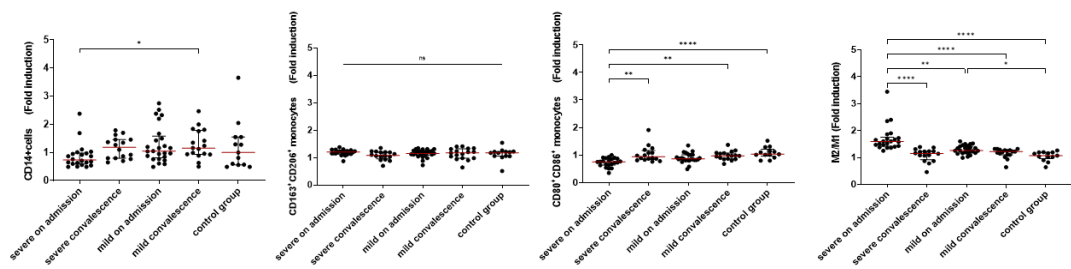


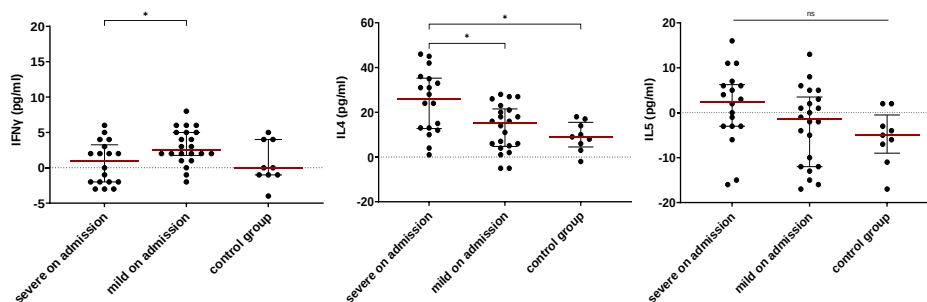
Figure 3.9. Sorted CD14⁺ monocytes and naïve CD4⁺T cells were isolated from healthy PBMCs via magnetic cell sorter. Purity, and viability of isolated (a) naïve CD4⁺T cells and (b) monocytes was measured using Zombie Aqua™ Live/Dead dye, (c) the protocol of isolation and coculturing of isolated cells with EVs was depicted (BioRender™ was used to illustrate), (d) unstimulated immature monocytes were in round shape on day 0. 15x10⁴ monocytes were seeded in 96 well flat-bottom plate and 15 µg EVs were added in each well. MCSF+IL4+LPS were applied for inducing M2 (round shape) and GMCSF+IFN γ for the induction of M1 macrophages (elongated) as positive controls. Some wells were left untreated (non-EV wells). Other wells were treated with EVs of the patients with severe (n=18) and mild (n=18) COVID-19 infection on admission and healthy controls (n=9) for 5 days. At the end of the 5th day, cells were visualized by live cell imaging (EVOS® FL Auto Live Cell Imaging System). MCSF and GMCSF performed cells were round and elongated, respectively. Cells applied severe disease EVs are inclined to have rounder shape compared to monocytes cultured with mild disease EVs, (e) Proinflammatory cytokines IFN γ and IL6 were lower, whereas anti-inflammatory cytokines IL10 and IL4 were higher in the supernatants of the monocyte-severe acute disease EV cultures compared to those in monocyte-mild acute disease EV cultures. Each dot represents one individual. Graphs show median levels (red horizontal lines) with interquartile ranges. The Kruskal-Wallis test with Dunn's multiple comparisons was used to determine the significance of differences between groups. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$), (f) the ratio of IFN γ to IL4 and to IL10 was depicted as bar graphs. Data are given as median with interquartile ranges, p values < 0.05 were given, (g) monocytes (15x10⁴/well) were MACS isolated from healthy PBMCs and incubated with patient derived EVs (15 µg EV/well) for 5 days in culture. At the end of the 5th day, cells were stained with CD68 were accepted as macrophages. Cells were stained for M1 (CD80-CD86) and M2 (CD163-CD206) specific surface markers and fold induction over EV-untreated wells was plotted. M1 (CD80-CD86) polarized macrophage, M2 (CD163-CD206) polarized macrophage over healthy EV treatment and M2/M1 induction ratio between treatment groups (severe acute=18, mild acute=18, healthy controls=9). Data are given median and interquartile ranges, (h) gating of M1 and M2 macrophage markers on monocytes stimulated with severe and mild COVID-19 patient and HC EVs, (i) results of the monocyte population in the PBMCs stimulated with the EVs of severe on admission (n=22), severe convalescence (n=16), mild on admission (n=25), mild convalescence (n=17), healthy controls (n=13) during 3 days were depicted. Data are given as median and interquartile ranges, and p values were determined with Kruskal-Wallis test with Dunn's multiple comparisons.

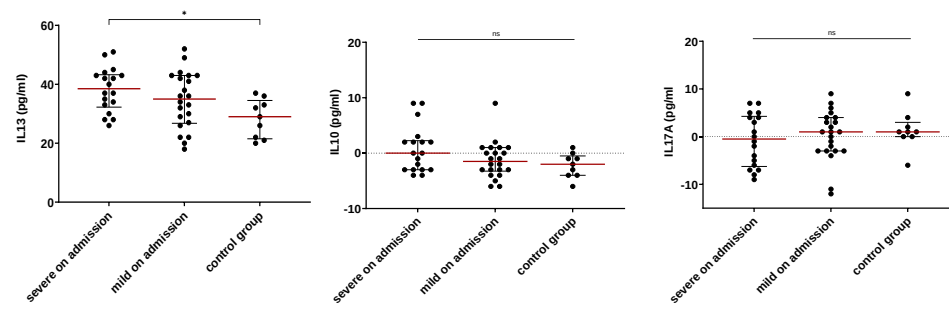
3.10. Extracellular vesicles modulate naïve CD4⁺T cell immune responses to a limited extent

Although EVs also triggered cytokine secretion from CD4⁺T cells, extremely lower amount of cytokines were obtained compared to the PBMC and monocyte responses. Higher amount of Th2 and lower levels of Th1 cytokines were produced by severe acute EVs (**Figure 3.10a and 3.10b**). We have shown that the EV uptake by naïve CD4⁺T cells was not favorable (**Figure 3.7**). As indicated in previous studies, naïve CD4⁺T cells require macrophages and other antigen presenting cells (APC) to be activated by EVs rather than direct internalization of these particles (329,330). This might give rise to less cytokine release from naïve CD4⁺T cells in our study.

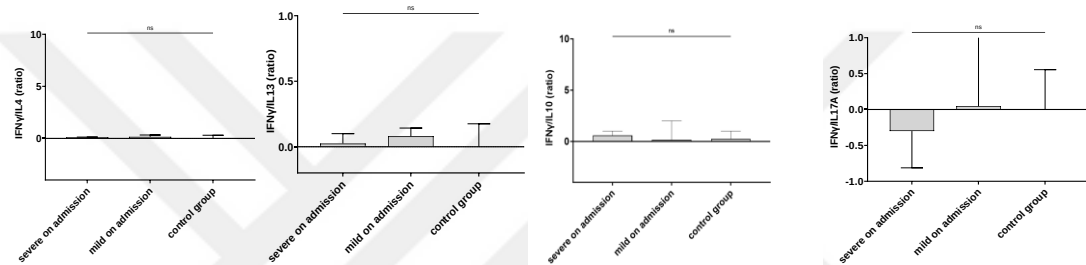
At the end of the 5th day of the incubation, EV stimulated CD4⁺T cells were harvested. According to intracellular staining (**Figure 3.11a and 3.11b**), IL4 (p=0.018) and IL10 (p=0.0005) producing T helper cells were significantly, whereas IFN γ ⁺Th cells were non-significantly elevated with severe patient EVs compared to HC ones (**Figure 3.10c**). In addition, Th1 (p=0.0039) and Th17 (p=0.047) cells were significantly decreased, whereas Th2 (p=0.017) and Treg cells were increased in the samples stimulated with severe patient EVs compared to HC EVs (**Figure 3.10d**). Interestingly, CD4⁺T cell frequency was decreased upon the severe EV stimulation (p=0.043). Gating strategy was given in **Appendix B (Appendix Figure 13, and Figure 14)**.

(a)

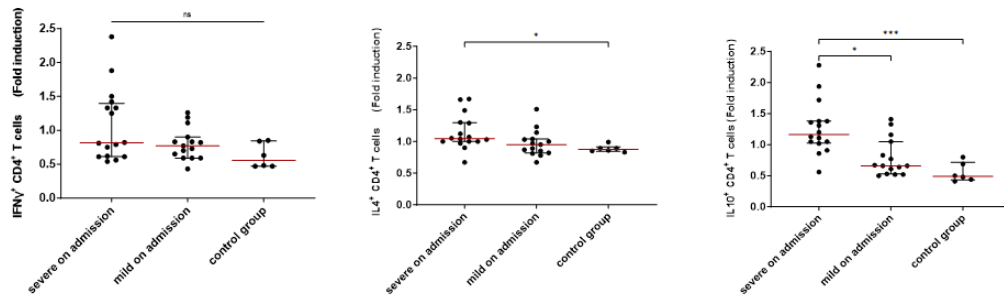




(b)



(c)



(d)

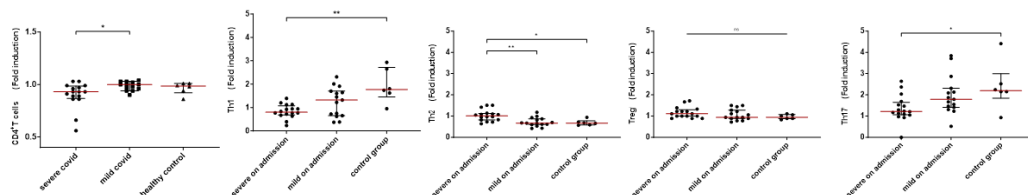
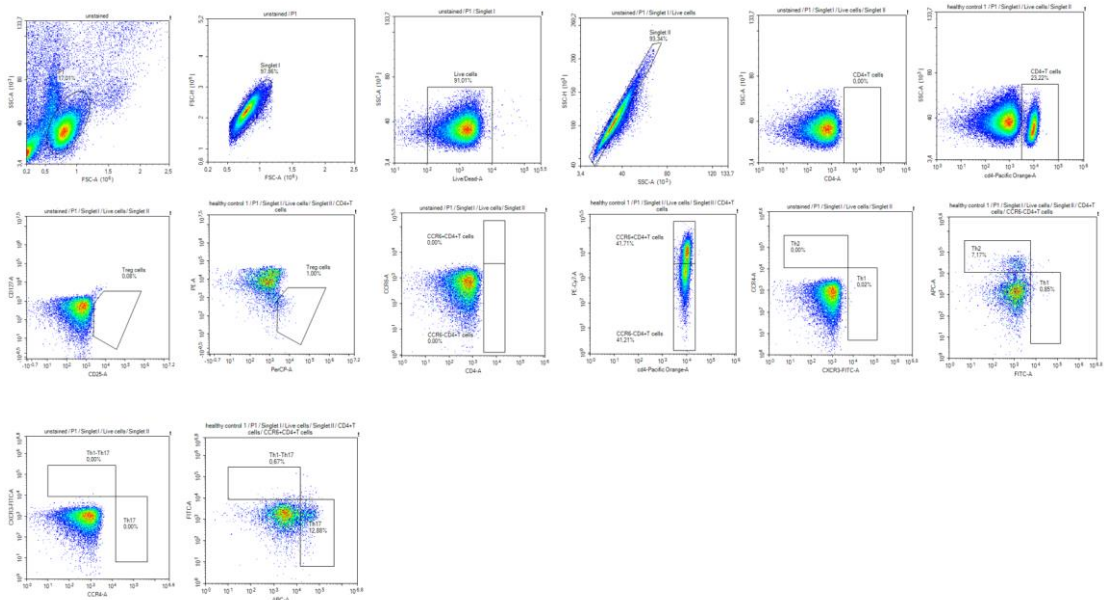


Figure 3.10. Stimulation of healthy human naïve CD4⁺T cells with plasma derived EVs of patients and healthy controls. Purified naïve CD4⁺T cells (75×10^3 T cells/well) were

stimulated with patient (severe on admission=18, mild on admission=22) and HC EVs (n=9) (15µg/well) for 5 days. Cytokine levels were measured by ELISA, **(a)** IFN γ , IL4, IL5, IL13, IL10 and IL17A levels were plotted. Scattered plots show median levels (red horizontal lines) with interquartile ranges. The Kruskal-Wallis test was performed following Dunn's multiple comparisons to evaluate the significance of differences between groups, **(b)** cytokine dependent variations in responses from naïve CD4⁺T cells following stimulation with patient EVs (severe patients on admission (n=18), mild patients on admission (n=22)) and HCs (n=9) for 5 days. Ratios of cytokines to IFN γ were shown as bar graphics with median values and interquartile ranges. **(c)** Intracellular IFN γ , IL-4 and IL-10 secretion from the EV-stimulated CD4⁺T cells (by 2h stimulation with PMA and ionomycin following 4 h Brefeldin-A to stop the reaction). The data are given as fold induction. p values were calculated by using Kruskal-Wallis test. For this experiment, EVs were used from severe on admission (n=16), mild on admission (n=15) and HCs (n=6). **(d)** Chemokine flow staining data were plotted for Th1 (CXCR3⁺CCR4⁻CCR6⁻), Th2 (CXCR3⁻CCR4⁺CCR6⁻), Th17 (CXCR3⁻CCR4⁺CCR6⁺ and CXCR3⁺CCR4⁻CCR6⁺) and Treg (CD25⁺CD127^{low/-}) subsets. Th17 cells were calculated as the summation of CXCR3⁻CCR4⁺CCR6⁺ (Th17) and CXCR3⁺CCR4⁻CCR6⁺ (Th1Th17) (EVs of the patients with severe acute disease (n=16), mild acute disease (n=15) and HCs (n=6)).

