

**DEVELOPMENT OF A COMBINATION VACCINE AGAINST N.
MENINGITIDIS AND SARS-COV-2 AND ANALYSIS OF MIS-C
PLASMA AND EXTRACELLULAR VESICLES IN RELATION
TO DISEASE SEVERITY IN PEDIATRIC PATIENTS**

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By

Tuğçe Canavar Yıldırım

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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 beloved daughter Mira Ada, dear husband, mom, dad, and
brother who make me feel like the luckiest person in the world**



ABSTRACT

DEVELOPMENT OF A COMBINATION VACCINE AGAINST N. MENINGITIDIS AND SARS-COV-2 AND ANALYSIS OF MIS-C PLASMA AND EXTRACELLULAR VESICLES IN RELATION TO DISEASE SEVERITY IN PEDIATRIC PATIENTS

Tuğçe Canavar Yıldırım

PhD in Molecular Biology and Genetics

Advisor: İhsan Gürsel

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Neisseria meningitidis is the causative agent of invasive meningococcal disease (IMD). Serogroup B remains the leading cause of IMD, representing 62% of documented serogroup cases overall, and is the most prevalent across all age groups under 65. In Türkiye, Serogroups B and W are responsible for most cases, accounting for over 75%. IMD has a case fatality rate of 10%, and 10% to 20% of survivors experience lifelong, disabling complications. Coronavirus disease 2019 (COVID-19), caused by the highly contagious SARS-CoV-2 virus, has had a devastating impact globally, leading to over 7 million deaths. It has become the most significant global health crisis since the influenza pandemic in 1918. Since the introduction of the first COVID-19 vaccine in the U.S., it is estimated that over 18 million hospitalizations and 3 million deaths have been prevented. Like flu vaccines, COVID-19 vaccines are expected to be introduced seasonally. In this thesis, we aim to develop a combination vaccine for populations where the prevalence of both COVID-19 and meningitis presents significant health risks. Our previous data indicated that our OMV-based bivalent vaccine, targeting B and W serogroups of *Neisseria meningitidis*, generates a broader humoral response and exhibits strong bactericidal activity. Similarly, our lab has developed a virus-like particle-based SARS-CoV-2 vaccine incorporating all four virus structural proteins, eliciting both humoral and cell-mediated immunity. Herein, we combined these two platforms and assessed their respective protective potencies in mice. Data revealed that combining the OMV and VLP vaccines with Alum adjuvant produced the most robust anti-meningococcal and anti-SARS-CoV-2 responses.

The second part of the study focused on uncovering the immune parameters present in the plasma of MIS-C patients and investigating the role of extracellular vesicles in influencing disease severity. The data revealed that plasma from MIS-C patients contained significantly elevated levels of proinflammatory cytokines, including TNF- α , IFN- γ , IL-6, and IL-17. We also investigated the pathological role of extracellular vesicles (EVs) in MIS-C. Our findings showed that patient-derived EVs induced significantly higher levels of IRF9 in the THP1-dual reporter cell line. Contrary to our expectations, however, we did not observe EV-specific NF- κ B induction in the THP1 cells.

Keywords: SARS-CoV-2, COVID-19, *Neisseria meningitidis* vaccine, Outer membrane vesicles, MIS-C, Extracellular vesicles, Humoral and cell-mediated immune response

ÖZET

N. MENİNGİTİDİS VE SARS-COV-2'YE KARMA AŞI GELİŞTİRİLMESİ VE PEDIATRİK HASTALARDA HASTALIK ŞİDDETİ İLE İLİŞKİLİ OLARAK MIS-C PLAZMASI VE EKSTRASELÜLER VEZİKÜLLERİN ANALİZİ

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Neisseria meningitidis, invaziv meningokok hastalığının (IMD) etken maddesidir. Serogrup B, tüm yaş gruplarında en yaygın serogrup olup, belgelenen serogrup vakalarının %62'sini oluşturarak İMD'nin başlıca nedeni olmaya devam etmektedir. Türkiye'de vakaların çoğunluğundan sorumlu olan Serogrup B ve W, vakaların %75'inden fazlasını oluşturmaktadır. İMD'nin vaka ölüm oranı %10'dur ve hayatta kalanların %10 ila %20'si ömür boyu kalıcı, engelleyici komplikasyonlar yaşamaktadır. 2019 yılında ortaya çıkan koronavirüs hastalığı (COVID-19), son derece bulaşıcı SARS-CoV-2 virüsünün neden olduğu, dünya çapında yıkıcı etkiye sahip bir hastalıktır ve 7 milyondan fazla ölüme yol açmıştır. 1918'deki grip pandemisinden bu yana en önemli küresel sağlık krizi haline gelmiştir. ABD'de ilk COVID-19 aşısının uygulanmaya başlamasından bu yana, tahmini olarak 18 milyondan fazla hastaneye yatış ve 3 milyon ölüm önlenmiştir. Grip aşılarında olduğu gibi, COVID-19 aşılarının da mevsimsel olarak uygulanması beklenmektedir. Bu tezde amacımız, COVID-19 ve menenjit prevalansının önemli sağlık riskleri oluşturduğu popülasyonlar için bir kombinasyon aşısı geliştirmektir. Önceki verilerimiz, *Neisseria meningitidis*' in B ve W serogruplarını hedefleyen OMV bazlı Bivalent aşımızın daha geniş bir humoral yanıt oluşturduğunu ve güçlü bakterisidal aktivite sergilediğini göstermektedir. Benzer şekilde, laboratuvarımız SARS-CoV-2 virüsünün dört yapısal proteinini içeren, virüs benzeri partikül (VLP) tabanlı bir COVID-19 aşısı geliştirmiş olup, bu aşı hem humoral hem de hücresel bağışıklık yanıtını uyarmaktadır. Bu çalışmada, bu iki platformu birleştirip farelerde koruyucu etkilerini test

ettik. Veriler, OMV ve VLP aşılarının Alum adjuvanıyla birleştirilmesinin, en güçlü anti-meningokok ve anti-SARS-CoV-2 yanıtlarını oluşturduğunu gösterdi.

Çalışmanın ikinci kısmı, MIS-C hastalarının plazmasında bulunan bağışıklık parametrelerini ortaya çıkarmaya ve hastalık şiddetini etkileyen ekstraselüler veziküllerin rolünü araştırmaya odaklandı. Veriler, MIS-C hastalarının plazmasında TNF- α , IFN- γ , IL-6 ve IL-17 gibi proinflamatuvar sitokinlerin önemli ölçüde yükseldiğini gösterdi. Ayrıca MIS-C'de ekstraselüler veziküllerin (EV) patolojik rolünü de araştırdık. Bulgularımız, hasta kaynaklı EV'lerin THP1-dual raportör hücre hattında önemli ölçüde daha yüksek IRF9 seviyeleri indüklediğini gösterdi. Ancak beklentilerimizin aksine, THP1 hücrelerinde EV'ye özgü NF- κ B indüksiyonu gözlemlemedik.

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

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ABBREVIATION

Atomic Force Microscopy	AFM
Angiotensin-converting enzyme 2	ACE2
Antigen-presenting cells	APCs
Acute respiratory distress syndrome	ARDS
Brain Heart Infusion	BHI
Bronchoalveolar lavage fluid	BALF
Capsular polysaccharide	CPS
Cationic antimicrobial peptides	CAMPs
CC-chemokine ligand 17	CCL17
Cerebrospinal fluid	CSF
C-reactive protein	CRP
Cytotoxic T lymphocytes	CTLs
Damage-associated molecular patterns	DAMPs
Dendritic cells	DCs
Envelope	E
Extracellular vesicles	EVs
Fetal bovine serum	FBS
Green fluorescent protein	GFP
Gastrointestinal tract	GIT
Granulocyte monocyte colony stimulation factor	GM-CSF

Genome-wide association studies	GWAS
human angiotensin-converting enzyme 2	hACE-2
Healthy control	HC
Hepatitis C virus	HCV
Human immunodeficiency virus	HIV
Interferon	IFN γ
Immunoglobulin E	IgE
Interleukin 4	IL4
Invasive meningococcal disease	IMD
Interferon regulatory factor 1	IRF1
International Society of Extracellular Vesicles	ISEV
Interferon-stimulated genes	ISGs
Kawasaki disease	KD
Membrane	M
Neisseria meningitidis	Men
Neisseria meningitidis serogroup B	MenB
Neisseria meningitidis serogroup W	MenW
N-acetylmannosamine	ManNAc
Membrane Attack Complex	MAC
Middle East Respiratory Syndrome Coronavirus	MERS-CoV
Mean fluorescence intensity	MFI
Multisystem Inflammatory Syndrome in Children	MIS-C

Minimal Information for Studies of Extracellular Vesicles	MISEV
messenger RNAs	mRNAs
Nuclear Factor Kappa B	NF- κ B
Nucleocapsid	N
neutralizing antibodies	nAbs
NOD-like receptor family	NLR
Open reading frames	ORFs
Outer Membrane Vesicle	OMV
Pathogen-associated molecular patterns	PAMPs
Peripheral blood mononuclear cells	PBMCs
plasmacytoid dendritic cells	pDCs
para-Nitrophenylphosphate	<i>pNPP</i>
Pattern recognition receptors	PRRs
Phosphoinositide 3-Kinase	PI3K
Receptor binding domain	RBD
Real-time reverse transcription-polymerase chain reaction	RT-PCR
Serum bactericidal assay	SBA
Spike	S
Severe acute respiratory syndrome coronavirus	SARS-CoV
SARS-CoV-2 non-structural protein 13	NS
single-stranded RNA	ssRNA
Tetanus Toxoid	TT

T cell receptor	TCR
Transmission Electron Microscopy	TEM
Transforming growth factor	TGF- β
Follicular helper	Tfh
T helper	Th
Toll-like receptors	TLR
Tumor necrosis factor	TNF
Tumor susceptibility gene 101	TSG101
Untranslated region	UTR
Virus-like particles	VLP
Variants of Concern	VOC
White blood cell	WBC
World Health Organization	

1 CHAPTER I

INTRODUCTION

1.1 INFECTIOUS DISEASES

1.1.1 *Neisseria Meningitidis* and Invasive Meningococcal Disease

Neisseria meningitidis is a gram-negative β proteobacterium first identified in 1887 within the cerebrospinal fluid (CSF) of a patient with meningitis (1). It is a fastidious, aerobic diplococcus that can be cultured on enriched media such as Brain Heart Infusion (BHI), Mueller-Hinton media, chocolate agar, or blood agar (2). *Neisseria meningitidis* is a member of the genus *Neisseria*, comprising at least 25 species that share 1,111 conserved gene families. Most of the 11 *Neisseria* species colonizing the nasopharynx of humans are non-pathogenic. In rare instances, the nasopharynx colonization of *Neisseria meningitidis* can cause invasive meningococcal disease (IMD) upon invasion.

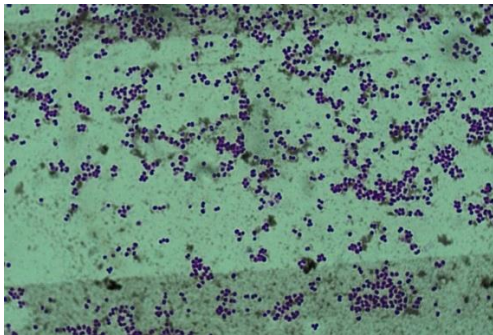


Figure 1.1. Gram-negative, encapsulated *Neisseria meningitidis* visualized under a light microscope at 40 $\times$ magnification.

The genome sequence of *N. meningitidis* strain MC58 (serogroup B) was first reported in 2000, with 2,158 genes being identified. Approximately 70% of the meningococcal genome constitutes the core region responsible for essential metabolic functions. Meningococci genomes have large genetic islands predicted to encode virulence factors and surface proteins. *Neisseria* species can

acquire DNA fragments from related organisms, co-colonizing the mucosa through horizontal genetic transfer, resulting in large antigenic variability and genetic diversity (3–6).