(a)



(b)

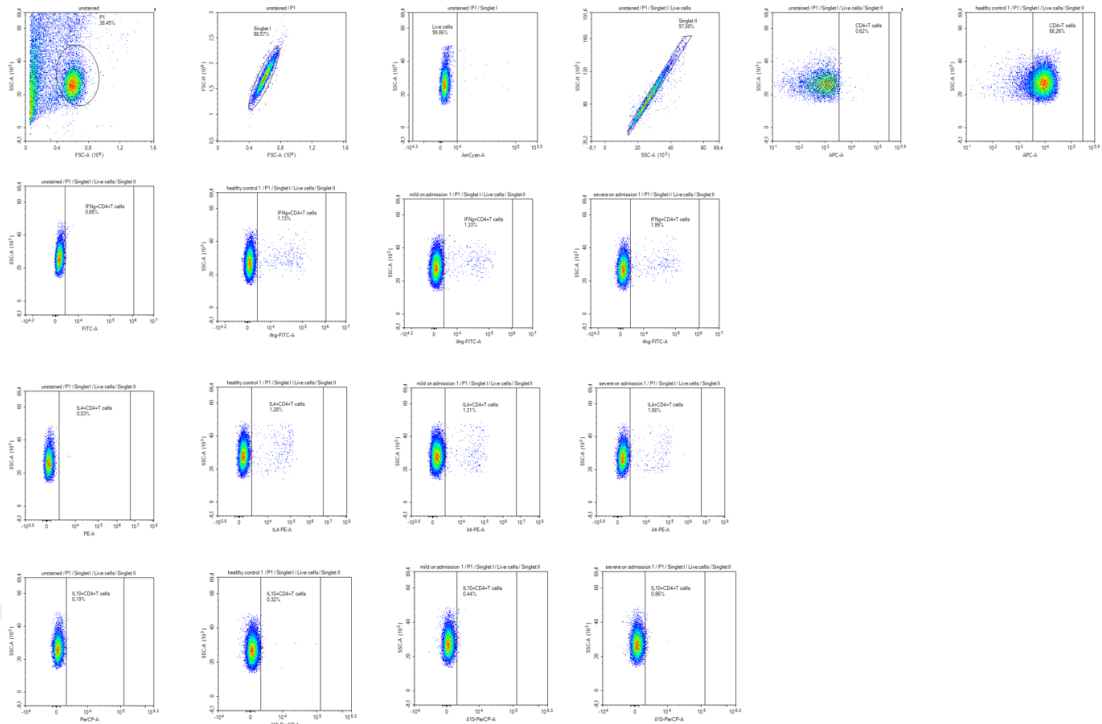


Figure 3.11. Gating strategy for (a) Th1 (CXCR3⁺CCR4⁻CCR6⁻), Th2 (CXCR3⁻CCR4⁺CCR6⁻), Treg (CD25⁺CD127^{low/-}), and Th17 (summation of Th17 (CXCR3⁻CCR4⁺CCR6⁺) and Th1Th17 (CXCR3⁺CCR4⁻CCR6⁺)) cells, (b) ICS of IFN γ , IL10 and IL4 in CD4⁺T cells.

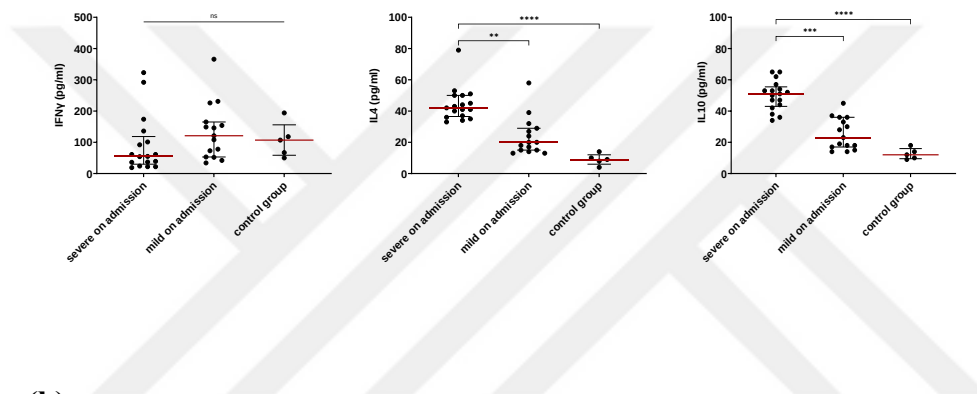
3.11. Naïve CD4⁺T cells need to be cocultured with monocytes to mount adequate immune response triggered by EVs

Naïve CD4⁺T cells had extremely limited EV uptake capacity. The activation of T cells by EVs is mostly through APCs, therefore we cocultured purified naïve CD4⁺T cells with monocytes, which present antigens to CD4⁺T cells and uptake EVs more efficiently (326). Cytokine levels were elevated in coculture compared to the culture including only naïve CD4⁺T cells. IFN γ was non-significantly low ($p=0.210$ vs $p=0.067$), but IL4 ($p=0.0012$ vs $p<0.001$) and IL10 ($p=0.002$ vs $p<0.0001$) were significantly increased in monocyte-CD4⁺T cell coculture treated with severe patient EVs compared to those in mild and HC ones (**Figure 3.12a**). Activation markers (CD69 and HLADR) were non-significantly elevated, whereas inhibitory marker PDL1 ($p=0.013$ vs $p<0.0001$) was increased on CD4⁺T cells cocultured with monocytes with severe patient EVs (**Figure 3.12b**). Furthermore, activation markers were non-significantly suppressed; however, PDL1 was significantly upregulated on monocytes

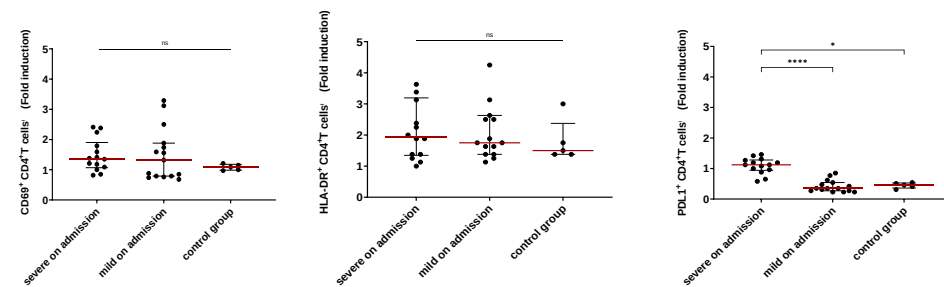
in coculture treated with severe patient EVs ($p=0.028$ vs $p=0.048$) (**Figure 3.12c**). Gating strategy was given in **Appendix B (Appendix Figure 15, and Figure 16)**.

Briefly, PDL1 expression, IL4 and IL10 secretion were increased after the stimulation with severe patient EVs. Additionally, $\text{IFN}\gamma$ was non-significantly depressed in samples stimulated with severe patient EVs compared to mild and healthy ones. A similar trend was obtained with cells incubated with mild patient EVs; however, it was not as significant and remarkable as seen in severe patient EVs.

(a)



(b)



(c)

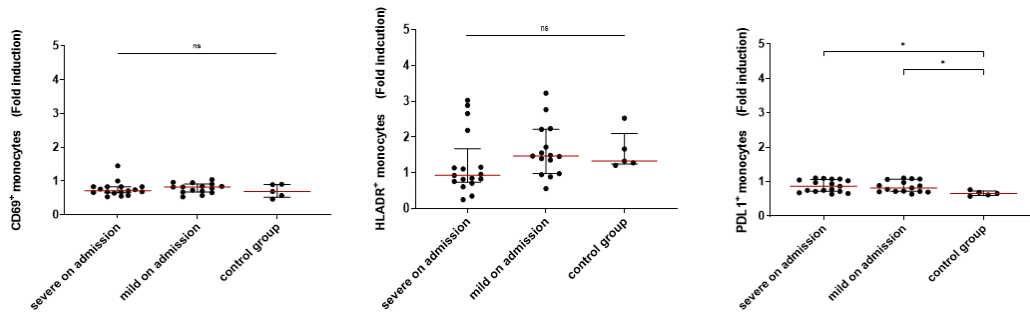


Figure 3.12. Stimulation of monocytes co-cultured with naïve CD4⁺T cells with plasma derived EVs of patients and healthy controls. Cells (monocytes/CD4⁺T cells: 150x10³/75x10³) were stimulated either with HCs (n=5) or patient (severe on admission=14, mild on admission=15) EVs (15 µg/well) for 5 days. **(a)** IFN γ , IL4 and IL10 cytokine secretions were measured by ELISA. IL4 and IL10 were significantly elevated in wells treated with severe EVs; however, IFN γ levels were similar between the groups. Each dot represents a donor. Results were given as median (red horizontal line) with interquartile ranges. **(b)** PDL1 was increased on the CD4⁺T cells treated with severe EVs. Data are presented as fold induction. **(c)** CD69, HLADR and PDL1 on monocytes were analyzed in flow cytometry, **(e)** all the markers were shown collectively among the groups. Bar graphics were given as median with interquartile ranges. The Kruskal-Wallis test was performed following Dunn's multiple comparisons to evaluate the significance of differences between groups.

4. CHAPTER 4

DISCUSSION

Extracellular vesicles (EVs) are gradually emerging as a novel intercellular communication mediators both in healthy and diseased conditions (331). Although many studies have showed the role of EVs in pathogenesis of viral infections (332–334), the results are conflicting. Some reports have demonstrated that EVs promote viral stability and favor viral propagation (335), others have suggested that EVs trigger antiviral immune responses and control viral replication (336). Throughout the pandemic, numerous studies on cell, antibody, and cytokine profiling in COVID-19 have been published. However, data on EVs in SARS CoV-2 infection (160,314,337–343) are limited. Herein, we studied the function of plasma derived severe and mild COVID-19 pediatric patient EVs, which may have a potential role in the disease pathogenesis.

Our results showed that the immunological response of children against COVID-19 did not vary by the presence of asthma. Conversely, the results differed depending on the severity and stage of COVID-19. All cytokines, except for IFN γ were higher in the plasma of severe acute patients than those in mild acute ones. When PBMCs of severe patients were stimulated with SARS CoV-2 specific peptide pool, they expressed not only more activation and inhibitory markers, but also less amount of IFN γ compared to mild ones. EVs were obtained by differential ultracentrifugation, which is the most studied method for the EV isolation (322). Accordingly, increased protein concentration of EVs was determined in the plasma of COVID-19 patients, and this was the highest in the severe acute cases. Since the monocytes CD4⁺T cells were shown to be highly reactive peripheral immune cells in SARS CoV-2, we investigated the function of EVs on these two cell populations (226,315,344). According to our results, white blood cell (WBC)-derived EVs were frequently secreted from T cells, monocytes, and B cells, respectively. Furthermore, inhibitory marker PDL1 was found to be higher in T cell originated EVs of severe acute patients compared to those in mild ones. Treg and Th2 subpopulations were increased, whereas Th1 and Th17 were suppressed in healthy

CD4⁺T lymphocytes stimulated with acute COVID-19 EVs. Additionally, activation and especially inhibitory markers were upregulated following the stimulation of healthy CD4⁺T lymphocytes with severe acute patient EVs. Furthermore, patient EVs suppressed the differentiation of healthy monocytes to M1 type macrophages. This suppression was evident in monocytes treated with severe acute EVs. Moreover, CD4⁺T lymphopenia and monocytopenia were significant in severe acute patients, and EVs from these patients also triggered CD4⁺T lymphopenia and monocytopenia in healthy cell populations.

The starting point of this study was to compare the SARS CoV-2 immune responses of children with and without asthma. During the early stages of the pandemic, asthma was identified as a potential risk factor for COVID-19 (305,345). Therefore, our hypothesis was that the patients with asthma would show more exaggerated immune responses. However, our results were quite similar between the groups with and without asthma. Upon further investigation, we discovered that the immune responses in COVID-19 was mostly affected by the severity of the disease rather by the presence of asthma. It should also be noted that, patients in this study had well-controlled asthma, and almost half of them did not use inhaled corticosteroids (ICS). Although our medical center is a tertiary reference hospital, and severe cases are referred to both pediatric allergy and pediatric infectious diseases departments, patients with asthma were not observed to be more severely affected or suffer more from COVID-19 than those without asthma during the recruitment period. These data suggest that controlled asthma does not provide an increased risk for the severity of SARS CoV-2 infection. As the pandemic progressed, it has been well-documented that the course of COVID-19 in the patients with and without asthma are similar, and asthma is not a risk factor for SARS CoV-2 infection (346). Several mechanisms have been proposed to explain the possible reasons. One of them is that the use of ICS lead to decreased expression of ACE2 receptors in the respiratory tract and suppression of IL6 and IL8 production (305,347). The other possible reasons may be the high compliance of personal distance, face mask, hygiene rules and annual vaccination for the protection against the seasonal influenza and pneumococcus in patients with asthma (306).

There are many conflicting data on the plasma level of IFN γ in SARS CoV-2 infection. Some studies have shown the high level of IFN γ in severe disease (348), whereas the others have presented the contradictory results (349,350). In our study, plasma IFN γ

levels were suppressed in severe acute patients compared to mild ones. Similar results have been published, and the possible mechanism was explained by Yang et al (324). Since IFN γ is an important cytokine for viral clearance, inadequate IFN γ response in acute phase results in suboptimal activation of the adaptive immune system and high viral load. These elevated virus load leads to significant tissue damage, trigger a delayed surge of IFNs, and an intensified inflammatory reaction. An intriguing study showed that T lymphocytes of asymptomatic COVID-19 patients had more IFN γ and other proinflammatory cytokines in early phase, compared to symptomatic cases (351). Thus, a deficient early IFN response followed by an exaggerated inflammatory condition is the hallmark of severe disease (324). The low level of IFN γ in early phase in severe patients has been attributed to some genetic mutations which impairs IFN γ production such as IFN γ -874*A polymorphisms (352–354) and X-linked TLR7 deficiency (355). Taken together, delayed and insufficient amount of IFN γ may cause ineffective antiviral responses and contribute to the severe clinical phenotype in COVID-19.

In our study, the increased IL10 was also remarkable in severe acute patients. Studies have shown that in the early stage of COVID-19, there is a drastic increase in the anti-inflammatory cytokine IL10 before the elevation in other cytokines, and this increase is proportional to the severity of the disease (356,357). The increase in other proinflammatory cytokines despite high IL10 levels suggests that IL10 may fail to suppress the proinflammatory cytokines (358) or have an unexplained proinflammatory role (359). However, these propositions need to be clarified. In our study, IL4 and IL13 were also increased in severe patients. These cytokines have been previously shown to be associated with the disease severity and mortality (232,360–363). A study has documented that increased IL13 promotes the accumulation of proinflammatory molecule hyaluronan in the lungs of SARS CoV-2 infected mice, and hyaluronan accumulation has been reversed by IL13 blockade (363). In contrast to the proinflammatory effects of Th2 cytokines, they also have anti-inflammatory functions such as suppressing Th1 responses and inducing Treg formation (364,365). In recent studies, it has also been shown that severe COVID-19 patients with low levels of IFN γ probably have an immunosuppressive state (344,366,367). Increased IL10 and Th2 cytokines, which are remarkably increased in severe patients both may cause hazardous proinflammatory consequences and may also contribute to this immunosuppressive condition.

There are many publications regarding T cell functions in COVID-19 after the stimulation with SARS CoV-2 specific peptides. As also shown in our study, these reports have indicated that CD4⁺T cells generally respond better to the SARS CoV-2 specific peptides than CD8⁺T cells, and peptides trigger the secretion of cytokines, and upregulation of the activation markers (315,362,368). After the stimulation with SARS CoV-2 peptides, our patients exhibited higher IFN γ responses compared to healthy donors, similar to previous results (369,370). Contrary to our assumption, we obtained less amount of IFN γ from severe patients than mild ones after peptide stimulation. In the meanwhile, expression of both activation and inhibitory markers showed a significant upregulation on the T cells of severe patients compared to mild counterparts. All these results were compatible with the prior findings about SARS CoV-2 specific peptide stimulation of T cells (315,369,371,372). Briefly, upon the incubation of patient T cells with SARS CoV-2 peptide pool, responses seemed to be in parallel with their pre-stimulated states.

The current study showed that the amount of EVs was higher in the plasma of COVID-19 patients compared to healthy controls, and this was proportional to the disease severity (373,374). During viral infections, host immune cells are activated, and the amount of EVs released by these cells are increased (375). Depending on the severity and stage of the disease, diversity of the plasma EV pool varies. In COVID-19 and healthy conditions, EVs mostly originate from platelets, erythrocytes, and endothelial cells (376,377). In the healthy state, lymphocyte- and monocyte-derived EVs contribute to the plasma EV pool to a limited extent (378). As these cells are highly activated and have important roles in SARS CoV-2 infection, these two cell-derived EVs are increased in COVID-19 (378). In fact, Burrello *et al*, showed that T lymphocytes, B lymphocytes, and other inflammatory cell derived EVs were increased in COVID-19 patients and associated with poor disease prognosis (374). In our study, T cell and subsequently monocyte derived EVs were found to be abundant in the plasma. Prior findings and our results indicate a possible lymphocyte and monocyte predominant immune response in SARS CoV-2 infection.

We found that activation markers were increased in patient EVs similar to previous reports (374,379). Besides, inhibitory marker Programmed cell death 1 ligand (PDL1) was also increased in T cell derived plasma EVs of severe patients compared to that of EVs from mild patients. PDL1 is mainly expressed on antigen-presenting cells (APCs),

binds to its receptor PD-1 on T cells, and contributes to the suppression of the immune responses by induction of Tregs (380). In recent years, PDL1 has been shown to be expressed not only on APCs but also on T cells; however, its function has not been fully understood. PDL1 on T cells inhibits the effector function of T cells and macrophages by depressing TCR-mediated signaling and self-activation (381–383). Proinflammatory cytokines including, IL6, TNF α , IL1 α , IL1 β and specifically IFN γ increase PDL1 expression (384). Nevertheless, the amount of IFN γ in the plasma of severe patients was low according to our results. One of the possible reasons of high levels of PDL1 in severe patients despite low levels of IFN γ may be due to other increased proinflammatory cytokines that have potential to induce PDL1 expression in severe patients. Another possible mechanism may be related to the protein Chemokine-like factor (CKLF)-like MARVEL transmembrane domain containing family member (CMTM6), which is ubiquitously expressed on the cell surfaces and defined as the only regulatory protein of PDL1 by preventing its degradation. Moreover, CMTM6 can induce the expression of PDL1 in the absence of IFN γ (385). Therefore, such increased and strong PDL1 expression in severe COVID-19 patients even at low levels of IFN γ , may be due to the sustained synthesis of CMTM6 protein in these patients (386). However, the studies describing the relationship between CMTM6 and COVID-19 are quite scant (387–389). Furthermore, PDL1 on T cells induces a highly suppressive and unique peripheral induced Treg (iTreg) population which is a potent source of IL10 (383). By upregulation of PDL1 on Treg, the hazardous results of effector T cells and inflammation can be limited (383). The direct interaction of PDL1 on the Tregs with PD-1 on B cells triggers the apoptosis of autoreactive B cells (390). As far as is known, severe COVID-19 is associated with autoreactive B cell activation that leads severe symptoms such as autoantibody-linked blood clotting, anti-interferon antibodies, connective-tissue disease associated interstitial lung disease (391). Increased PDL1 on Tregs may have a role in immune modulation, elimination of autoreactive cells and balancing of exaggerated immune responses in severe patients. From another perspective, high levels of IL10 in severe COVID-19 patients induces PDL1 expression. Subsequent binding of PDL1 with PD-1 promotes the inhibition and exhaustion of T cells, facilitates virus replication and poor T cell effector functions (392). Taken together, increased inhibitory markers in severe patients may have a dual effect, protecting patients from excessive immune response, on the one hand, contributing to the worsening clinical condition of COVID-19 patients. Considering the

documented immunosuppressive features in severe COVID-19 patients (344,366,367) and the findings of our study, it can be deduced that immune balance in severe acute patients may probably shift to the suppressive direction which cause the deterioration of the clinical manifestations.

Our study strongly supports that plasma derived EVs of COVID-19 patients play a substantial role in the immune responses. The EVs boosted the expression of surface markers, chemokine receptors, activation of cells and secretion of various cytokines (342). Conversely, significant increase in inhibitor markers, IL10 and Th2-related cytokines especially in severe acute patients supported the suppressive aspect of EVs. However, during the convalescence period, this suppressive effect gradually disappeared. Some immunosuppressive effects were also observed in mild patients, but that was not as prominent as in severe patient EVs. Notably, the suppressive effect caused by EVs of mild patients is more pronounced on monocytes. There are few studies on the function of plasma derived EVs associated with SARS CoV-2 infection (314,341,342). One of these studies emphasized that plasma derived EVs triggered the same set of cytokines as before EV stimulation and caused the similar immune profile as seen during the infection (342). However, detailed information about the clinical severity of the patients were not given in that study (342). Although a study composed of mild COVID-19 patients showed that response from the healthy cells after the stimulation with plasma derived EVs exhibited a similar cytokine pattern as before EV stimulation, TNF α secretion was decreased after the stimulation with acute patient EVs (341). In fact, this result underlines some suppressive effect of SARS CoV-2 related plasma derived EVs (341). Our results are compatible with this study due to a certain amount of immunosuppressive effect of mild patient EVs specifically on monocytes (341). Profound suppressive outcomes of severe patient EVs on both monocytes and T cells were clearly observed in our study. Another study presenting the effects of mild and severe convalescent patient EVs on immune cells demonstrated the immunosuppressive consequences of EVs, especially those in the mild ones (393). In contrast, suppressive effect of EVs was more pronounced in severe convalescence patients according to our findings. Furthermore, EVs of convalescent patients had suppressive functions only to a limited extent compared with acute patients. Apart from the effects of EVs on T cells, we could not find a study on the roles of SARS CoV-2 related plasma derived EVs on monocytes. Since the monocytes are antigen presenting

cells (APCs) and extensively uptake EVs, they probably have a critical role in the EV-mediated responses. As shown in our study, T cells uptake EVs to a limited extent and cannot be directly stimulated by EVs without the presence of APCs contrary to monocytes (326,327). EVs dramatically suppressed proinflammatory M1 type macrophages, and this was more prominent with severe patient EVs. This result chiefly revealed the suppressive properties of severe acute patient EVs. Considering the suppressive effect of EVs, especially in severe patients, it can be suggested that EVs may also contribute to the possible immunosuppressive condition in severe patients. In this respect, the current study may provide a different and new perspective on COVID-19 immunity.