There are both encapsulated and non-encapsulated *N. meningitidis* serogroups. Nearly all invasive strains have a polysaccharide capsule. The classification of meningococci into serogroups is based on capsular structure. While *Neisseria meningitidis* expresses 12 distinct capsular polysaccharides (7), only six of these—namely, serogroups A, B, C, W, X, and Y—are associated with invasive diseases. Serogroup A (MenA), the dominant causative agent for large pandemic outbreaks in sub-Saharan Africa, has a capsular polysaccharide (CPS) composed of repeating units of α 1→6-linked ManNAc 1-phosphate that is O-acetylated. The capsular polysaccharide of serogroup X, which has also recently caused endemic disease in the same region, is a homopolymer of α 1→4-linked ManNAc 1-phosphate (8). The CPS of serogroups B, C, Y, and W-135 structurally differs from serogroups A and X and contains sialic acid. Serogroup B and C capsular polysaccharides are both homopolymers of sialic acid but with different linkages: partially O-acetylated α 2→9-sialic acid for serogroup C and α 2→8-sialic acid for serogroup B. The serogroup W capsular polysaccharide comprises partially O-acetylated sialic acid and an alternating D-galactose (9). Sialic acid, mannosamine, or glucosamine-based capsular polysaccharides are important virulence factors in meningococcal infections. They are essential for various aspects of meningococcal disease progression. Because meningococcal capsules mask the adhesion ligands, it is difficult for encapsulated strains to attach to mucosal surfaces, making transmission of meningococci easier (10). Furthermore, the polysaccharide capsule is essential for the survival of meningococci in the intracellular environment by protecting this microorganism from cationic antimicrobial peptides (CAMPs), important components of the host innate defense system against microbial infections. The structure of meningococcal capsules participates in the activation of classical and alternative complement pathways on the bacterial surface. The insertion of membrane attack complex (MAC) on encapsulated strains was less, while a consistent increase was monitored on non-encapsulated strains in kinetic studies (11).

Invasive meningococcal disease (IMD) is a serious life-threatening disease associated with high morbidity and mortality caused by *Neisseria meningitidis*. IMD is characterized by a wide range of clinical symptoms with a 6-10% case fatality. The distribution of serogroups that cause IMD differs across regions and changes over time in many parts of the world (12). MenA is the main

causative agent in Africa's sub-Saharan meningitis belt and is still a dominant serogroup in Asia, including India. Meanwhile, MenC is a threat to Europe and North America. Recently, MenB has represented the highest percentage of IMD cases worldwide, and MenW has accounted for major epidemics in Africa and the Middle East, including Türkiye (13).

Vaccination can prevent meningococcal disease outbreaks, particularly in high-risk environments such as schools and military barracks. There are several licensed meningococcal vaccines used globally to protect against various serogroups. These vaccines can be categorized into 3 main groups: polysaccharide, conjugate, and protein-based. Polysaccharide vaccines induce weak immune response and cannot provide long-term immunity. Therefore, polysaccharides were chemically conjugated to a protein carrier such as tetanus toxoid (TT) and diphtheria toxoid (DT), able to stimulate T-cell response. Licensed quadrivalent vaccines Menactra® (MenACYW-DT) and Menveo® (MenACYW-CRM) are examples of conjugate vaccines (14). Details in protein-based vaccines, including outer membrane vesicles, are given in Section 1.4.1.

Due to rare occurrences of IMD, it is difficult to conduct clinical efficacy studies to get approval for the licensure of meningococcal vaccines. Consequently, the effectiveness of *N. meningitidis* vaccines is assessed by detecting serum bactericidal antibodies against the meningococcal strains. Serum bactericidal assay (SBA) is a key laboratory test to detect the ability of vaccine-induced antibodies to kill bacteria. The assay relies on the complement proteins to mediate bacterial killing. human complement (hSBA) or baby rabbit complement (rSBA) are complementary sources. hSBA titers of ≥ 4 or rSBA titers of ≥ 8 have been considered as a threshold for the licensure of meningococcal vaccines (15).

1.1.2 SARS-CoV-2 and COVID-19

COVID-19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in millions of cases and more than 5 million deaths globally (16). Although developed vaccines against the virus reduced the disease impact, the accumulation of mutations in the viral genome has continued and led to quickly evolving variants, which can provide immune escape. Therefore, it is important to fully comprehend this virus and develop more inclusive vaccines against variants that could be more lethal in the future.

SARS-CoV-2 belongs to the genus Betacoronavirus, comprising Middle East Respiratory Syndrome-Coronavirus (MERS-CoV) and SARS-CoV-1. It is a positive-sense RNA virus with a large genome of ~30 kilobases and composed of the four main structural proteins: membrane (M), envelope (E), a helical nucleocapsid (N), and an exterior decorated with several spike (S) proteins. Spike facilitates the infection of host cells by binding them to the host cell receptor, human angiotensin-converting enzyme 2 (hACE2). The spike protein is a trimer of three identical subunits, S1 and S2. The S1 subunit includes the receptor-binding domain (RBD), which is exposed and ready to bind to the ACE2 receptor in its prefusion conformation (17). After binding, the spike protein undergoes a significant conformational change to the postfusion state, where the S2 subunit undergoes a change that brings the viral membrane and the host cell membrane close together, allowing them to fuse and the viral genetic material to enter the host cell. In the postfusion state, conformational changes cause S1 subunit disengagement from S2 (18).

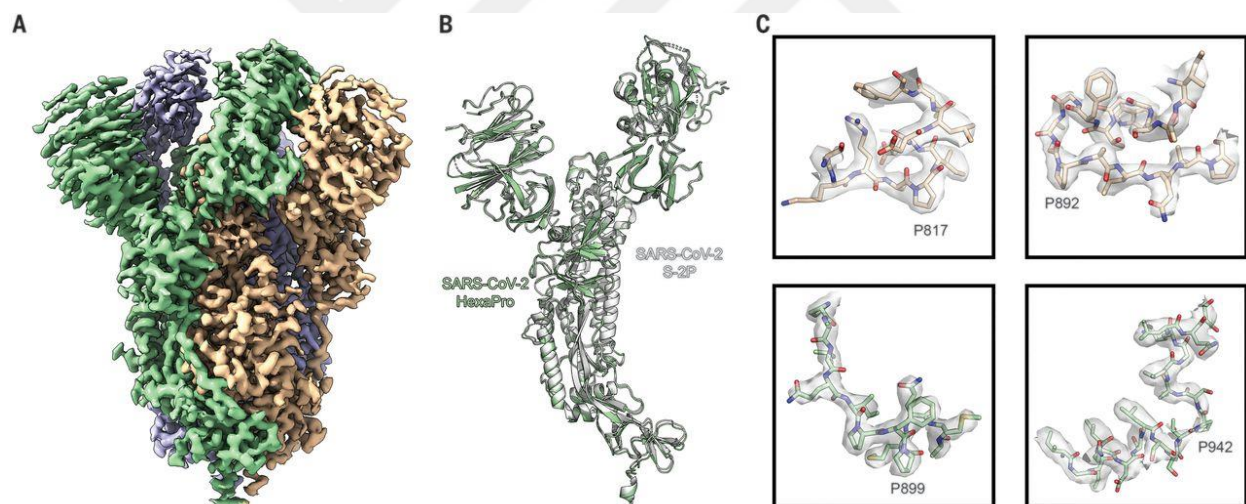


Figure 1.2. HexaPro (6P)-stabilized SARS-CoV-2 Spike protein (Adapted with permission from Ref. 19). The SARS-CoV-2 spike protein undergoes a conformational change when it binds to the ACE2 receptor in human cells, shifting from an inherently unstable prefusion state to a postfusion state. However, the prefusion state of the SARS-CoV-2 Spike protein is critical for vaccine development since the immune system needs to recognize it to prevent infection. Six proline mutations in the S2 subunit of SARS-CoV-2 spike stabilize the protein in its prefusion state.

SARS-CoV-2's evolutionary rate is rapid (9×10^{-4} substitutions per site per year) (20), and its high transmissibility allows pile-up mutations in its genome. Mutations have been identified in all parts of the viral genome. Still, mutations related to the spike, especially RBD, are particularly interesting, as they render fitness advantage due to increased RBD affinity with ACE2 and immune escape (21). Different nomenclature systems have described several emerging variants according to the mutations at key sites in the spike protein. The SARS-CoV-2 variants that lead to adverse changes in clinical disease severity requiring major public health interventions or variants resisting available vaccines are defined by the World Health Organization (WHO) as “variants of concern” (VOC). Previously circulating VOCs were Pango lineages B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), and B.1.617.2 (Delta) and Omicron parent lineage B.1.1.529. Since 2023, Omicron subvariants have been under monitoring. As of July 15, 2024, the SARS-CoV-2 Omicron variants KP.2, KP.3, and LB.1 are highly prevalent in the US.

SARS-CoV-2 infection induces the activation of multiple intracellular pathways such as NF- κ B, PI3K/AKT, Type I and III interferons, TLRs, and TGF- β pathways. NF- κ B activity leads to a dominant immune profile in severe COVID-19 patients. Many SARS-CoV-2 proteins like NSP13, ORF9c, and ORF7a induce the secretion of NF- κ B related proinflammatory cytokines and chemokines (22,23). SARS-CoV-2 infection can also trigger a severe immune response characterized by the overproduction of cytokines via critical signaling pathways like NF- κ B, JAK-STAT, and MAPK. This uncontrolled release of pro-inflammatory cytokines is termed a “cytokine storm”, which leads to severe tissue damage, multi-organ failure, and death (24). Several pro-inflammatory cytokines, including IL-6, IL-1 β , TNF- α , IL-8, and IFN- γ , are excessively secreted by immune cells like macrophages, DCs, monocytes, and T-cells (25). Furthermore, SARS-CoV-2 could hamper the production of IFN, the first crucial antiviral cellular response, inhibiting the IFN-I signaling pathway through viral proteins ORF6, ORF8, and nucleocapsid (26).

1.1.3 COVID-19 Vaccines

The COVID-19 pandemic has profoundly affected global health, daily life, and economies since its emergence in late 2019. In response to this crisis, the rapid development of COVID-19 vaccines has become a keystone of the worldwide approach to alleviating the spread and severity of the SARS-CoV-2 virus. Several COVID-19 vaccines have been developed, including inactivated,

attenuated, DNA, mRNA, protein subunit, and VLP vaccines. As of August 2024, 64 of SARS-CoV-2 vaccines are approved by one or more national regulatory authorities (27). The choice of antigen in SARS-CoV-2 vaccine development is highly crucial. The spike protein and receptor-binding domain (RBD) directly interact with the ACE2 receptor and are primary targets for most SARS-CoV-2 vaccines (28). Neutralizing antibodies predominantly produced against the prefusion form of the spike protein are mostly effective because this is the form present on the virus's surface when it is outside the host cell. The integrity of virus particles is protected in vaccines, where in vitro-cultured viruses are inactivated by chemicals (29). Therefore, inactivated COVID-19 vaccines have the advantage of using the entire virus as immunogens, but β -propiolactone used in the inactivation process results in the loss of all Spike S1 subunits, leaving only the postfusion S2 (30). This may cause reduced binding affinity of neutralizing antibodies. When the spike protein shifts to its postfusion state, many of the epitopes these antibodies recognize are altered or hidden.

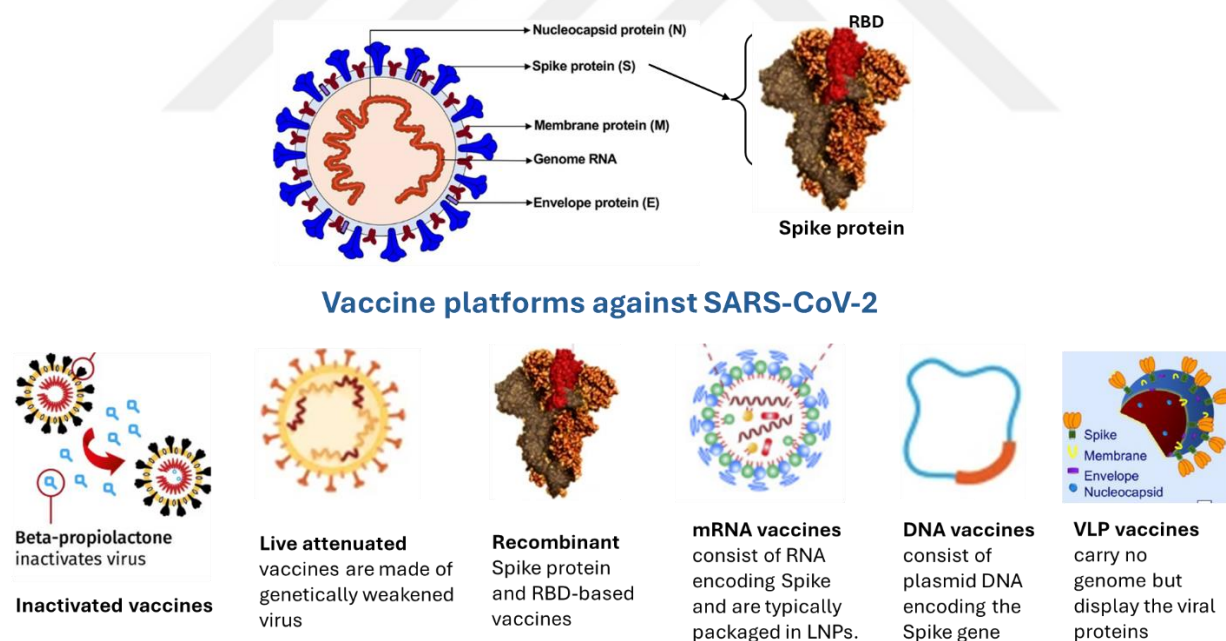


Figure 1.3. Vaccine strategies against SARS-CoV-2. (Adapted with permission from Krammer, 2020 (31) and Zhang, 2021 (32).