Although the lymphopenia in COVID-19 and its association with disease severity are well-known, the exact mechanism has not been fully elucidated (394). Pontelli et al explained the lymphopenia in SARS CoV-2 by apoptosis of T cells (395). The other possible reason was explained by the tissue sequestration of T cells (396). T cells of severe patients expressed large amounts of activation markers (HLADR, CD69, CD25) and chemokine receptors, such as CCR6, CCR4 and CXCR3, as also shown in our study. These receptors and markers recruit the lymphocytes into the tissues (386). Nonetheless, this theory may not be sufficient to explain the lymphopenia in mild patients. According to another study, T cell lymphopenia results from apoptosis caused by proinflammatory cytokines secreted from inflammatory monocytes (397). Furthermore, increased amount of activated highly suppressive monocytes called myeloid derived suppressor cells (MDSC) in COVID-19 may also lead lymphopenia (394). In addition to lymphopenia, monocytopenia is seen in severe COVID-19 patients. Despite monocytopenia, monocytes have inflammatory phenotype which cause severe clinical manifestations, cytokine storm and T cell lymphopenia (398). In our study, lymphopenia and monocytopenia were seen in COVID-19 patients, mainly in severe acute cases. Monocytopenia and lymphopenia were noted not only in patient PBMCs, but also in healthy cells following the stimulation with EVs, specifically with severe acute ones. This suggests that EVs may have a potential role in SARS CoV-2 induced cytopenia.

The current study has some limitations. First, our study population was consisted of severe and mild patients. Ideally, including children with asymptomatic, moderate, and even multisystem inflammatory syndrome would contribute to understand the functions

of plasma derived EVs. But we did not have patients with those clinical presentations during the recruitment process. Secondly, we incubated EVs only with healthy cells. The culturing of patient EVs not only with healthy cells but also with patient PBMCs would also provide information on EVs. However, it is not straightforward to perform study with pediatric patients in practice, and we worked with very small amount of blood samples. Finally, it would be appropriate to investigate the proliferative effect of EVs on cells and their role in cytopenia. Despite all these limitations, including high number of children who have been relatively less studied throughout the pandemic, not pooling and well categorization of the patients according to disease severity and stage are the strengths of the current study.

In conclusion, to the best of our knowledge, this is the most comprehensive study revealing the immune functions of plasma derived EVs on two immune cell populations which have crucial functions in SARS CoV-2 infection. The study also clarified that plasma derived EVs of COVID-19 patients can provide fascinating information about the disease severity and reflect the immune status of the host cells from which EVs originated. The distinct immunosuppressive properties of EVs from severe patients, who had immunosuppressive properties during acute phase by impaired IFN γ secretion, increased IL10 and Th2-related cytokines, increased inhibitory surface marker expression and marked cytopenia, are evidence of this argument. Taken together, our findings contributed to elucidate the effects of EVs on different cell populations. However, information about the effects of plasma derived EVs on immunity is still limited and contradictory. In the future, clarifying the exact immunological roles of these promising particles may provide great benefits in predicting the progression and treatment of the diseases.

5. CHAPTER 5

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6. CHAPTER 6

APPENDICES

6.1. Appendix A

Homemade buffer recipes

10X Phosphate Buffered Saline (PBS) (for ELISA and FACS Buffer)

- 80 g NaCl
- 2 g KCl
- 15.2 g Na₂HPO₄·2H₂O
- 2 g KH₂PO₄
- 1 L of dH₂O
- pH is adjusted to 6.8, and autoclave the buffer.
- 10X PBS is diluted to 1X PBS with dH₂O.
- pH is adjusted to 7.4 for 1xPBS, then it is autoclaved.

Blocking Buffer (for ELISA)

- 25 g Bovine Serum Albumin (BSA) (5%)
- 250 µL Tween[®]20 (0.025%)
- Complete to 500 mL with 1X PBS, mix well until BSA is totally dissolved.

Wash Buffer (for ELISA)

- 4.5 L dH₂O
- 0.5 L 10X PBS

- 2.5 mL Tween[®]20

Fluorescent Activated Cell Sorting (FACS) Buffer (for Flow Cytometry)

- 5 g BSA - 250 mg NaN₃
- 500 ml 1X PBS
- Store at +4°C

RIPA Buffer (SDS Page-Western Blot)

- 2 M NaCl
- 1 M Tris (pH 8)
- Nonidet P-40
- 10% SDS
- Protease inhibitor cocktail (Thermo Fisher)
- Fresh phosphatase inhibitors (Thermo Fisher)

10X Running Buffer (SDS Page-Western Blot)

- 144 g Glycine
- 30 g Tris-Base
- 10 g SDS
- 1 L dH₂O
- To prepare 1X Running Buffer, dilute 10X buffer with dH₂O.

10X Transfer Buffer (SDS Page-Western Blot)

- 144 g Glycine

- 30 g Tris-Base
- 1 L dH₂O
- Store at +4°C
- To prepare 1X Transfer Buffer:
 - ✓ 700 ml dH₂O
 - ✓ 200 ml Methanol
 - ✓ 100 ml 10X Transfer Buffer
 - ✓ Store at -20°C

Blocking Buffer (SDS Page-Western Blot)

- 2.5 g Milk powder
- 50 µl Tween[®]20
- 50 ml 1X PBS

PBS-T Buffer (SDS Page-Western Blot)

- 999 ml 1X PBS
- 1 ml Tween[®]20

Antibody Dilution Buffer (SDS Page-Western Blot)

- 2.5 g Milk Powder
- 25 µl Tween[®]20
- 50 ml 1X PBS

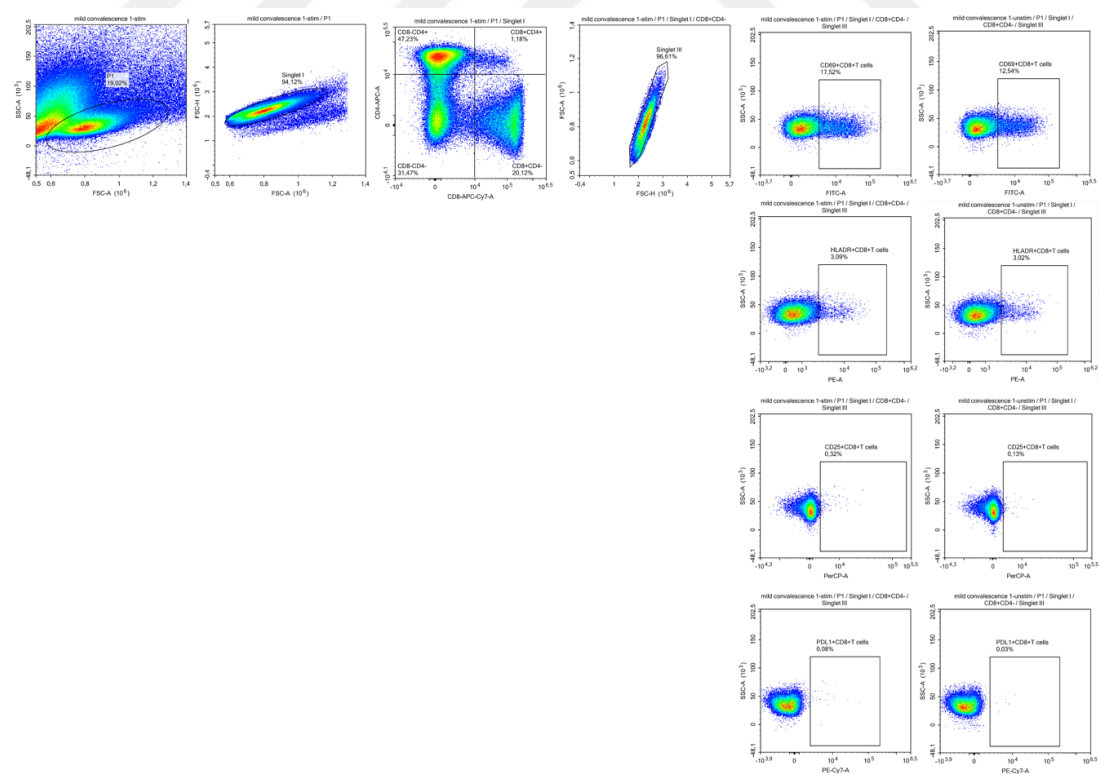
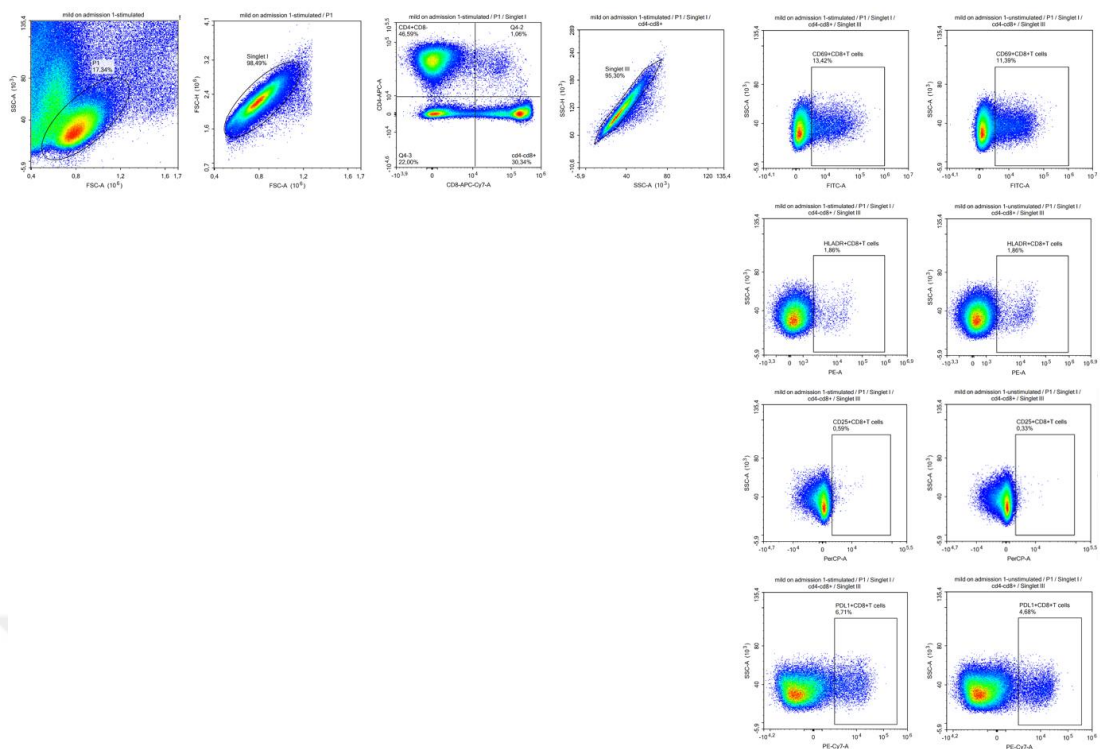
MACS Buffer (for isolation of monocytes and naïve CD4⁺T cells)

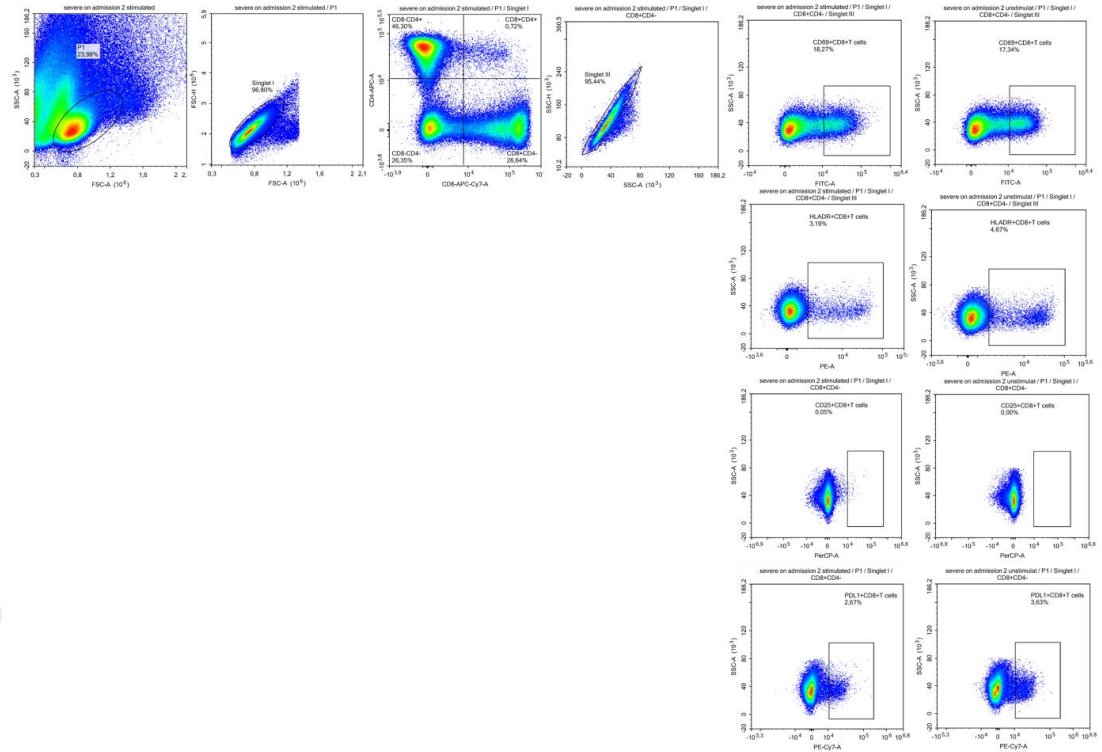
- 2.5 g BSA (0.5%)

- 2 nM EDTA
- 500 mL 1xDPBS
- Store at +4°C

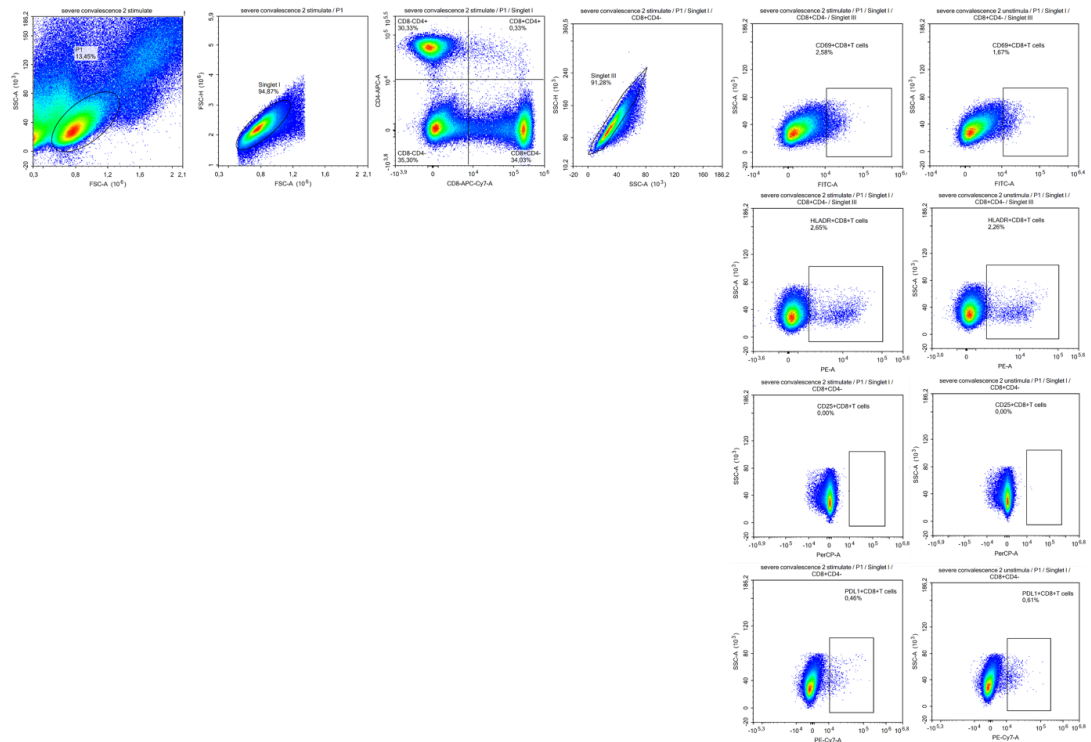






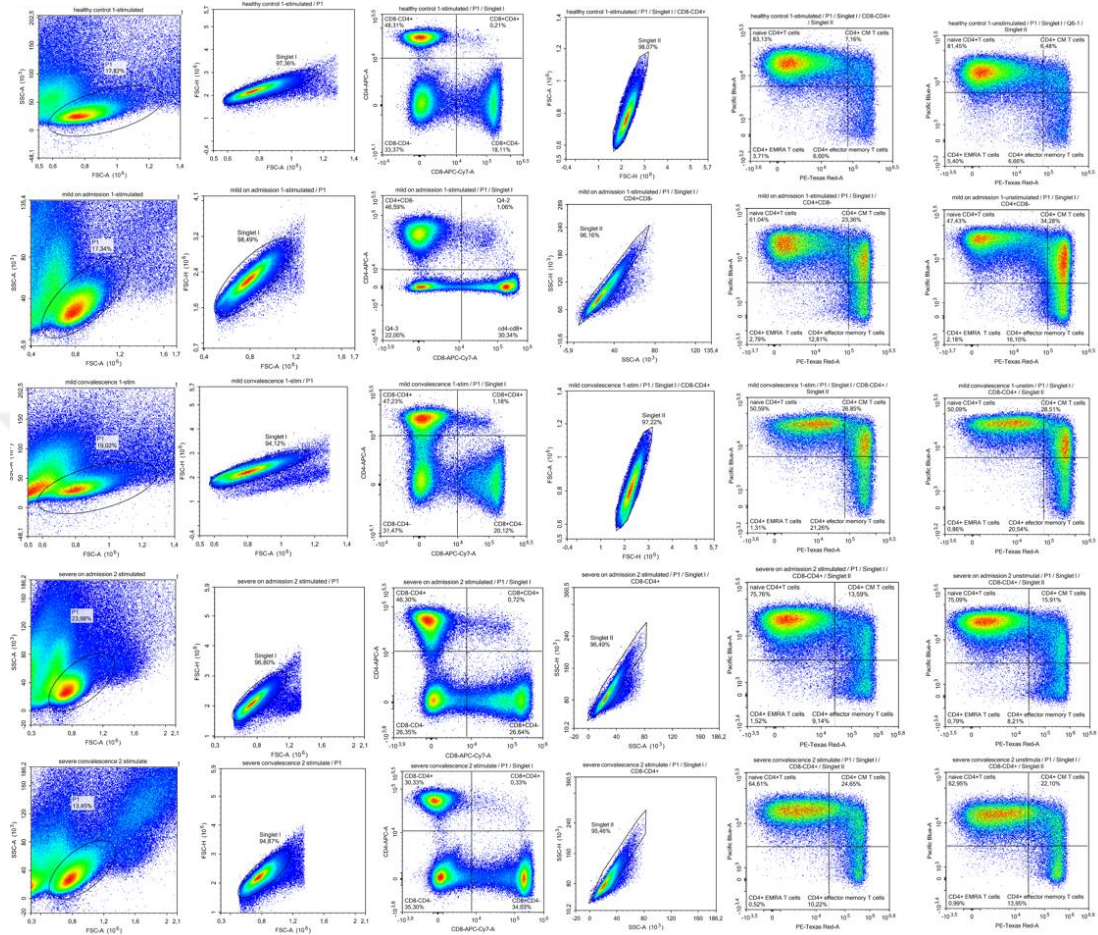


(e)

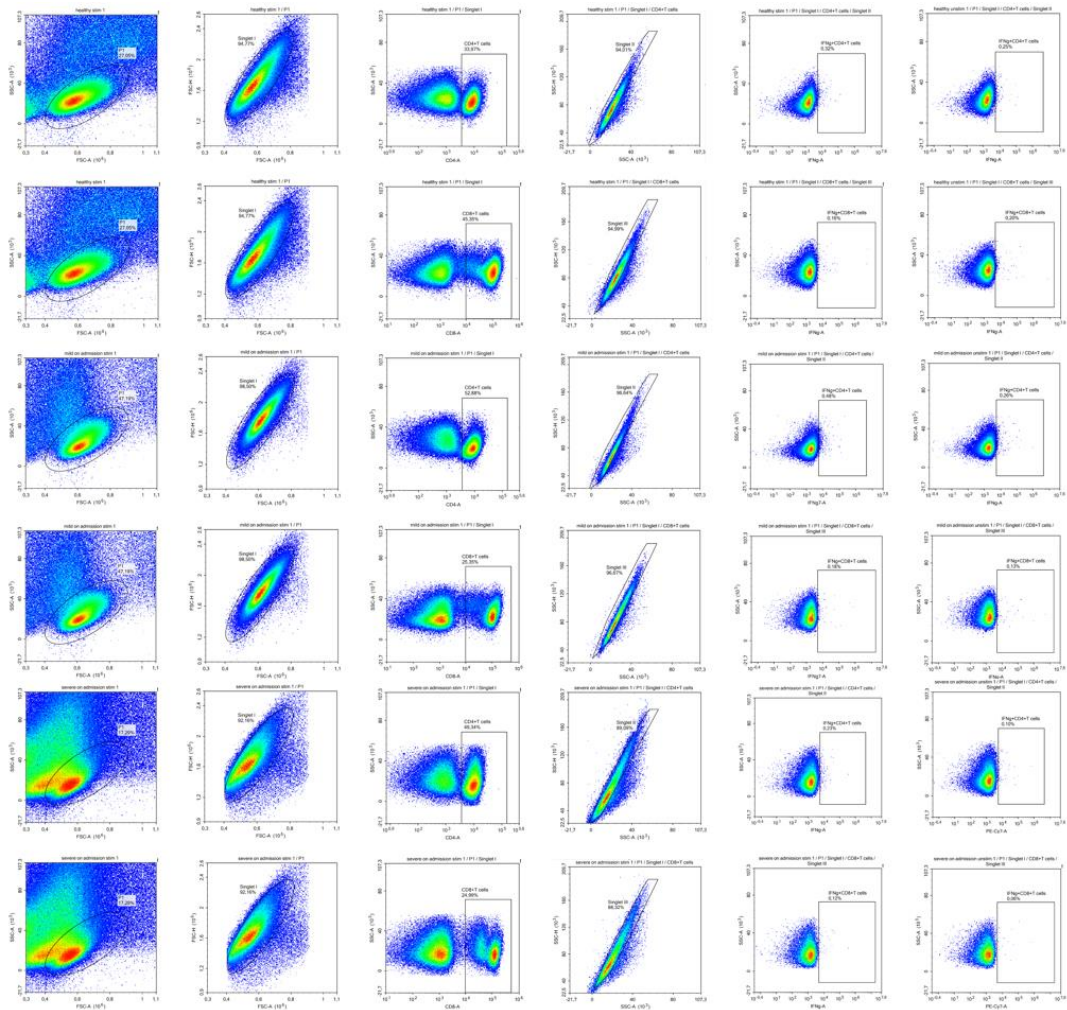


Appendix Figure 2. SMNO stimulated CD8⁺T cells and gating strategy of CD69, HLADR, CD25 and PDL1 of (a) stimulated and unstimulated healthy control, (b) stimulated and unstimulated mild on admission, (c) stimulated and unstimulated mild convalescence, (d)

stimulated and unstimulated severe on admission, (e) stimulated and unstimulated severe convalescence patients.

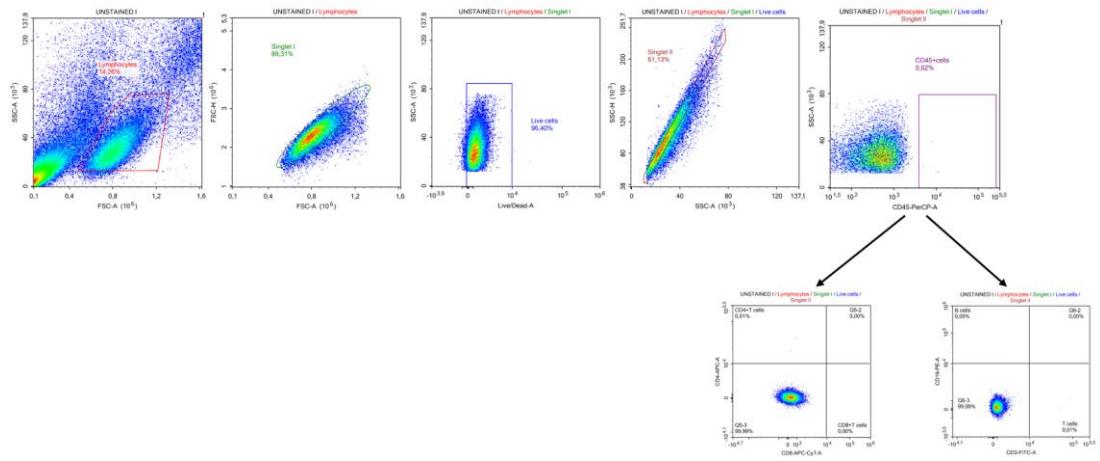


Appendix Figure 3. SMNO stimulated CD4⁺T cells and gating strategy of naïve, central memory, effector memory and EMRA population of stimulated and unstimulated healthy control, stimulated and unstimulated mild on admission, stimulated and unstimulated mild convalescence, stimulated and unstimulated severe on admission, stimulated and unstimulated severe convalescence patients.