Viral vector vaccines utilize engineered, replication-attenuated viruses to transmit viral proteins to host cells, initiating an immune response (33). Examples include vaccines such as Sputnik V and ChAdOx1 nCoV-19, which use adenovirus vectors expressing full-length S protein. While these

vaccines are safer to produce than live attenuated virus vaccines and can induce Th1 cell responses(34), they can cause side effects like thrombocytopenia (35).

DNA vaccines utilize recombinant plasmids to encode viral antigens in host cells (36). DNA vaccines are more stable and can be stored longer than mRNA vaccines but have lower immunogenicity. Furthermore, injection routes, like intramuscular or electroporation, can influence their effectiveness (31). Another concern about DNA vaccines is the risk of genomic integration. Nevertheless, the DNA-based COVID-19 vaccine, ZyCoV-D, received emergency use authorization in India in 2021. This is the first DNA vaccine approved worldwide (37).

mRNA vaccines have had a fundamental role in controlling the COVID-19 pandemic. Pfizer-BioNTech and Moderna COVID-19 vaccines comprise mRNA encoding prefusion-stabilized spike protein and lipid nanoparticles. These vaccines represented a significant advancement in immunology. They induced the highest level of neutralizing antibodies among all SARS-CoV-2 vaccine candidates in phase III clinical trials (38–40). While the original mRNA vaccines were shown to be less efficient against new VOCs like Omicron, bivalent vaccines targeting both Wuhan-Hu-1 and Omicron BA.4/BA.5 have demonstrated improved effectiveness. These updates enhance protection, particularly against severe disease and hospitalization. Ongoing research on the efficacy of mRNA vaccines against evolving subvariants suggests that mRNA vaccines still provide substantial protection, especially when booster doses are administered (41–44). However, some critical side effects, like myocarditis, have been associated with mRNA vaccines. A study by Yonker et al. has shown that individuals developed myocarditis after vaccination with either the Pfizer BNT162b2 or Moderna mRNA-1273 COVID-19 mRNA vaccine carry increased levels of full-length Spike protein in their plasma, indicating a potential underlying cause (45).

Virus-like particle (VLP) vaccines are designed to resemble the virus they target but lack the genetic material required for replication. This makes them non-infectious and safe. They display viral proteins on their surface, closely mimicking the virus's natural structure. This structural similarity makes VLPs more accessible for APCs such as DCs, resulting in a strong humoral response and a robust cellular response. (46). VLPs are highly immunogenic due to their repetitive and multivalent nature. This causes a strong and long-lasting immune response, potentially reducing the need for booster doses. They are adjuvant compatible, enabling them to be used with various adjuvants (46–49). VLP vaccines can present conserved viral epitopes and multiple

epitopes on the spike protein, potentially offering broader protection against future coronavirus outbreaks (47,50). For example, a SARS-CoV-2 vaccine named RBDM utilized VLPs to display the wild-type SARS-CoV-2 RBD in an array produced using split protein Tag/Catcher technology. In mice, RBDM produced significantly higher IgG responses and elicited strong neutralizing antibodies against most SARS-CoV-2 variants, including Alpha, Beta variant, Delta, Gamma, Lambda, Omicron BA.1, BA.4/5, and XBB.1 variant. RBDM protected K18-hACE2 transgenic mice from wild-type and Delta SARS-CoV-2 strains, with no detectable virus in lung tissues. In rhesus macaques, the vaccine produced higher neutralizing antibodies than other vaccines produced by Moderna, Pfizer, AstraZeneca, and Sinovac, and robust T-cell responses, protecting against Delta variant challenges (51).

In summary, SARS-CoV-2 vaccine research proceeds to be critical in the global effort to control and ultimately eradicate COVID-19. The development of several vaccine platforms has shown remarkable adaptability in addressing emerging variants. These advancements offer strong protection against the COVID-19 outbreak and lay the foundation for future vaccine innovations that deal with various infectious diseases.

1.1.4 Multisystem Inflammatory Syndrome in Children (MIS-C)

The infection due to SARS-CoV-2, named COVID-19, commonly has led to mild upper respiratory illness in children (52,53). However, at the beginning of May 2020, pediatric cases with severe hyperinflammation and multiple organ involvement were published in London (54). Subsequently, an increasing number of post-COVID-19 cases were reported worldwide, and the entity called multisystem inflammatory syndrome in children (MIS-C). After 1 year of follow-up, Kapoor et al. showed that 54 pediatric patients with MIS-C tested positive for SARS-CoV-2 antibodies (55). Other studies also have supported that children with COVID-19 infection have been at risk of MIS-C. The syndrome primarily has been observed in children aged 6-12, with male predominance (56). The major presentation of cases included persistent fever, rash, conjunctivitis, and symptoms of organ involvement such as cardiovascular (57,58) and gastrointestinal systems (59,60). Among all clinical presenting symptoms, gastrointestinal symptoms are the most frequently seen in COVID-19 and 90% of MIS-C patients (61–63). This might cause diagnostic challenges and require detecting more characteristic symptoms with careful

evaluation. One study showing 13.5% of MIS-C patients with cervical inflammation suggested cervical symptoms as diagnostic criteria (64). Studies, including cytokine profiling and analyses of blood immune cells, were conducted to identify patients with MIS-C and those with severe COVID-19. These studies have demonstrated that the inflammatory responses, primarily the sum of IL-10 and TNF- α levels, distinguished MIS-C from the cytokine storm of severe acute COVID-19 and share multiple aspects with Kawasaki disease, despite differences in T cell subsets, IL17-A, and biomarkers linked to arterial damage (65,66). Complementing the findings of Pierce et al. Ravichandran et al. found that IL-2, IL-6, IL-10, IL-13, and IL-17 are the main pro-inflammatory cytokines to determine the severity of COVID-19, while patients with MIS-C have high TNF, which might be the result of a cleared viral infection or decreasing antiviral cytokine levels (67,68). Healthcare providers considered immunomodulatory drugs like IVIG, glucocorticoids, and IL-6 inhibitors as early treatments (69). However, MIS-C is a rare but potentially life-threatening condition that is not comprehended fully and is still relatively new for medical professionals. Therefore, researchers should continue their investigations on this topic.

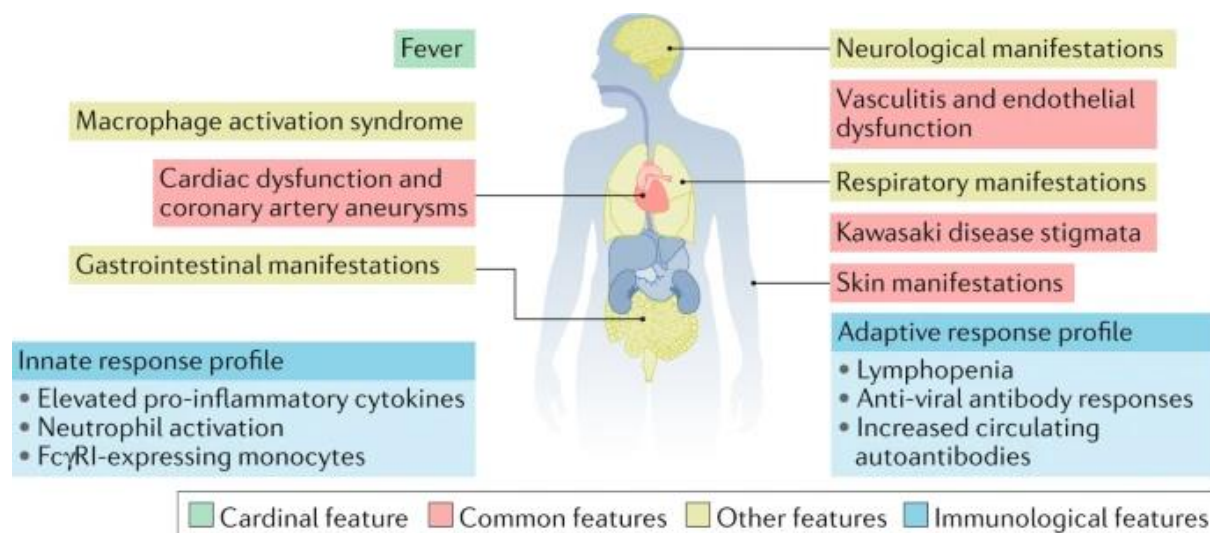


Figure 1.4. Clinical and immunological features of MIS-C. (Adapted with permission from Ref. 70).

1.2 OUTER MEMBRANE VESICLES (OMVs)

Extracellular vesicles are released naturally by all life forms, including eukarya, archaea, and bacteria. The vesicles, ranging in size from 20-350 nm, formed by budding from the outer membrane of Gram-negative bacteria during all stages of growth are called outer membrane vesicles (71–74). OMVs, initially thought of as bacterial artifacts, first began to attract attention when they were identified in primary cultures of spinal fluid taken from patients with meningococcal disease. This was the first study to show OMVs secreted from pathogenic bacteria in a physiologically relevant setting (75). Today, it is known that OMVs are purposely secreted by gram-negative bacteria to serve diverse functions, including biofilm formation, regulating interactions within bacterial communities, and promoting inflammation and disease progression (76–79). OMVs have a similar bilayer structure to their parent cells, with the outer layer containing lipopolysaccharide (LPS) while the inner layer consists of phospholipids (80). Well-characterized OMV cargoes contain DNA, RNA, virulence factors, surface and cytoplasmic proteins, antibiotic-degrading enzymes, and peptidoglycan (81). Specific components within OMVs, such as lipids or proteins, can be enriched or reduced compared to their abundance in the bacterial envelope. Notably, the production of OMVs takes place without cell lysis or membrane leakage, indicating that OMV formation is a deliberate process rather than a stochastic event. Additionally, factors like temperature, nutrient availability, oxidative conditions, quorum sensing, and antibiotics that target the bacterial envelope can influence the levels of vesicle production (82–84).

1.2.1 Biogenesis of Outer Membrane Vesicles

The cell envelope of Gram-negative bacteria consists of three main components: the inner membrane, the outer membrane, and a thick peptidoglycan (PG) layer within the periplasmic space (85). Embedded proteins, lipids, lipoproteins, and LPS facilitate the integrity and fluidity of the membrane. There is no universal mechanism underlying how OMVs are produced and released in external surroundings, but several mechanisms have been proposed. One of the mechanisms is based on the disruption of linkage between the peptidoglycan layer and outer membrane in Gram-negative bacteria. For OMVs to form protrusions and ultimately be released entirely, the connections between the membrane and the PG layer must be disrupted at some locations. Braun's lipoprotein (Lpp) is one of the most prevalent membrane proteins (86) found in Gram-negative

bacteria and is covalently linked to diaminopimelic acid (DAP) found in the peptidoglycan layer. Notably, when Lpp-PG binding is reduced, as seen in *E. coli nlpI* mutants, it leads to induction in hyper-vesiculation. Similar outcomes are observed in *Salmonella typhimurium* when the *lppAB* operon is disrupted (87). The porin outer membrane protein A (OmpA) is another player in envelope cross-linkage. It is the most abundant protein in the outer membrane of many Gram-negative bacteria and provides structural integrity by non-covalently binding to PG (88). In *E. coli* and *S. typhimurium* *Lpp/OmpA* double mutants, OMV release is higher than in single *Lpp* mutants (89). The Tol-Pal complex is another bridge between the outer membrane and PG. Deletion of *tolA* in *Pseudomonas putida* and *tolB* in *Helicobacter pylori* resulted in increased OMV production (90,91).

The second proposed mechanism is based on the turgor pressure of misfolded protein accumulation between PG and outer membrane (92). This pressure is thought to create localized spaces, causing the OM to protrude and eventually release vesicles. Supporting evidence for this hypothesis includes a study showing that specific mimics of misfolded proteins can be found within OMVs, suggesting that bacteria may use these vesicles to eliminate cellular waste products (93). This research also indicated that deleting DegP, a periplasmic chaperone and protease responsible for removing misfolded and unfolded outer membrane proteins, increases vesicle formation. Furthermore, another study on extracellular vesicles from a mutant *E. coli* strain that hyper-produces vesicles due to the absence of DegP revealed an accumulation of misfolded DegP substrate outer membrane porins.

A third proposed mechanism involves the accumulation of specific substances that can induce curvature in the bacterial membrane. These substances include B-band lipopolysaccharides (LPS) and the *Pseudomonas* Quinolone Signal (PQS). In the case of *Pseudomonas aeruginosa*, the natural production of extracellular vesicles (EVs) is linked to the presence of B-band LPS (94). These EVs were found to contain this type of LPS, which is notably different from A-band LPS due to its long O-side chains that extend up to 40 nanometers from the membrane (95). These side chains carry a negative charge, and depending on the presence of counterions, they may repel each other sufficiently to cause the membrane to curve. If a localized region of the outer membrane accumulates enough B-band LPS, it may protrude and form a vesicle.

Likewise, Pseudomonas Quinolone Signal (PQS) is believed to have the ability to induce membrane curvature. EV production in *P. aeruginosa* is dependent on PQS, even when the receptor for PQS is deleted (77). This suggests that PQS may physically trigger the formation of EVs rather than solely serving as a signaling molecule (96). It's worth noting that PQS production is tied to the presence of molecular oxygen, and EV production is significantly decreased under anaerobic conditions (97). The evidence supporting these suggested mechanisms for vesicle formation primarily comes from experiments involving deletion mutations; however, the OMV production level varies between WT organisms and growth conditions. This suggests that vesicle formation is a multifaceted process that relies on a combination of intricate subcellular mechanisms and pathways.

1.3 OMV VACCINES

1.3.1 OMV-based Meningococcal Vaccines

N. meningitidis has 12 serogroups based on their polysaccharide capsules, six of which (A, C, B, W, Y, and X) can cause epidemics (98). A Conservative vaccine strategy against these serogroups is to formulate toxoid proteins conjugated capsule polysaccharides. For example, MenACWY-DT (Menactra) comprises capsular polysaccharides from each serogroup individually conjugated to diphtheria toxoid protein. MenA-TT (MenAfriVac) and MenACWY-TT (NIMENRIX), extensively recommended for infants in our country, contain capsular polysaccharides coupled with tetanus toxoid. To date, several conjugated meningococcal vaccines have been approved for human use. However, this strategy is unsuitable for the serogroup B capsule polysaccharide due to its structural similarity with neural cell-adhesion molecules (N-CAM) in the human brain (99). This similarity creates poor immunogenicity and autoimmunity concerns. Therefore, researchers have focused on developing protein-based vaccines against MenB, and outer membrane proteins (OMPs) have gained great attention. PorA has been revealed as a highly abundant OMP, and antibodies against PorA have shown bactericidal activity (100). However, OMPs are insoluble, resulting in difficulties in vaccine formulation. Since PorA is naturally found in OMVs isolated from *N. meningitidis* strains, vaccine studies for MenB strains have directed attention to OMVs derived from capsule-deficient bacteria. In 1987, the first detergent-treated OMV-based vaccine (VA-MENGOC-BC) against MenB was licensed in Cuba. Then, outbreaks were prevented using OMV-based vaccines in Norway and New Zealand (MeNZB™) (101). However, PorA is

extremely variable between strains, although it is the main immunogenic antigen in meningococcal OMVs (102). Therefore, additional conserved antigens in *N. meningitidis* were identified in silico using a whole genome sequencing technology termed reverse vaccinology. These antigens were added to the OMV formulation after being tested for immunogenicity. A multi-component meningococcal B vaccine, 4CMenB (Bexsero), was developed, composed of MenZB OMVs and three recombinant proteins: a fragment of NadA, NHBA, and fHbp. Bexsero was approved by the FDA in 2013, and since then, its effectiveness against other serogroups and strains has been investigated (103,104).

Subsequently, OMVs have been employed as antigen sources for vaccine preclinical studies of other serogroups. For example, mice immunized with bivalent A+W meningococcal OMV-based vaccine produce significant levels of IgG antibodies against both A and W and higher bactericidal titers against MenW than mice immunized with the ACYW-conjugate vaccine (Menveo) (105).

Using genetic engineering with detergent-free methods to detoxify LPS in OMVs and induce hyper-vesiculation paves the way for novel meningococcal vaccines.

1.3.2 OMV-based COVID-19 Vaccines

Numerous vaccines have been developed since the beginning of the COVID-19 pandemic. Some have been approved for human use, and several are still in clinical development (106). However, developing affordable and accessible vaccines against SARS-CoV-2 for all countries remains important. As discussed in Section 1.2.3, OMVs are cost-effective and can stimulate the humoral and cellular arms of the immune response. Their potential as an adjuvant in other viral vaccines has been explored before SARS-CoV-2 by many research groups. For example, *E. coli* OMVs have been used as an adjuvant for Influenza in many studies. OMV adjuvanted groups induced IFN- γ showing a Th1-dependent immune profile, although Alum adjuvanted groups were more efficient in improving the antibody titers. All ferrets administered with OMV adjuvanted formulations in this study were protected from a lethal viral challenge (107). In another study, using *E. coli* OMVs as adjuvants resulted in a balanced Th1/Th2 humoral response besides promoting DCs activation and maturation (108). Martins et al. have used *N. meningitidis* OMVs to fuse with virus particles secreted from Zika virus-infected cell lines. Mice vaccinated with the resulting nanoparticles can produce enriched IgG titers, Th1/Th2-based cytokines, immunologic memory, and neutralizing antibodies against Zika virus (109). Furthermore, a multivalent vaccine

composed of *E. coli* OMVs genetically decorated with Influenza (H1) and MERS-CoV (RBD) antigens has induced neutralizing antibodies against both viruses, indicating the potential of OMV adjuvants in dual vaccine strategies (110).

According to WHO, there are few OMV-used COVID-19 vaccines in clinical trials. SOBERANA 02 and SOBERANA Plus, developed by The Finlay Vaccine Institute in Cuba, are in phase III clinical trials and have been approved in Cuba, Iran, and Belarus. They have utilized dimeric SARS-CoV-2 RBD as antigen, *N. meningitidis* serogroup B OMVs, and Alum as adjuvants (111–113). The RBD+OMV+Alum formulation was more efficient in mice than RBD, which was only absorbed in Alum. The vaccine could generate strong B-cell memory and neutralizing antibodies capable of recognizing mutant RBD from other VOCs in animal studies (114).

On the other hand, preclinical studies on developing COVID-19 vaccines containing OMVs have highlighted promising results. Van der Ley et al. developed a vaccine composed of six-proline-stabilized Spike of SARS-CoV-2 linked via the LPS-binding peptide sequence to OMVs from *N. meningitidis* showing a strong humoral response with neutralizing antibodies and protection against viral challenge (115). Additionally, only intranasal administration of the vaccine produced IgA in the lungs and the nose of hamsters. Another intranasal vaccine, including *Salmonella typhimurium* OMVs decorated with recombinant RBD via Spytag-SpyCatcher technology, induced high IgG antibodies and detectable mucosal responses. The vaccine-induced neutralizing antibodies against the wild-type and the Delta variant. Hamsters immunized with RBD-decorated OMVs had lower virus titers in bronchoalveolar lavage fluid with milder lung pathology (116,117). Thaba et al. compared the adjuvanticity of *E. coli* and *Vibrio cholera* OMVs in RBD vaccine preparations. Robust immune responses and effective neutralizing antibodies were obtained when OMVs of each species were used separately. Interestingly, the alternating immunization approach using OMVs from each bacterium resulted in the most potent outcomes (118).

Although these SARS-CoV-2 vaccine studies have shown promising results, most target only the Spike antigen. However, the emergence of VOCs associated with altered Spike sequences highlights the importance of developing novel vaccines targeting other SARS-CoV-2 antigens capable of further improving humoral and cellular responses.

1.4 INNATE IMMUNITY

Innate immunity is the evolutionarily conserved first line of defense against pathogens that breach one of the host's anatomic barriers and include phagocytes such as neutrophils, macrophages, and dendritic cells. It uses a limited number of innate receptors and secreted proteins encoded by genes directly inherited from an individual's parents. These germline-encoded receptors are called pattern-recognition receptors (PRRs) and can recognize one or more different molecular 'patterns' present in microbes called pathogen-associated molecular patterns (PAMPs) (119). Different PRRs, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), react with specific PAMPs, activating distinct signaling pathways and leading to specific antipathogen responses (120).

Toll-like receptors (TLRs) are one of the major sensors that play a key role in innate immunity. TLRs are characterized by an extracellular domain containing leucine-rich repeats (LRRs) responsible for ligand recognition, a transmembrane domain anchoring the receptor in the cell membrane, and an intracellular Toll/interleukin-1 receptor (TIR) domain essential for initiating signaling cascades (121). The diversity within the TLR family arises from variations in the LRR domain, allowing for the recognition of distinct pathogen-associated molecular patterns (PAMPs). This structural versatility enables TLRs to detect a wide array of microbial components, including lipopolysaccharides, lipoproteins, nucleic acids, and carbohydrates (122). There are 10 TLRs in humans and 12 in mice. TLR11, TLR12, and TLR13 are expressed only in mice but not in humans. (123). The expression of TLRs is dynamic and mediated immediately in response to pathogens and various cytokines. TLRs can be expressed either on the cell surface (TLRs 1,2,4,5 and 6) or located intracellularly in the membranes of endosomes (TLRs 3,7,8,9,11,12 and 13) (124). Ligands of TLRs expressed on the endosome must be internalized into cells by phagocytosis, receptor-mediated endocytosis, or micropinocytosis. Microbes or their components taken into cells are degraded by proteases found in the acidic lumen of the endosome, and TLRs then recognize their released nucleic acids. Each TLR subfamily has a distinct recognition pattern for related PAMPs. Specifically, mammalian TLR1, TLR2, and TLR6 form a subfamily that recognizes lipids and lipoproteins like diacyl and triacyl lipoproteins in both Gram-positive and Gram-negative bacteria (125). In contrast, mammalian TLR3, TLR7, TLR8, TLR9, and TLR13 specialize in recognizing nucleic acids. TLR7 is expressed only by B cells and plasmacytoid dendritic cells (pDCs) in humans, while it is expressed by various innate immune cells in mice. TLR7 and TLR8 recognize

small fragments of single-stranded RNA (ssRNA) released into endosomes when many viruses carry ssRNA genomes like SARS-CoV-2, influenza, and dengue virus (126,127). TLR9 recognizes ssDNA sequences that have unmethylated CpG dinucleotides (CpG ODN), which are often methylated in the genomic DNA of mammals. TLR3 can recognize double-stranded RNA (dsRNA) after direct endocytosis of viruses or by phagocytosis of dying cells in which viruses replicate. Some members of TLRs can identify structurally unrelated ligands. For instance, TLR4 can recognize lipopolysaccharide (LPS), respiratory syncytial virus (RSV) fusion protein, heat-shock proteins, and fibronectin (128).

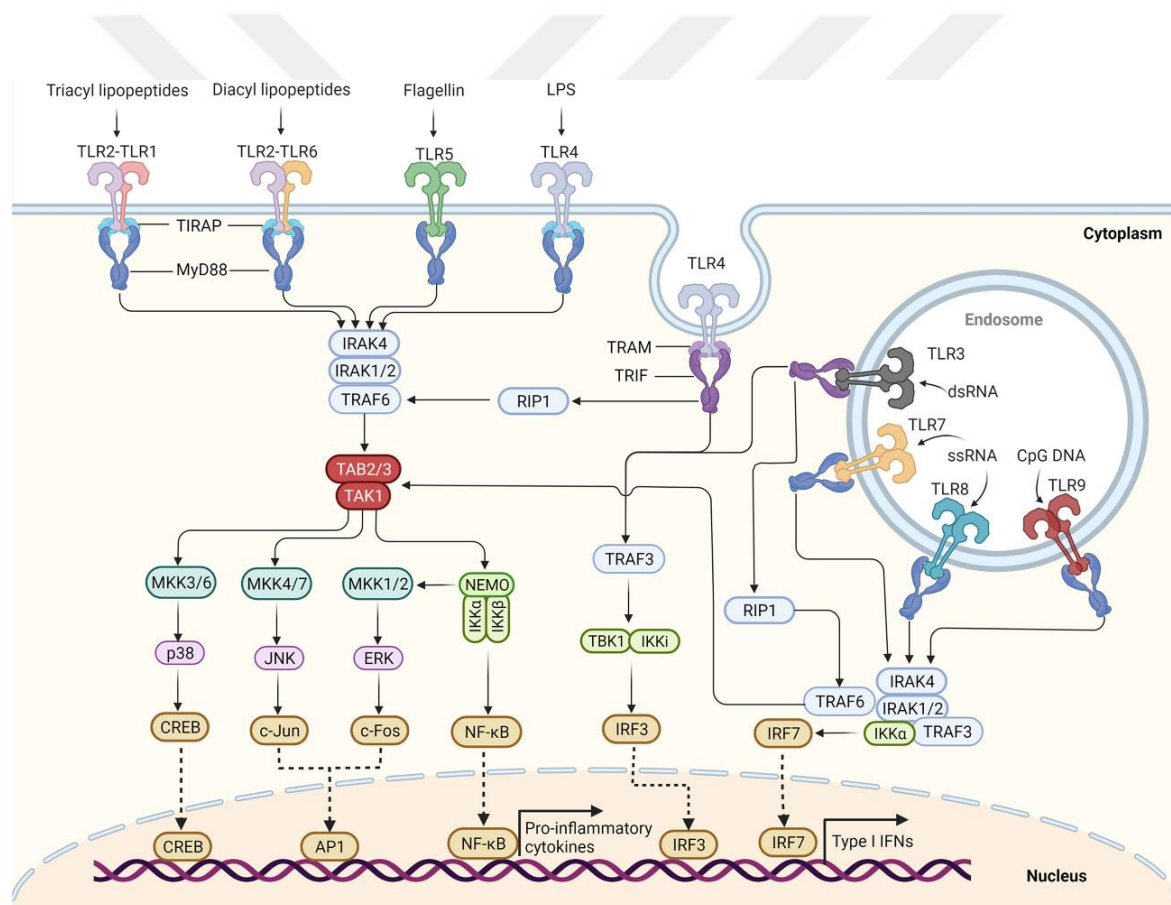


Figure 1.3. TLR signaling pathways. (Adopted from Ref. 123).