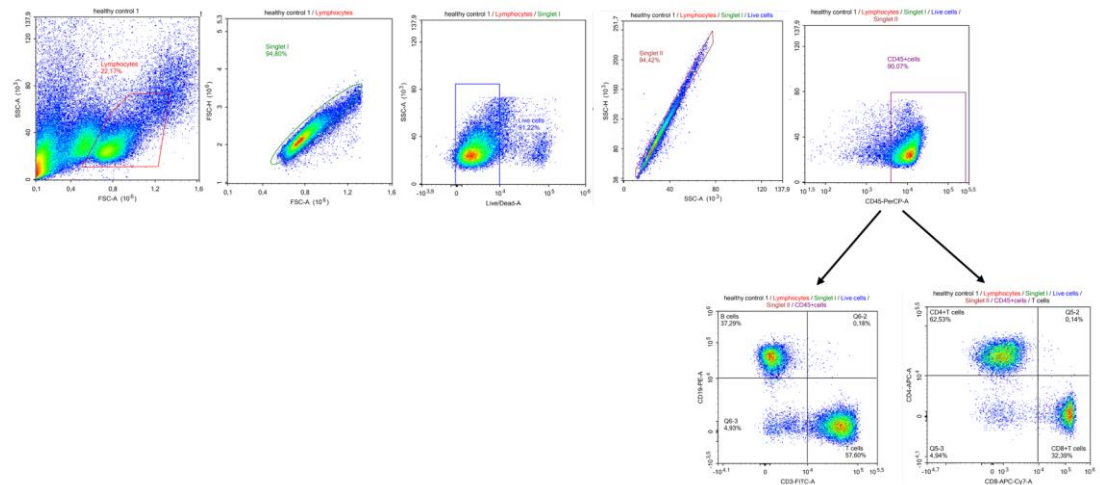


Appendix Figure 4. Gating of intracellular IFN γ secretion from CD4⁺T cells upon the SMNO peptide pool stimulation in healthy controls, mild on admission and severe on admission patients.

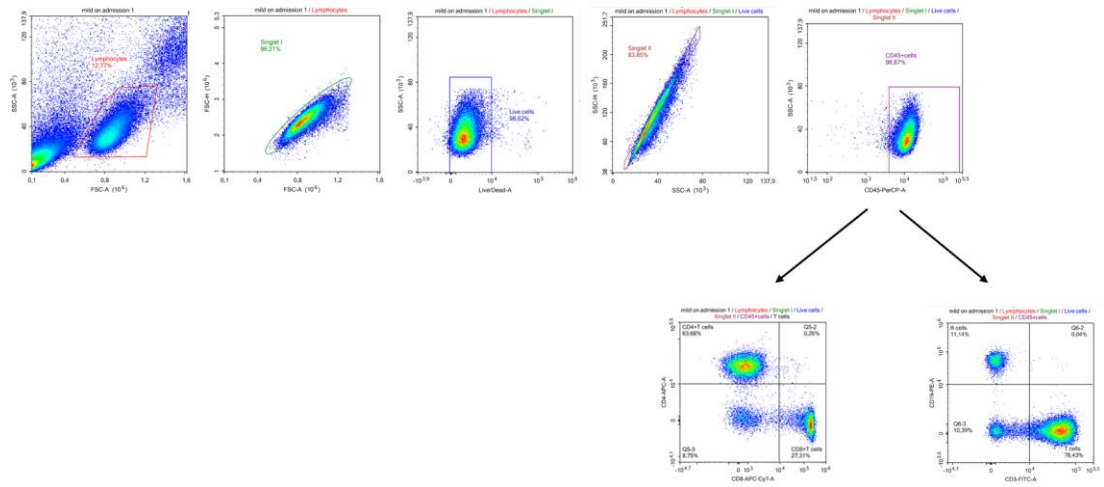
(a)



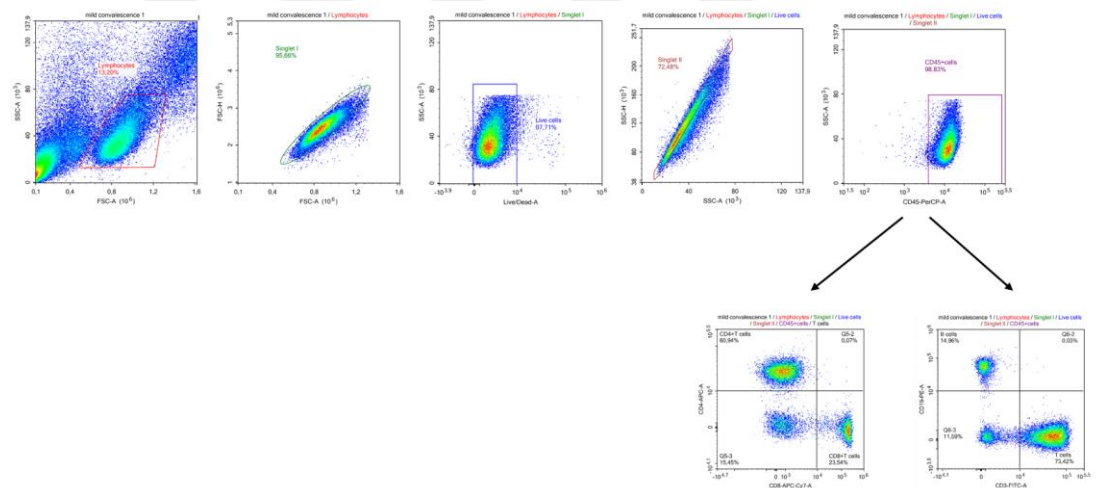
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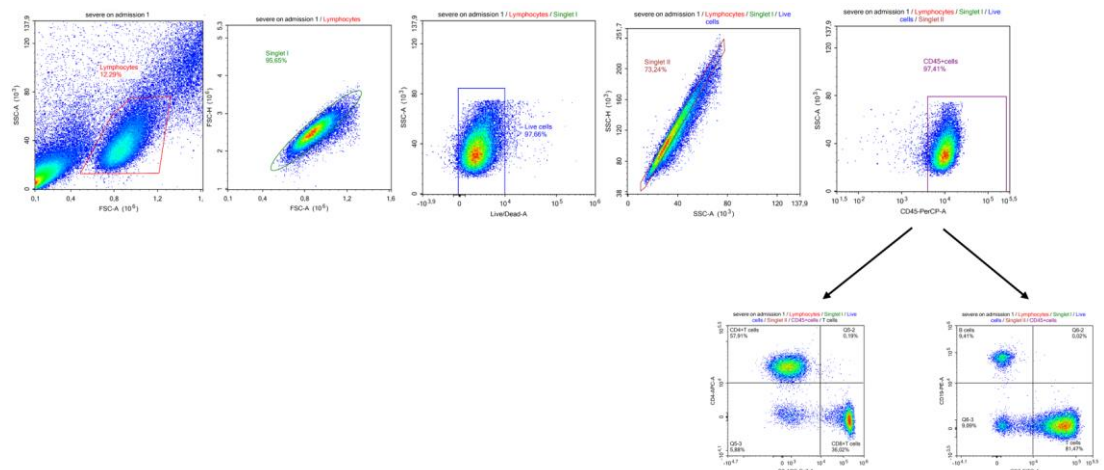
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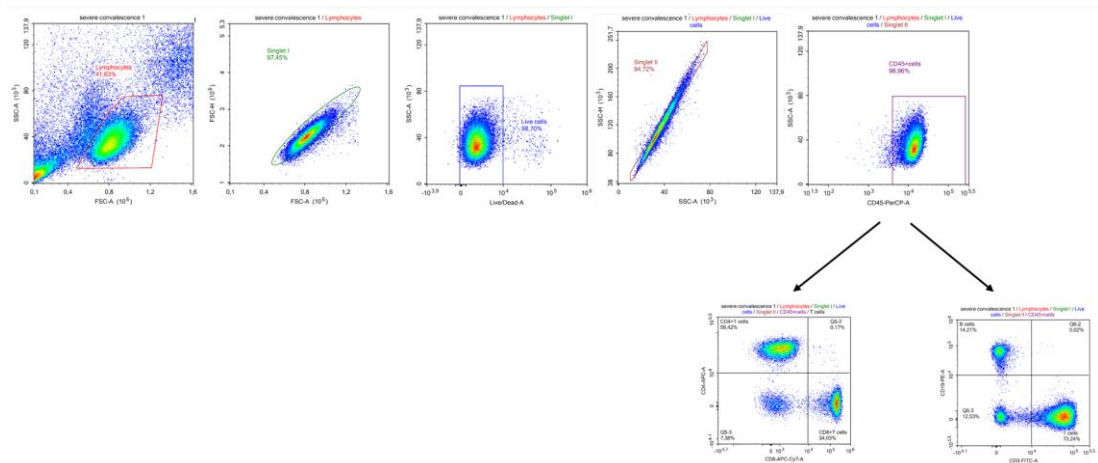
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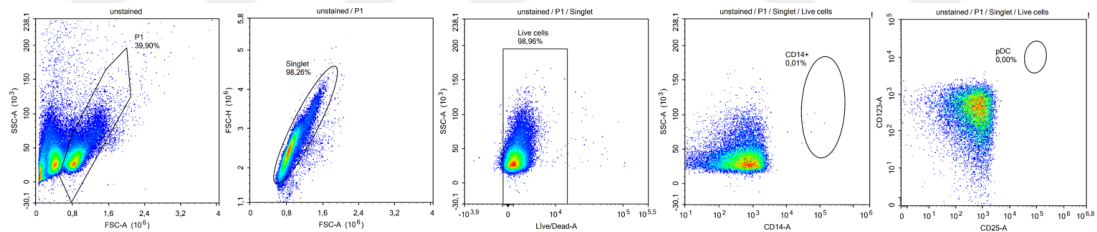


(f)

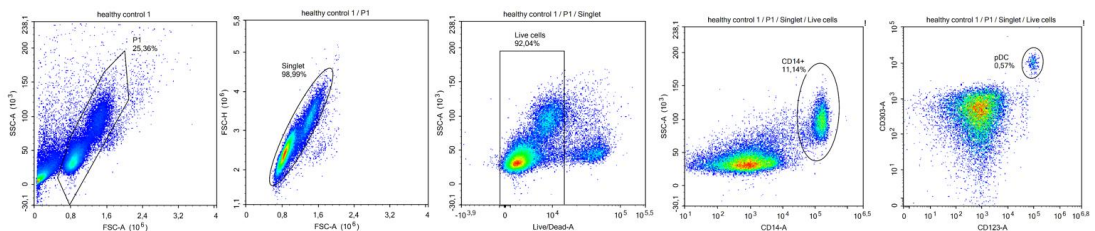


Appendix Figure 5. Gating strategy of CD4⁺T, CD8⁺T cells and B cells for (a) unstained, (b) healthy control, (c) mild on admission, (d) mild convalescence, (e) severe on admission, (f) severe convalescence patients.