The binding of microbial ligands to TLRs triggers signaling pathways that activate transcription factors, specifically interferon regulatory factors (IRFs) and nuclear factor- κ B (NF- κ B). This activation results in the expression of proinflammatory cytokines, type I interferons (IFN- α and IFN- β), and other immune mediators, generating a robust immune response. Following ligand

binding, dimerization brings together cytoplasmic TIR domains of TLRs, which recruits TIR-domain-containing adaptor molecules to the TLR (129). There are five such mammalian adaptors: MyD88, TRIF, TIRAP, TRAM, and BCAP. The selective usage of these adaptor molecules determines downstream signals and is mediated by different TLR ligands. MyD88 is responsible for the responses of most TLRs except TLR3, which interacts only with TRIF (130). TLR4 recruits both TRIF and MyD88. The MyD88-dependent and TRIF-dependent pathways are central in mediating the recruitment of various molecules, ultimately causing the activation of specific transcription factors. The signaling pathways activated by TLRs are not isolated entities; instead, they display crosstalk, creating a complex regulatory network. Crosstalk between TLRs and other PRRs and feedback loops within the TLR cascades contribute to fine-tuning immune responses (131).

Toll-like receptors (TLRs), expressed on the cellular plasma membrane or within endocytic vesicles, function primarily as detectors for microbial products in the extracellular environment. Other families of innate sensors recognize pathogen products in the cytosol, with NOD-like receptors being one of them. NOD-like receptors (NLRs) are characterized by the presence of a nucleotide-binding and oligomerization domain (NOD) along with leucine-rich repeats (LRRs) (132). Distinct members of the NLR family, such as NOD1 and NOD2, exhibit specificities in recognizing fragments of bacterial cell-wall peptidoglycan responding to γ -glutamyl diaminopimelic acid and muramyl dipeptide, respectively. Activation of NLRs can lead to diverse outcomes: some initiate NF- κ B-mediated inflammatory responses akin to Toll-like receptors (TLRs), while others initiate a distinctive cascade resulting in programmed cell death and the release of varied pro-inflammatory cytokines (133).

Another cytosolic sensor family is RIG-I-like receptors (RLRs) which recognize viral RNAs produced within a cell. RNA helicase-like domain of these receptors binds to viral dsRNAs. Since the capping process in eukaryotic transcription modifies the initial nucleotide of the RNA, RNA genomes of the viruses that do not replicate in the nucleus and do not undergo this modification can be recognized by RIG-I-like receptors (134).

While host DNA must be located in the nucleus, foreign DNA may be present in the cytosol during infection. The primary sensor for cytosolic DNA is an enzyme known as cGAS (cyclic GAMP synthase). Direct binding of cGAS to cytosolic DNA results in the production of a second

messenger molecule named cyclic guanosine monophosphate–adenosine monophosphate (cGAMP). Subsequently, cGAMP activates STING (stimulator of interferon genes), a protein localized within the endoplasmic reticulum membrane. STING exists as an inactive homodimer. The binding of cGAMP to STING induces a conformational change, leading to the recruitment of TBK1, activation of IRF3, and subsequent production of type I interferons (135).

1.4.1 The Roles of OMVs in Innate Immunity

In the interaction between pathogens and the immune system, OMVs play a pivotal role by carrying PAMPs that can be sensed by the host's immune cells (136). This recognition triggers a rapid and crucial inflammatory response, a cornerstone of innate immunity. Generally, OMVs engage in inflammation triggered by TLR signaling and NLR signaling. TLR4 recognizes LPS in bacterial OMVs. The interaction begins with LPS-binding protein (LBP) binding to LPS, which then extracts and transfers lipid A molecules to CD14. This process leads to the presentation of lipid A to myeloid differentiation factor 2 (MD-2), activating TLR4 through dimerization (137). TLR4 can initiate two signaling pathways: one involving MyD88, leading to the production of inflammatory mediators (IL-6, IL-1b, IL-8, and TNF-a) through NF-kB and MAPK signaling pathways and another involving TRIF, inducing IFN secretion via IRF3. LPS serves as an important virulence factor carried within OMVs of bacteria such as *E. coli*, *Neisseria meningitidis*, *Salmonella typhimurium*, and *B. fragilis* (138,139). TLR2 can recognize several virulent factors like peptidoglycan, lipopeptide/proteins, FomA, and OmpA in OMVs. For example, FomA in *F. nucleatum* OMVs, and OmpA in *A. baumannii* OMVs have been shown to initiate a strong proinflammatory response through TLR2 activation (140). H7 flagellin on EHEC OMVs has been found to produce IL-8 by human intestinal epithelial cells in a TLR-5-dependent manner (141).

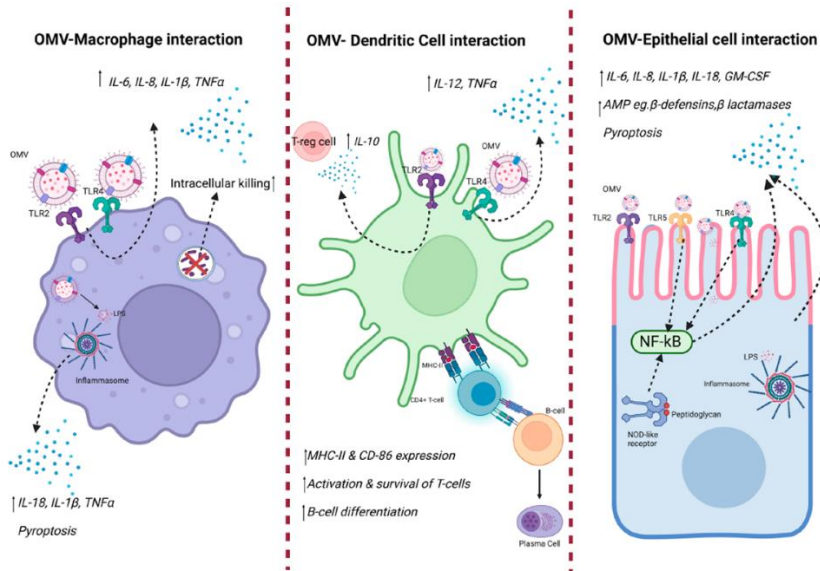


Figure 1.4. OMVs induce innate immunity. Their cargo includes various PAMPs and DAMPs such as LPS, DNA, RNA, and OMPs that activate host PRRs, resulting in downstream immune signaling. (Adapted from Ref. 142).

After endocytosis, OMVs can also be detected by cytosolic PRRs. TLR7 and TLR8 in the endosome can recognize ssRNA on bacterial OMVs, leading to nuclear translocation of IRF5/7 and activating the NF- κ B signaling pathway. This causes the production of IFN- β , IFN- γ , TNF- α and IL-12 (143). Deoxyribonuclease treatment of vesicles resulted in a proportion of intact DNA, indicating vesicle-located DNAs. TLR9 in the endosomes recognizes DNA on bacterial OMVs. Expression of TLR9 in the endosomes of B cells is increased by OMV stimulation, and DNA in OMVs promotes IL-6 and IgM secretion (144). NLRs in the cytosol can recognize peptidoglycan in OMVs derived from *H. pylori*, *P. aeruginosa*, and *Neisseria gonorrhoeae* upon entering host cells and activate NF- κ B signaling (145). In summary, due to OMVs inheriting PAMPs of native bacteria, they can be effectively internalized, recognized, and initiate inflammatory responses.

1.4.2 Vaccine Adjuvants and OMVs as an Emerging Vaccine Platform

Since most highly purified recombinant antigens are poorly immunogenic, adjuvants are necessary in vaccines to increase immune protection and duration. Adjuvants in vaccine formulations can be various components, such as aluminum salts, synthetic small molecules, microbial products, and lipid nanoparticles, which can enhance immunogenicity when administered with vaccine antigens (146). Adjuvants are indispensable constituents of vaccines, though their precise mechanisms of

action remain incompletely understood despite widespread usage. Recent advancements in comprehending how the innate immune system regulates antigen-specific responses have contributed to exploring the mechanisms underlying the function of adjuvants. Adjuvants can be broadly classified into three categories: (I) immunostimulants, (II) delivery systems, and (III) vehicle adjuvants (147). Immunostimulants comprise danger signal molecules that induce the maturation and activation of antigen-presenting cells (APCs) by engaging Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). This activation promotes the production of antigen signals and co-stimulatory signals, thereby enhancing adaptive immune responses (148–151). Conversely, delivery systems consist of carrier materials that facilitate antigen presentation by protecting the presence of loaded antigens and directing them toward lymph nodes or APCs (152–154).

Adjuvants improve vaccines in several ways. First, they could reduce antigen doses and prolong the duration of immune responses (155). Second, they mediate the immune phenotype to propagate the most convenient immune responses against each specific pathogen (156). Third, some adjuvants could increase the uptake of several infectious pathogens by mucosal epithelial cells, which triggers inflammatory responses and helps immune cells reach the infection side (157). Therefore, using traditional adjuvants and developing novel ones have always been critical to vaccine design.

Up to date, MF-59, AS03 (oil-in-water emulsions), Alum, AS04 (composed of detoxified LPS and alum), CpG ODN 1018, AS01, and lipid nanoparticle (LNP) are classical adjuvants that have been licensed in human vaccines (158).

1.4.2.1 Aluminum hydroxide (Alum)

Aluminum salts were the first discovered adjuvants in 1926 that were approved for human vaccines (159). Aluminum hydroxide (AH) and aluminum phosphate (AP) are two adjuvants that are well-known and widely used in licensed vaccines, though their mechanisms of action are complex and incompletely understood. The absorption of antigens with aluminum makes them particulate, which is critical to their action. Particulates are easily phagocytosed by dendritic cells. Absorption increases antigen-presentation, indicated by high activation of CD4 T cells and MHC-II peptide complexes (160). It leads to the slow antigen release into the injection site, thus promoting local inflammation and cytokine production, called the “depot effect” (161). Aluminum adjuvants facilitate the differentiation and activation of monocytes into dendritic cells in the injection site,

thus improving antigen transportation to the draining lymph node. Aluminum hydroxide also initiates the complement cascade, which is very important for humoral immune response, resulting in the recruitment of immune cells to the injection site via released complement effectors and the formation of membrane attack complexes (162). LPS and other PAMPs on OMVs are of great concern for vaccine developers due to the potential risk of systemic reactogenicity. Some studies show that Alum absorption results in decreased reactogenicity in vaccine formulations. Rosenqvist et al. have demonstrated that detergent treated-OMVs (dOMVs) absorbed by Alum have shown the slightest reaction in humans compared to free endotoxin, which causes the highest fever. Also, dOMVs formulated on Alum have reduced responses compared to free endotoxin when tested via rabbit pyrogenicity test and LAL assay (163,164). FDA-approved meningococcal B vaccines, Bexsero (GSK), contains 0.519 mg AH, and Trumenba (Pfizer) contains 0.25 mg AP per dose (165).

Vaccine adjuvants are also important in enhancing cell-mediated protective immunity against the antigens. However, Alum has a strong Th2 bias and induces weak Th1 and Th17 responses (166), which are necessary against certain infectious diseases like HIV, influenza, tuberculosis, and malaria. Combining Alum with other adjuvants inducing distinct mechanisms to activate innate and adaptive immunity can lead to more effective, long-lasting responses and dose sparing. Immunostimulatory molecules, especially the TLR ligands, like poly (I: C) and CpG ODN, are splendid candidates for combinations as they are adsorbed strongly to aluminum hydroxide and activate signaling pathways through MyD88 and TRIF.

1.4.2.2 CpG ODN

Bacterial DNA is an excellent immunostimulatory that induces innate immune responses because unmethylated CG motifs present at high frequency in prokaryotic DNA but are rare in eukaryotic DNA. Synthetic ODNs expressing unmethylated CpG motifs (CpG ODNs) are utilized to mimic the immunostimulatory activity of bacterial DNAs (167). Upon uptake by target cells, bacterial DNA and CpG ODNs reach the late endosomal compartment, where TLR-9 recognizes them. TLR-9 is the only responsive receptor to CpG ODNs (168). In humans, TLR-9 is expressed primarily in B cells and plasmacytoid DCs (169). To date, four classes of CpG ODN have been depicted: K, D, C, and P-type CpG ODNs. K-type ODNs encode multiple CpG motifs on a phosphorothioate backbone. Using phosphorothioate makes CpG ODNs more stable against

nucleases and prolongs in vivo half-life. D-type ODNs comprise a mixed phosphodiester/phosphorothioate backbone and a single CpG motif. While K-type ODNs activate pDCs and B cells, D-type ODNs only affect pDCs. K-type triggers pDCs to differentiate and produce TNF- α , whereas D-type ODNs activate pDCs to secrete IFN- α (170). B-cells stimulated by CpG DNA produce IL-6, IL-12, and IP-10 and secrete IgM. The expression of costimulatory molecules, MHC-II, CD80, CD86, and CD40, increases in CpG ODN-stimulated B cells, and eventually, they differentiate into plasma and memory cells (171,172). CpG ODN stimulates pDCs to secrete Type-I IFNs, providing broad protection against viruses and bacteria (173). All these various aspects of CpG ODNs make them excellent adjuvant candidates for vaccines developed using new technologies.

So far, using CpG ODNs as vaccine adjuvants in numerous preclinical studies has shown promising results. They improve both humoral and cellular responses in different animal models. Antibody titers against pathogens like influenza and hepatitis B in mice significantly increase upon CpG ODN use. CpG ODNs improve CD4⁺ and CD8⁺ T-cell responses in murine models, especially leishmania infections (174). When CpG ODNs are administered intranasally or intravaginally, they enhance mucosal and systemic immunity against HSV-2 and Salmonella (175,176). Furthermore, CpG ODNs boost cytotoxic CD8⁺ T-cell responses against tumor antigens, indicating potential for cancer vaccines (177). HIV-infected macaques and immunodeficient animals show improved reactions to vaccines when CpG ODNs are included (178). Our group recently conducted a pre-clinical vaccine study against SARS-CoV-2, including K-type CpG ODN as an adjuvant mixed with VLPs. In this study, compared to VLP alone, only the CpG ODN adjuvanted groups have significant anti-Spike, anti-RBD, and anti-N IgG2a, showing the preferential Th1-biased immunostimulatory activity of CpG ODN. Moreover, when splenocytes from immunized mice were re-stimulated with recombinant SARS-CoV-2 antigens, only mice immunized with CpG ODN or CpG ODN+Alum gave a Th1-biased IFN- γ response but no Th2-related cytokines (28). Considering the role of Th1 responses in viral clearance mechanisms, using CpG ODN provides an advantage over non-adjuvanted and/or Alum adsorbed vaccine formulations against the SARS-CoV-2 virus (179).

Many clinical trials have proved the potential of CpG ODN as a vaccine adjuvant. CpG ODNs produce significantly higher antibody responses than the vaccine alone in the Hepatitis B (Engerix

B) vaccine trial (180). Subjects vaccinated with the AMA1 malaria vaccine candidate composed of CpG ODNs got 5-5-fold increased antibody titers, with sustained responses observed for over 200 days (181).

When the safety profile of CpG ODN is searched, there are some concerns, like CpG ODN could cause autoimmune diseases and predispose subjects to toxic shock. Under certain circumstances, CpG ODNs are suspected to trigger organ-specific autoimmune diseases and lead to more frequent and adverse systemic side effects (182). However, various studies in which mice were repeatedly vaccinated with immunostimulatory doses of CpG DNA demonstrated the insufficiency of CpG ODNs to induce autoimmunity, although dramatic increases in secretory B cells and IgG levels (183). Additionally, there are no records of toxic shock when CpG ODN is injected at concentrations typically present in vaccines. Overall, CpG ODNs are considered reasonably safe as an adjuvant.

1.4.2.3 OMVs as Potent Vaccine Antigens and Adjuvants

OMVs naturally have essential characteristics that are preferred for vaccine designs. First, its manufacture is easy and cost-effective. Gram-negative bacteria naturally secrete OMVs up to several milligrams per liter under optimized conditions. Second, OMVs comprise species-specific antigens and PAMPs that both innate and adaptive immune pathways can recognize. Therefore, OMVs are ideal vaccine components that can function as immunogenic antigens and inherent adjuvants in one package. PAMPs like LPS, flagellin, peptidoglycans, lipoproteins, and other OMPs can be recognized by PRRs, mainly TLRs, activating APCs and naïve T cells (184). Third, since their natural size is between 20-300 nm, OMVs have a structural advantage, while many efforts are made to develop numerous nanoparticle-based vaccine delivery vehicles today (185). By their small size, OMVs efficiently translocate antigens from the injection site to the draining lymph node, where they are taken up by dendritic cells and processed for presentation (186). Finally, they can be genetically modified to improve their production, immunogenicity, and safety.

LPS causes excessive release of pro-inflammatory cytokines in humans, which is the central issue regarding OMVs in adjuvant development. Thus, modifying the structure of LPS or reducing its content is essential to mitigate the toxicity of OMVs to acceptable levels. One conservative strategy is to treat OMVs with a detergent, most commonly sodium deoxycholate (187). This method reduces LPS content and increases OMV yield; however, it also reduces antigen variety

by disrupting membrane proteins (188). Therefore, ongoing investigations aim to figure out LPS structure and modify bacteria to express a less toxic version of this. The ultimate goal of these studies is to enhance the safety profile of OMVs and optimize their ability to elicit robust and sufficient antibody responses. The main component responsible for the toxicity of LPS is lipid A (189). Its penta-acetylated form, rather than hexa-acetylated, is not toxic, as is shown in human TLR4 experiments (190). Deletion of *lpxL1* and *lpxL2* genes generates mutant strains expressing penta-acetylated Lipid A and OMVs, which are still immunogenic (191). However, additional adjuvants might be required to enhance innate immunity due to the loss of TLR4 stimulation in humans. The difficulty of utilizing OMV-based adjuvants in human vaccines is finding the optimal balance between maintaining an efficient level of immune response and limiting toxic side effects. The expression of a distinct non-toxic LPS structure that can still stimulate human TLR4 has been achieved by heterologous expression of the *pagL* gene encoding lipid A 3-O-deacylase from *Bordetella bronchiseptica* (192). *pagL* is found in many Gram-negative bacteria but not in *Neisseria meningitidis*. The heterologous expression of this gene in *N. meningitidis* synthesizes a different penta-acetylated form of LPS. While deleting *lpxL1* removes the secondary C12:0 acyl chain at the 2'-position, *pagL* enzyme specifically deacylates lipid A at the 3-position (193). This resulting form can activate TLR4 signaling and initiate human monocytes for cytokine production.

Recent developments in bioengineering promise to modify OMVs as ideal antigens and adjuvants for next-generation vaccine designs. Previous studies on *N. meningitidis* serogroup B have demonstrated the great potential of OMVs after genetic modifications, showing the increase in OMV yield, LPS detoxification, and introduction of multiple antigens.

1.5 AIM OF STUDY

The aim of this study is twofold. The first part focuses on developing a combination vaccine against *Neisseria meningitidis* and SARS-CoV-2 to overcome the challenges of managing and administering multiple vaccines. This approach is expected to simplify vaccination procedures, reduce costs, improve acceptance, and increase the likelihood of disease eradication.

The second part of the study seeks to understand the changes in humoral responses, cytokine levels, and extracellular vesicle profiles that correlate with disease severity in MIS-C. This research aims to enhance diagnostic and therapeutic strategies for managing the condition by analyzing plasma samples from pediatric patients.

2 CHAPTER 2

MATERIALS & METHODS

2.1 MATERIALS

2.1.1 Bacteria Cell Culture Media, Reagents and Filters

Brain Heart Infusion (BHI) Media (HIMEDIA) and Mueller Hinton Broth (Millipore, Sigma) were used for *N. meningitidis* culture. OMVs were concentrated using Amicon® Ultra-15 Centrifugal Filters purchased from Merck (Darmstadt, Germany). Sodium deoxycholate (#SE1833003) was purchased from Serva (Heidelberg, Germany). Denarase^R was obtained from c-LEcta (Leipzig, Germany).

2.1.2 Cell Culture Media, Kits, and Solutions

RPMI 1640 with L-glutamine (#BI01-100-1A), High Glucose DMEM with L-Glutamine (#01-052-1A), and Cell Culture Grade Water (#03-055-1A) were obtained from Biological Industries, Sartorius (Kibbutz Beit-HaEmek, Israel). Heat Inactivated FBS (Gibco, #10270-106), Penicillin-Streptomycin (10,000 U/mL, #15140-122), 10X Trypsin-EDTA without phenol red (0.5%, #15400-054), Sodium Pyruvate (100mM, #11360-070), MEM Non-Essential Amino Acids (100X, #11140-050), and DPBS (#14190-250) were obtained from Gibco, Thermo Fisher Scientific (Waltham, MA, USA). Complete RPMI for THP1-Dual™ Cells (#thpd-nfis, Invivogen, France) and DMEM for HEK-Blue™ hTLR4 cells (#hkb-hltr4, Invivogen, France) was prepared by supplementing the base media with %10 FBS and Penicillin-Streptomycin (Invivogen, # ant-nr-2). QUANTI-Blue™ SEAP detection reagent (#rep-qbs) and QUANTI-Luc™ 4 Lucia/Gaussia luciferase detection reagent (#rep-qlc4lg1) were obtained from Invivogen. Lymphocyte Separation Medium (density 1.077 g/ml, #LSM-A) for plasma and PBMC isolation was purchased from Capricorn (Ebsdorfergrund, Germany). Lipopolysaccharides from *Escherichia coli* O55:B5 (LPS), used in endotoxicity experiments, were obtained from Sigma-Aldrich (St. Louis, MO, USA). For bead-based cytokine analyses, LEGENDplex™ COVID-19 Cytokine Storm Panel 1 (14-plex) w/VbP (#741089) and LEGENDplex™ HU Th Cytokine Panel (12-plex) w/ VbP V02 (#741028) kits were purchased from Biolegend (San Diego, USA).

2.1.3 Western Blot Antibodies and Reagents

All antibodies and reagents used in Western-Blot analyses are listed in Table 1.

Table 1. Human-specific Western Blot antibodies used throughout the study.

Antibodies	Product Name	Catalog#	Company
	Anti-TSG101 antibody [EPR7130(B)]	ab125011	Abcam
	anti-SARS-CoV-2 Spike RBD antibody	40592-T62	SinoBiological
	Anti-rabbit IgG, HRP-linked Antibody	7074S	Cell Signaling
Reagents	Amersham ECL Prime Detection Reagents	RPN2236	Cytiva
	PageRuler™ Prestained Protein Ladder,180 kDa	26616	Thermo Scientific
	PageRuler™ Prestained Protein Ladder,250 kDa	26619	Thermo Scientific

2.1.4 ELISA Antibodies and Reagents

Antibodies and reagents used for IgG ELISA experiments and their working conditions are listed in Table 2.

Table 2. Monoclonal antibodies and reagents used for ELISA throughout the study.

Product	Catalog#	Company	Working Concentration
Anti-mouse IgG-AP	1030-04	Southern Biotech/USA	1:1000
Anti-mouse IgG1-AP	1070-04	Southern Biotech/USA	1:1000
Anti-mouse IgG2a-AP	1080-04	Southern Biotech/USA	1:1000
Anti-mouse IgG2c-AP	1079-04	Southern Biotech/USA	1:1000
Anti-human IgA-AP	2050-04	Southern Biotech/USA	1:1000
Anti-human IgG-AP	2040-04	Southern Biotech/USA	1:1000

Anti-human IgM-AP	2020-04	Southern Biotech/USA	1:1000
PNPP	34047	Thermo Scientific	1 tablet/5 ml

2.1.5 Adjuvants used in immunization studies

Aluminum Hydroxide gel adjuvant (Alhydrogel^R 2%) was purchased from Croda (Frederikssund, Denmark). K-type CpG ODN (K3- 5'- ATCGACTCTCGAGCGTTCTC- 3') was synthesized by Alpha DNA (Columbia, USA).