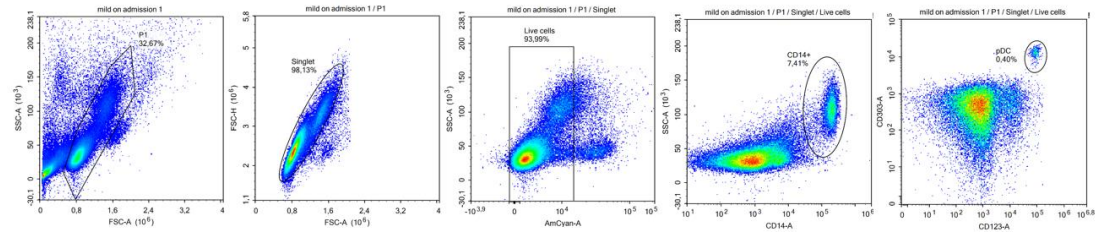
(a)



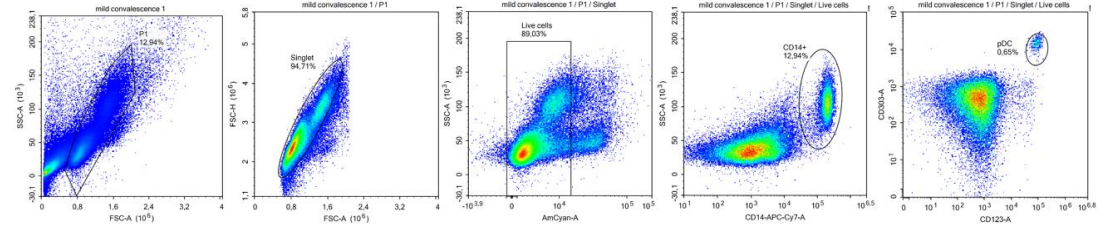
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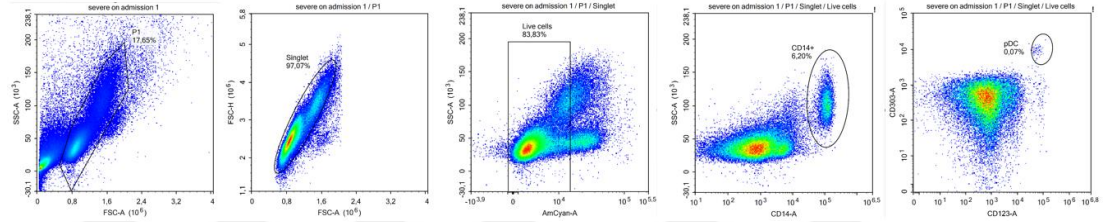
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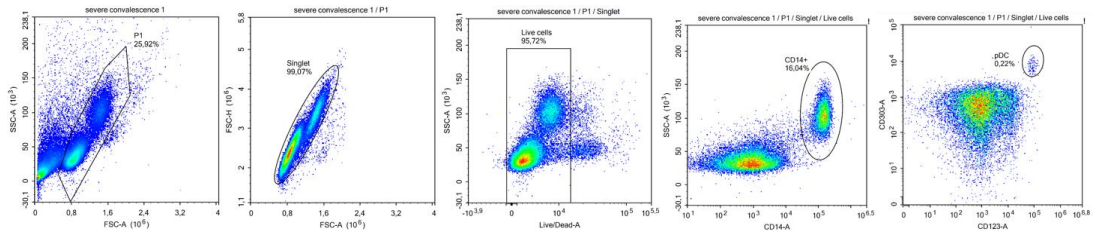
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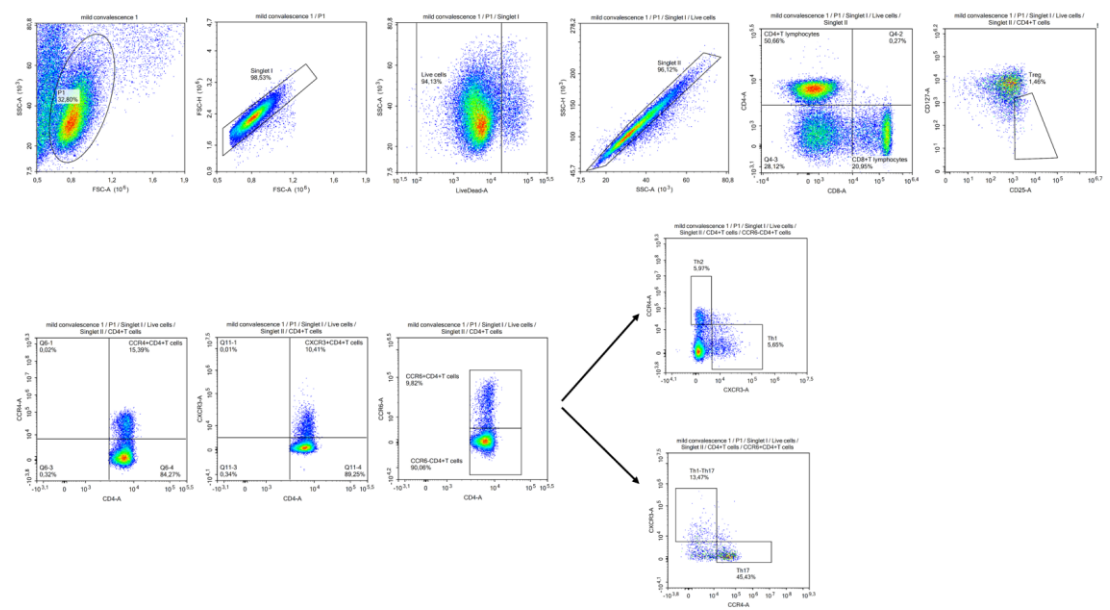
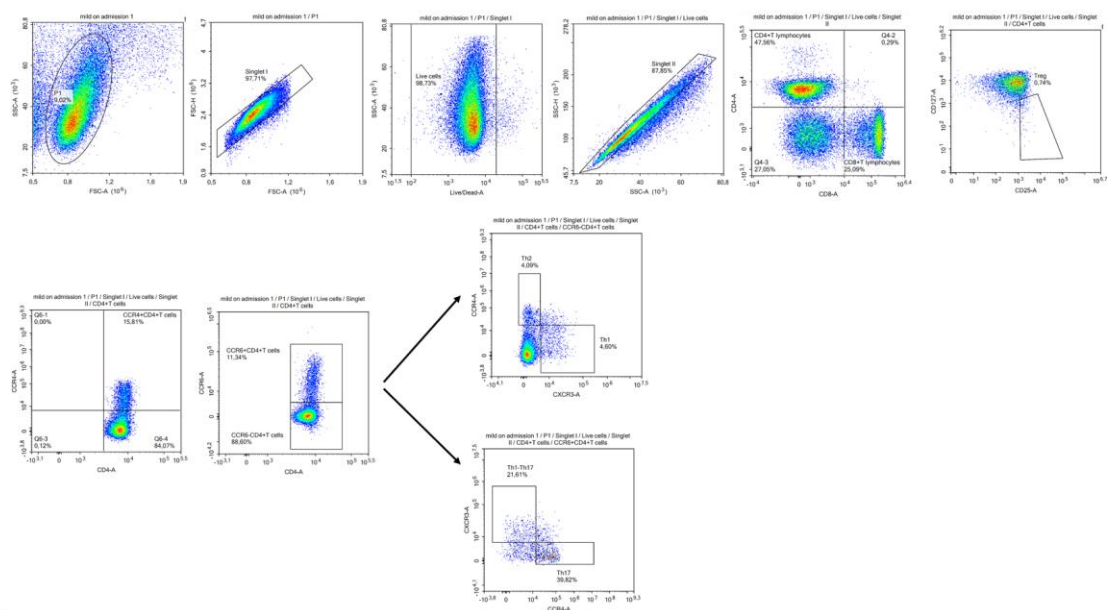


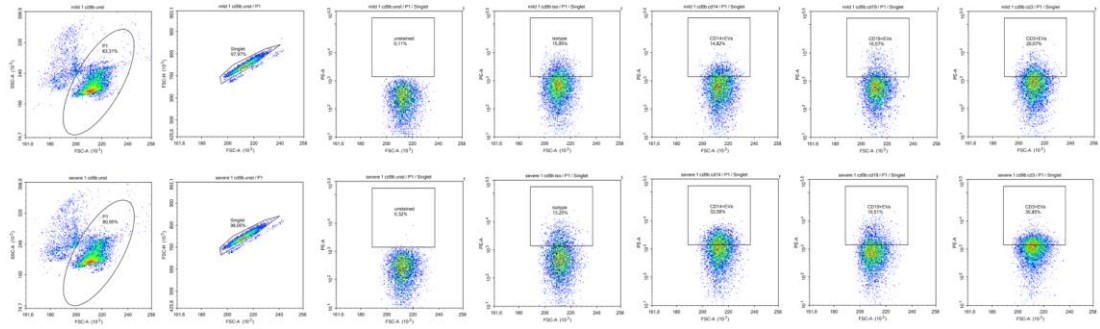
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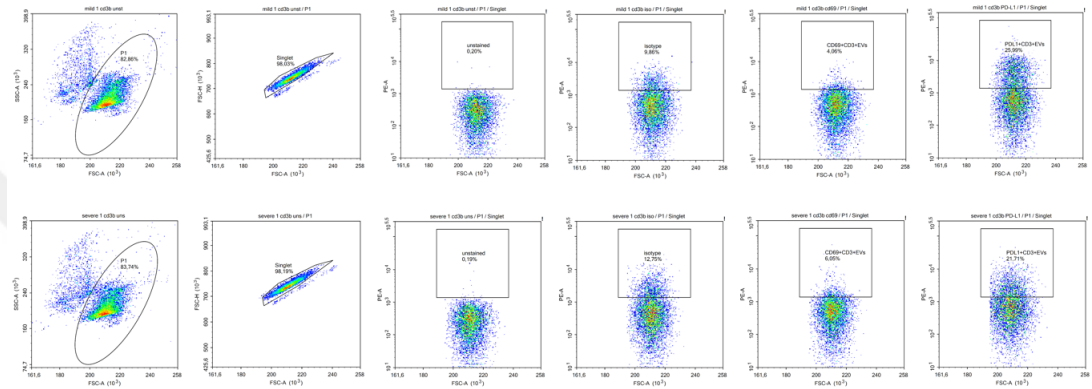
Appendix Figure 6. Gating strategy of plasmacytoid dendritic cells (pDC) and monocytes for (a) unstained, (b) healthy control, (c) mild on admission, (d) mild convalescence, (e) severe on admission, (f) severe convalescence patients.

(a)