2.2 METHODS

2.2.1 PART I: The Development of a Combined Vesicle-Vaccine against SARS-CoV-2 and *Neisseria meningitidis*

Table 3. The *Neisseria meningitidis* ST-11 (serogroup W) and ST-32 (serogroup B) used for OMV production are representative isolates from clinical cases of meningococcal diseases in Türkiye.

PubMLST								
ID	Isolate	Origin/Year	Genogroup	PorA_VR1	PorA_VR2	FetA-VR	Sequence Type	Clonal Complex
60860	16	Turkey/2014	B	7-11	16	F5-43	32	ST-32 complex
60745	58	Turkey/2017	W	5	2	F1-1	11	ST-22 complex

2.2.1.1 *Neisseria meningitidis* growth conditions for outer membrane vesicle (OMV) isolation

The bacteria were inoculated into 5 ml of brain heart infusion (BHI) broth (NIMEDIA) and cultured in a rotary shaker at 37°C, 5% CO₂ overnight. Afterward, the culture medium was diluted 1:50 with fresh BHI broth, and the bacteria were cultured for 36 hours. *N. meningitidis* were pelleted by centrifugation at 4000 g for 30 min, and the supernatant was filtered using 0.22-µm CA (cellulose acetate) membrane vacuum filters (Corning). The filtered supernatant was stored at +4 °C for further steps in the OMV isolation procedure.

2.2.1.2 Outer Membrane Vesicle (OMV) isolation from *Neisseria meningitidis* serogroup W and B strains

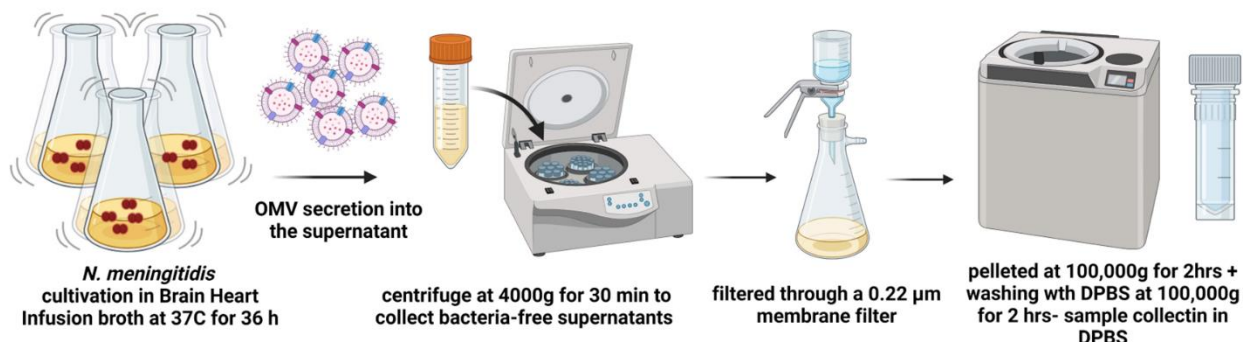


Figure 2.1 Schematic drawing of outer membrane vesicle (OMV) isolation from *N. meningitidis* using differential ultracentrifugation method.

2.2.1.2.1 Ultracentrifugation Method

The OMVs in filtered supernatant were pelleted by ultracentrifugation at 100,000g for two h, followed by an additional washing with PBS at 100,000g for two h. Finally, the membrane vesicle pellets were resuspended with DPBS. Protein content was quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce) following the manufacturer's instructions, and the OMVs were stored at -80°C for further use.

2.2.1.2.2 Isolation of OMVs by protein liquid chromatography system after detergent treatment on culture supernatant

The bacterial culture supernatants were harvested by centrifugation at 10000 g for 20 min and filtered through a 0.22µm CA (cellulose acetate) membrane vacuum filter. The harvest was treated with Denarase at $+4^{\circ}\text{C}$ overnight, then concentrated using a 10 kDa Amicon Ultra-15 filter. After washing once with DPBS, one volume of 100 mM Tris, 2 mM EDTA, pH 8.5, containing 1% sodium deoxycholate, was added and incubated in a rotary shaker at 37°C for 30 min. OMVs were resuspended in DPBS, followed by the concentration step using a 10 kDa Amicon Ultra-15 filter.

2.2.1.3 Characterization of outer membrane vesicles released by clinical isolates of *Neisseria meningitidis*

2.2.1.3.1 Nanoparticle analysis of OMVs using Tunable-resistive pulse sensing (TRPS)

Tunable-resistive pulse sensing (TRPS) measurements were performed using the qNano Gold system (IZON S/N 601A, UK) and analyzed with the IZON Control Suite 3.4.2.48 software (IZON Science LTD, UK). The calibration particles, IZON coating solution, wetting solution, and nanopores (NP150 and NP250) were utilized following the manufacturer's protocols. For each measurement, 35 µl of either 1:1000 diluted calibration particles (CPC100, IZON Reagent Kit, RK3-167) or sample liquid was loaded, and each sample reading was repeated three times.

2.2.1.3.2 Imaging OMVs with Scanning Electron Microscopy (SEM)

Ten µl aliquot of the OMV solution was deposited onto a silica surface, air-dried, and sputter-coated with 5 nm of Au/Pd alloy using a precision coating system. Omvs were visualized by environmental scanning electron microscopy (SEM) (Zeiss Sigma500 FESEM, Germany).

2.2.1.3.3 In vitro Endotoxicity Assay with *N. meningitidis* OMVs-stimulated HEK-hTLR4 cells

HEK-Blue™ hTLR4 cells (Invivogen, France) express an inducible reporter gene for SEAP (secreted embryonic alkaline phosphatase). TLR4-dependent NF-κB activation is triggered upon stimulating with LPS on OMVs and produces SEAP, whose level can be monitored by the detection reagent QUANTI-Blue™. HEK-Blue™ hTLR4 cells at a concentration of 200,000 cells/ml in 200 µl were incubated with various doses of LPS and OMVs for 24 hours at 37°C with 5% CO₂. Twenty µl of sample supernatants were mixed and incubated with 180 µl of QUANTI-Blue solution for 30 minutes. The optical density (OD) values were measured at 620 nm.

2.2.1.4 IMMUNIZATION STUDIES

2.2.1.4.1 Mouse Strains

The Bilkent University, Department of Molecular Biology and Genetics (Ankara, Türkiye) and the Vivarium Facility of the Izmir Biomedicine and Genome Center (IBG, Izmir, Türkiye) provided the animals (BALB/c, C57BL/6, and C57BL/6-ACE2) used in this study. All animal procedures were approved by the Animal Experiments Local Ethical Committee (AELEC) of Bilkent University and IBG by national laws and policies. All methods were carried out according to the approved guidelines and regulations. Animals were housed in individually ventilated cages under 22 ± 2 °C ambient temperature and 12:12 h light: dark cycle.

2.2.1.4.2 Comparator vaccines

The comparator vaccines used in this study are commercially available vaccines: ACYW conjugate vaccine (NimenrixTM, Pfizer) and the four-component serogroup B vaccine (4CMenB, Bexsero®, GlaxoSmithKline). These vaccines are licensed for one human dose of 0.5 ml each. NimenrixTM contains 10 µg/ml of each polysaccharide A, C, W, and Y conjugated to tetanus toxoid. Bexsero contains 50 µg/ml of OMV, including PorA P1.4 recovered from group B strain NZ98/254, along with 100 µg/ml of three recombinant meningococcal proteins: NHBA, NadA, and fHbp, respectively. During the study, mice were administered two doses, equivalent to 1/5th of a human dose for Bexsero and 2/5th of a human dose for Nimenrix, with a 2-week interval. The dose for NimenrixTM was 8 µg/0.2 ml, while for Bexsero, it was 5 µg/0.1 ml.

2.2.1.4.3 Bivalent Meningococcal OMV Vaccine Immunization

2.5 µg MenW OMVs plus 2.5 µg MenB OMVs were formulated only with Alum (20 µg/mouse) or K3 CpG ODN (30 µg/mouse) or their combinations. BALB/c mice (n=16/group) were subcutaneously (s.c) injected on days 0 and 14. Comparator BALB/c mice (n=8/group) were injected two weeks apart with 100 µl of 4CMenB vaccine (Bexsero®, GSK, includes 25 µg MenB OMV in 0.5 ml for human dose) or 200 µl of MenACYW conjugate vaccine (NimenrixTM, Pfizer). Before the day of the booster and two weeks post-booster injection, mice were bled, and sera were stored at -20°C until further use.

2.2.1.4.4 SARS-CoV-2 Delta Plus-RBD Vaccine Immunization

Three different formulations of Delta-RBD protein were prepared along with the negative control for animal immunizations: (a) formulation 1 = 1 µg Delta-RBD, 60 µg Alum, 30 µg K3 CpG, (b) formulation 2 = 5 µg Delta-RBD, 60 µg Alum, 30 µg CpG, (c) formulation 3 = 25 µg RBD-DP, 60 µg Alum, 30 µg CpG; and (4) placebo = 60 µg Alum, 30 µg CpG. Briefly, K3 CpG was added into Delta-RBD protein in PBS and then mixed with Alum by dropwise Alum addition. The solution was incubated at RT for 30 minutes in a tube rotator. BALB/c mice and K18-hACE2 transgenic mice (n=10/group) were intraperitoneally (i.p.) injected two weeks apart with these three different formulations. Sera were collected on days 14, 28, and 88 post-immunization and assessed for Delta RBD-specific IgG, IgG1, and IgG2a by ELISA.

2.2.1.4.5 Immunization studies with combination vaccine composed of bivalent N. meningitidis OMVs and SARS-CoV-2 Delta Plus-RBD

2.5 µg MenW OMVs plus 2.5 µg MenB OMVs were mixed with two different doses of SARS-CoV-2 Delta-RBD (low dose: 3 µg and high dose: 15 µg) and formulated only with K3 CpG or Alum, or their combinations. C57BL/6 mice (n=6/group) were intraperitoneally immunized with these formulations on days 0 and 14. Sera were collected on days 14 and 28 post-immunization and stored at -20°C until further use.

2.2.1.4.6 Immunization studies with a combination vaccine composed of bivalent N. meningitidis OMVs and SARS-CoV-2 6p prefusion stabilized Delta Plus and Omicron-Spike bearing VLPs

6p-Delta Spike /or Omicron Spike expressing VLPs (10 µg/mice) and MenB+MenW OMVs (5 µg/mice) were formulated only with K3 CpG (30 µg/mice) or Alum (60 µg/mice) or their combinations. In one of the groups, CpG ODN was encapsulated into the OMV-VLP mixture by lyophilization, and Alum was then absorbed into this mixture. Briefly, 10 µg of SARS-CoV-2 VLP, 5 µg of meningococcal OMVs, and 30 µg CpG ODN were snap-frozen and lyophilized overnight. After resuspending in PBS in a controlled manner, Alum was added dropwise and incubated for 30 minutes. C57BL/6 mice (n=6/group) were intraperitoneally (i.p.) immunized on

days 0 and 14 with these formulations. Mice sera were collected on days 14 and 28 post-immunization and stored at -20°C until further use.

2.2.1.5 IgG ELISA

Immulon 2HB ELISA plates (SPL Life Sciences, Korea) were coated with 50 μl /well in-house recombinant 6p-Spike (1 $\mu\text{g}/\text{ml}$), Delta-RBD (1 $\mu\text{g}/\text{ml}$), Omicron-RBD (2 $\mu\text{g}/\text{ml}$) and nucleocapsid (1 $\mu\text{g}/\text{ml}$) and isolated OMVs (0.5 $\mu\text{g}/\text{ml}$) in PBS at $+4^{\circ}\text{C}$ overnight. Plates were then blocked for 4 hours at room temperature in 200 μl /well of 5% BSA-PBS-T solution. The plates were washed twice with PBS-Tween (0.05%, PBS-T) and twice with dH₂O. 1:50 diluted mice sera were serially diluted four-fold with 5% BSA in PBS-Tween (0.05%, PBS-T) into wells. 50 μl of serially diluted sera was transferred into ELISA plates and incubated at $+4^{\circ}\text{C}$ overnight. Following subsequent washing, 50 μl of ALP conjugated goat anti-mouse IgG (Southern Biotech), anti-mouse IgG1 (Southern Biotech), anti-mouse IgG2a, or anti-mouse IgG2c (Southern Biotech) antibodies were plated at a 1:1000 dilution and incubated for 2 hours at room temperature. After the last washing step, the PNPP (Thermo Scientific, U.S.A.) substrate solution was prepared according to the manufacturer's instructions and added (50 μl /well) to develop plates. Consecutive optical density values at 405nm were measured using a microwell plate reader (SpectraMax Molecular Devices, U.S.A.) for 5 hours. Final log₁₀ titers were calculated in GraphPad Prism 9 software.