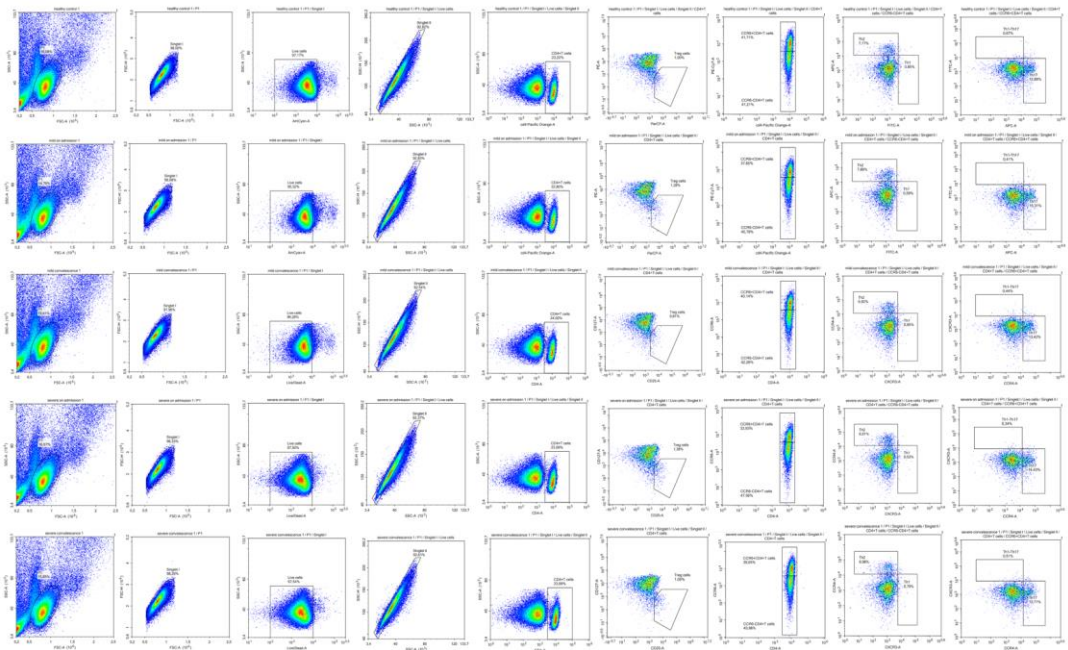




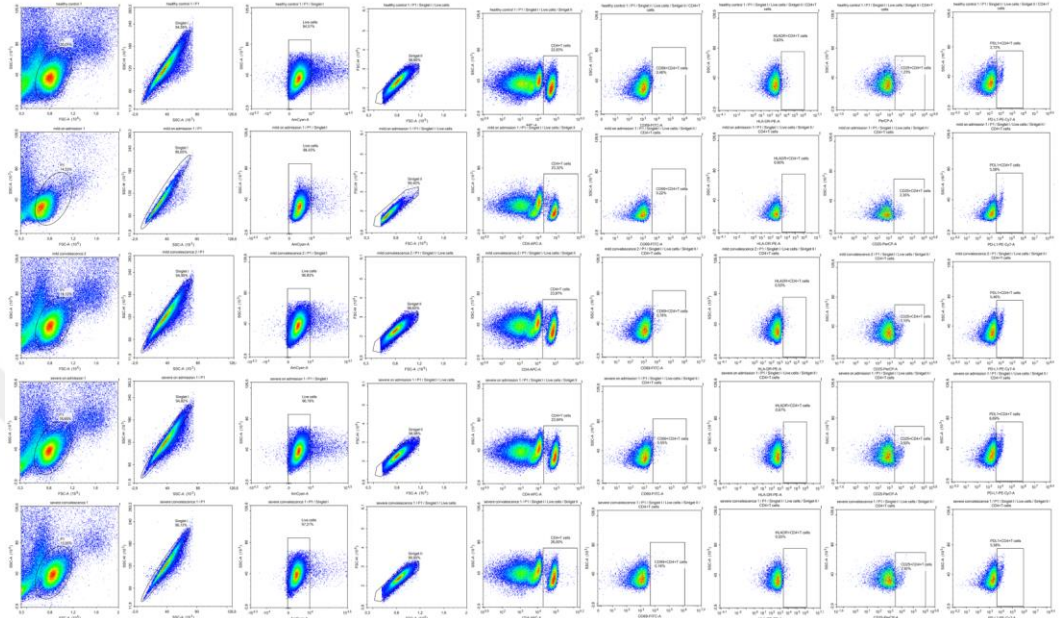
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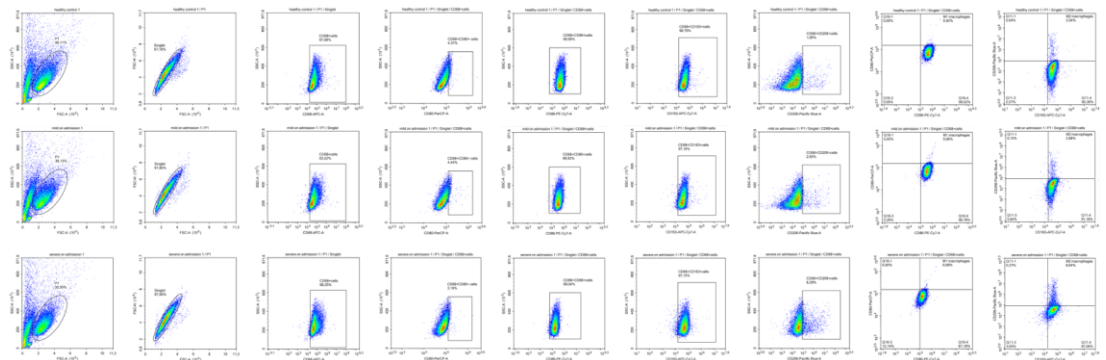
Appendix Figure 8. Origin of EVs obtained from (a) mild and severe on admission, activation, and inhibitory markers on EVs obtained from (b) mild and severe on admission patients.



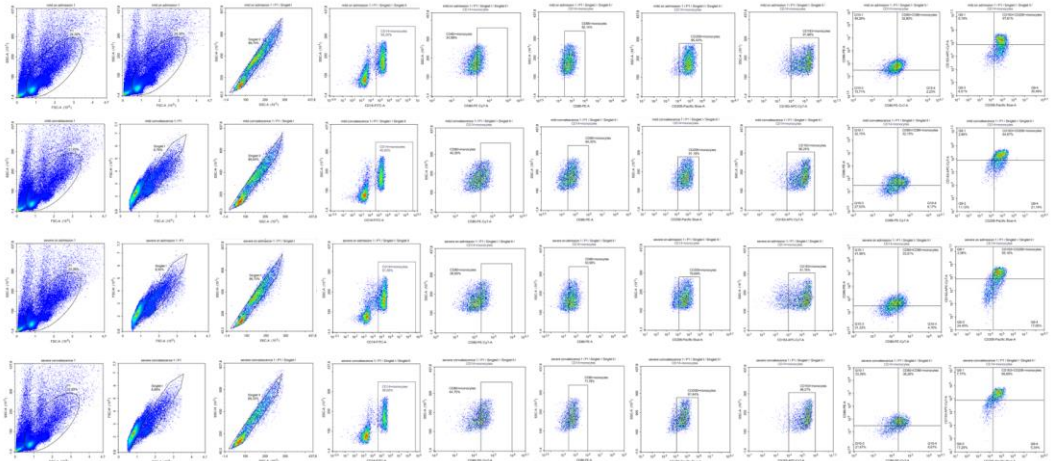
Appendix Figure 9. Gating of CD4⁺T cell subsets in healthy PBMCs which were stimulated with EVs of healthy controls, mild on admission, mild convalescence, severe on admission and severe convalescence patients.



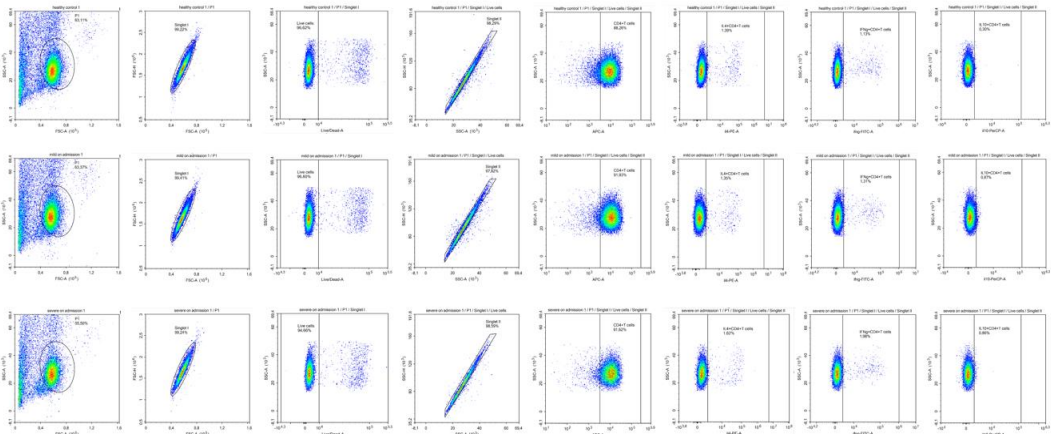
Appendix Figure 10. Gating of activation and inhibitory markers on CD4⁺T cells in healthy PBMCs which were stimulated with EVs of healthy controls, mild on admission, mild convalescence, severe on admission and severe convalescence patients.



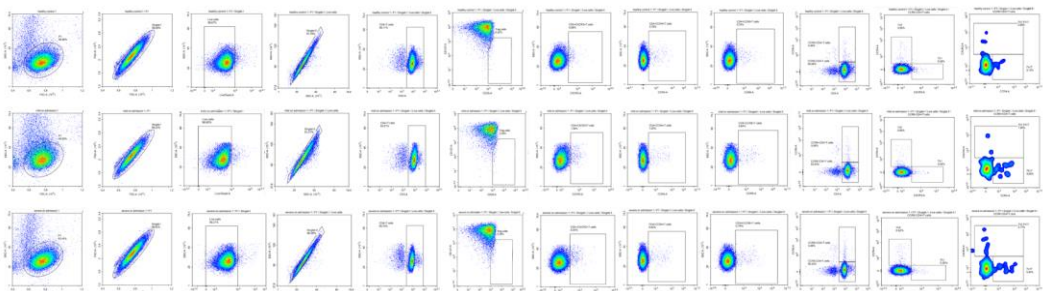
Appendix Figure 11. Gating of isolated healthy monocytes which were stimulated with EVs of healthy controls, mild on admission, and severe on admission patients.



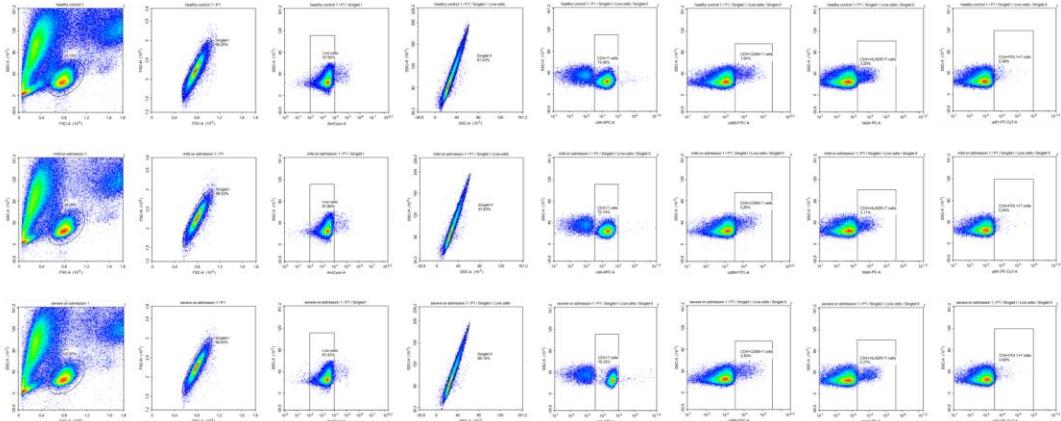
Appendix Figure 12. Gating of surface markers on monocyte population of healthy PBMCs that were incubated with EVs obtained from mild on admission, mild convalescence, severe on admission and severe convalescence patients.



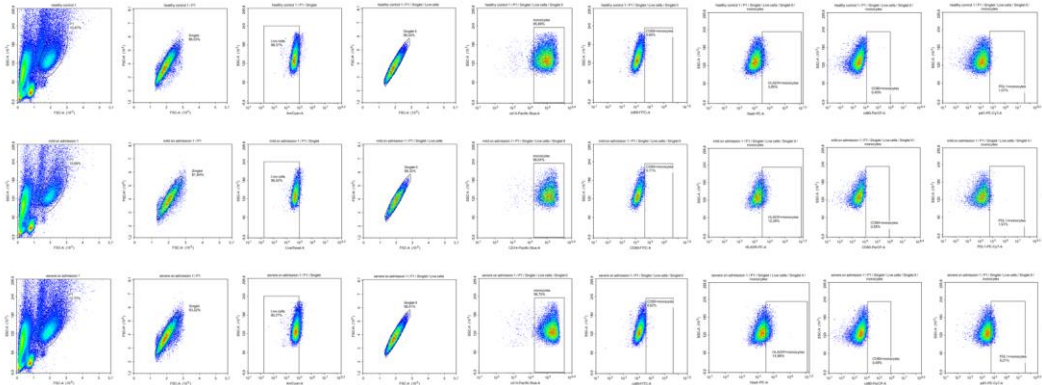
Appendix Figure 13. Gating of intracellular cytokine secretion from isolated healthy CD4⁺T cell subsets which were stimulated with EVs of healthy controls, mild on admission, and severe on admission patients.



Appendix Figure 14. Gating of surface markers on isolated healthy CD4⁺T cell population that were incubated with EVs obtained from healthy controls, mild on admission, and severe on admission patients.



Appendix Figure 15. Gating of surface activation and inhibitory markers on isolated healthy CD4⁺T cells cocultured with isolated healthy monocytes which were stimulated with EVs of healthy controls, mild on admission, and severe on admission patients.



Appendix Figure 16. Gating of surface activation and inhibitory markers on isolated healthy monocytes cocultured with isolated healthy CD4⁺T cells which were stimulated with EVs of healthy controls, mild on admission, and severe on admission patients.

7. CHAPTER

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