2.2.1.6 Serum Bactericidal Activity (SBA) Assay

Neisseria meningitidis MenB and MenW target strains were incubated with heat-inactivated animal sera and active complement to evaluate vaccine-induced antibodies' complement-mediated killing activity. Pooled baby rabbit serum (3-4 weeks sterile, Pel-Freez) was used as the complement source. Sera from non-immunized mice were used as negative controls.

Bacteria were subcultured on blood agar plates (BD™ Columbia agar with 5% sheep blood) and incubated for 18 hours at 37°C , 5% CO₂. Colonies were inoculated in 4 ml of Brain Heart Infusion broth (BHI) and incubated on a shaker at 37°C , 210 rpm until OD₆₀₀=0.3. A working dilution of bacteria (1:625 MenB; 1:1200 MenW) was prepared in PBS containing 1% (w/v) BSA and 0.1% (w/v) glucose (Assay buffer). All sera tested in SBA were previously heat-inactivated (HI) at 56°C

for 30 minutes to remove endogenous complement activity. Serial 3-fold dilutions of HI sera were prepared in a 96-well round bottom microtiter plate by adding 20 µl of pre-diluted sera (starting from 1/72 dilution for MenB; 1/8 dilution for MenW) to 40 µl of assay buffer previously added to the wells, mixing, and transferring 20 µl into the next well. The reaction mixtures were prepared in a 96-well flat bottom plate in a final volume of 40 µl, containing 10 µl of the target bacterial cells, 20 µl of HI-serum (3-fold serially diluted for MenB, 4-fold for MenW), and 10 µl of active baby rabbit serum (BRS) as an external source of complement. The plate was incubated for one hour at 37°C, 180 rpm, in a rotating shaker. The tilt method allowed 35 µl of reaction mixtures to flow down a blood agar plate. Agar plates were incubated overnight at 37°C with 5% CO₂. On the following day, colony-forming units (CFU) were counted to evaluate the bactericidal titer of each serum. The bactericidal antibody titer was expressed as the reciprocal of the final serum dilution, yielding $\geq 90\%$ killing after 1 hour compared to the average for controls.

2.2.2 PART II: Antibody Responses and Cytokine Profiling among Children with Multisystem Inflammatory Syndrome versus COVID-19 Infection

2.2.2.1 Study Setting

Study samples were collected from patients and healthy subjects between April 2020 and February 2021 at the Hacettepe University Faculty of Medicine Ihsan Dogramaci Children's Hospital. Plasma samples were stored at -80°C, and subsequent laboratory tests were conducted at the Department of Molecular Biology and Genetics, Bilkent University. Hacettepe University Ethics Board for Non-Interventional Studies reviewed and approved the study protocol (Decision no: 2021/09-37). Data regarding pediatric patients' demographic and clinical characteristics were collected via patients' charts and hospital laboratory databases.

2.2.2.2 Patients and Definitions

The study enrolled patients under 18 with confirmed COVID-19 who consented to provide blood samples. Confirmed cases were defined as those testing positive for SARS-CoV-2 via PCR. The study included all cases, regardless of whether the patients were symptomatic or asymptomatic, ensuring a comprehensive representation of the pediatric population affected by COVID-19. Seventeen age- and gender-matched pediatric healthy volunteers were enrolled as controls. We

obtained informed written consent from the guardians of all patients and controls. Confirmed cases were categorized into asymptomatic, mild/moderate, or severe/critical groups based on the classification system developed by Dong et al. (194) as follows: (a) Asymptomatic Infection: Patients who tested positive for SARS-CoV-2 via PCR but exhibited no clinical symptoms or radiologic findings. (b) Mild Disease: Patients presenting with symptoms of upper respiratory tract infections without evidence of pneumonia. (c) Moderate Disease: Patients diagnosed with pneumonia. (d) Severe Disease: Patients experiencing progressive respiratory symptoms, including dyspnea and central cyanosis. (e) Critically Ill: Patients suffering from acute respiratory distress syndrome (ARDS) or respiratory failure, as well as those experiencing shock and organ dysfunction. Using CDC and WHO criteria, MIS-C cases were identified as persistent fever, elevated inflammatory markers, and multi-organ dysfunction (195,196).

2.2.2.3 Sample Collection

For each patient, blood samples were collected at three-time points: at the time of admission, 10–20 days after admission (first interval), and 30–60 days (second interval) after the initial baseline collection.

2.2.2.4 ELISA for Serum Antibody Response

Recombinant hexaprotline stabilized (6p) Spike protein and nucleocapsid were produced in-house and optimized before use. Prof. Mehmet İnan (Izmir Biomedicine and Genome Center) kindly provided recombinant Delta SARS-COV-2 RBD. Anti-Delta-RBD, anti-spike, and anti-nucleocapsid IgG, IgA, and IgM antibody titers were measured by ELISA. Immulon 2 HB microtiter plates (Thermo Scientific, San Jose, CA, USA) were coated with the recombinant antigens and then blocked with BSA-PBS. Serially diluted human sera were added to the plates and incubated at +4 0C overnight. Bound antibody subclasses were detected using alkaline phosphatase streptavidin (ALP)-conjugated anti-human IgG (1:1000), anti-human IgA (1:1000), or anti-human IgM (1:1000) antibodies and plates were developed by the addition of PNPP substrate. Consecutive optical density values at 405nm were measured using a microwell plate reader (SpectraMax Molecular Devices, U.S.A.). log₁₀ titers were calculated by curve fitting in GraphPad Prism software with endpoint OD defined as three standard deviations higher than the average absorbance values of negative healthy control sera.

2.2.2.5 Bead-Based Cytokine Assay

Serum levels of cytokines were assessed using cytometric bead-based multiplex assay panels according to the manufacturer's instructions. LEGENDplex™ HU Th Cytokine Panel (12-plex) and COVID-19 Cytokine Storm Panel 1 (LEGENDplex™, Biolegend) were used for the simultaneous quantification of 19 human cytokines, including MCP-1, G-CSF, IL-6, IFN- α 2, IL-2, IL-8, IL-7, IL-1RA, TNF- α , IP-10, IFN γ , MIP-1 α , IL-10, IL-5, IL-13, IL-17A, IL-4, and IL-22 in the serum samples of COVID-19 and MIS-C pediatric patients. Serum samples were diluted with Assay Buffer in 1:2 dilution. 25 μ l of Assay Buffer (or Matrix B2 buffer for standards), 30 μ l of diluted samples/ standards, and 20 μ l of pre-mixed beads were incubated for 2 hours at room temperature in 96-well V-bottom plates. The plates were washed once with 1X Wash buffer and centrifuged at 400 g for 5 minutes. Immediately after centrifuging, the supernatant was discarded, and 20 μ l of detection antibodies were added to each well for incubation at 110 rpm on a plate shaker for 1 hour at room temperature. After incubation, 20 μ l of SA-PE was added to each well directly without washing the plate. The plates were washed once, and beads in each well were resuspended in 150 μ l of 1X Wash buffer. Samples and standards were read on the NovoCyt flow cytometer. LEGENDplex™ DATA Analysis and GraphPad Prism 9 software were used for data analysis. See Appendix for further details.

2.2.2.6 Plasma Isolation from Patients' Peripheral Blood

Blood samples collected in EDTA sprayed blood collection tube (BD-EDTA) were mixed with an equal volume of DPBS and slowly layered onto the lymphocyte separation medium with a 3:2 (blood: medium) ratio. Then, samples were centrifuged at 800 g for 20 minutes. The upper plasma fraction and the cloudy interface containing PBMC were separated. The plasma fraction was centrifuged at 2000 g for 10 minutes to remove platelets and dead cells. Supernatants were collected in new tubes, snap-frozen in liquid nitrogen, and kept at -80°C for further use.

2.2.2.7 Small Extracellular Vesicle Isolation from Patients' Plasma

Frozen plasma samples were thawed slowly overnight at +4°C to prevent EV rupture and vigorously vortexed before proceeding to isolation. Plasma samples were centrifuged at 10,000 g for 30 min at 4°C. Supernatants were transferred to size-exclusion chromatography (IZON qEV, 20 nm) columns for purification, and the manufacturer's instructions were followed. EVs collected in DPBS were stored at -80 °C for further use. Before being used in experiments, the Pierce BCA

Protein Assay Kit (Thermo Fisher Scientific) determined EVs' protein content according to the manufacturer's instructions.

Carboxyl-modified latex beads (10 μ l, Life Technologies) were incubated with 20 μ g LEAF-purified anti-human CD63 antibody (Biolegend) for 30 minutes. The volume was adjusted to 500 μ l with 1x PBS, and the mixture was rotated overnight. Beads were blocked with 5% BSA in 1x PBS for 4 hours. For EV analysis, 2 μ g EVs were incubated with anti-CD63-conjugated beads overnight. After washing (13,200 x g), bead-EV complexes were incubated with 2 μ g/ml anti-CD81-PE antibodies or isotype controls in sodium azide FACS buffer for 30 minutes, washed, and analyzed on a Novocyte D3000 (Agilent, USA). All steps were performed at room temperature.

2.2.2.8 Western-Blot Analysis

Total exosomal proteins were extracted using a RIPA lysis buffer containing 2 M NaCl, 1 M Tris (pH = 8), NP-40, 10% SDS, protease inhibitor cocktail (Thermo Fisher), and fresh phosphatase inhibitors (Thermo Fisher). After 30 min of incubation on ice and 15 min of sonication, lysates were centrifuged at 10,000 g for 20 min, and proteins were collected. Protein concentration was determined by the BCA Protein Assay Reagent Kit (Thermo Scientific). Forty μ g of exosome lysates were loaded on a 4–20% Mini-PROTEAN[®] TGX[™] precast protein gel (#4561094, BioRad). Electrophoresis was performed using a Mini-Protean Electrophoresis System (Bio-Rad) running at 110 V until the dye front reached the bottom of the gel. For Western-Blot analysis, gels were transferred to PVDF membranes by applying 100 V for 60 min and blocked with 5% BSA in 1X PBS-T. The proteins were incubated with primary anti-SARS-CoV-2 Spike RBD antibody (Rabbit PAb, SinoBiological, Cat no. HD15SE2802) and at room temperature for 1 hour. Following primary antibody incubation, membranes were washed and incubated with HRP-linked anti-rabbit IgG at RT for 1 hour. The blots were developed using the chemiluminescent detection kit, ECL+ from Amersham, according to the manufacturer's protocols and visualized by ChemiDoc MP (Bio-Rad).

2.2.2.9 Analysis of patient-derived EVs on IRF and NF- κ B activation

THP1-DUAL reporter cells (Invivogen #thpd-nfis) were used to monitor NF κ -B and IRF pathway activation to assess the potential intrinsic immunostimulatory activity of patient-derived EVs. Briefly, THP-1 Dual cells (100,000/well) were stimulated with 10 μ g/ml of healthy- or patient-derived EVs for 48 hours. The supernatants were then collected and mixed with QUANTI-Blue[™]

or QUANTI-LucTM detection reagents according to the manufacturer's instructions. OD655 values were measured on a multiplate reader.

2.2.2.10 Proteomics Studies and Mass Spectrometry

The protein extraction from plasma-derived EVs of pediatric patients with severe COVID-19 or MIS-C and LC-MS/MS analyses were conducted as described in our previous study. See [Canavar Yildirim et al., 2024] (197) for further details.

2.2.2.11 LC-MS/MS Data Analysis

An LFQ-based, bottom-up LC-MS/MS experiment, used for qualitative assessment of spectral counts of individual peptides with relative abundance estimates, was performed in data-dependent acquisition (DDA) mode. Using an in-house script written in Python, long strings of protein descriptions were parsed to yield protein symbols for downstream analysis. Within-group reproducibility among biological replicates in each sample group was confirmed through Venn diagrams generated using the “ggVennDiagram” function in the R package ggVennDiagram v1.2.3. Enrichment of functional terms in the query data for over-representation was performed using the R package ReactomePA v1.28.053. A protein-protein interaction network by STRING v12.0 was constructed with a minimal interaction score threshold of 0.9400 (and at a Fisher's Exact Test p-value of 5e-02). A hypergeometric test was intended to identify any meaningful difference of the overlap between sample groups given the two-set Venn diagram.

2.2.2.12 Statistical Analysis

LEGENDplexTM DATA Analysis and GraphPad Prism 9 software were used for bead-based cytokine assay. Statistical differences for all other experiments were analyzed using GraphPad Prism 9 statistical software. Groups were compared using a one-way ANOVA with Kruskal-Wallis test. Additional comparative statistical analyses were mentioned in the figure legends. In all analyses, a P value below 0.05 was considered statistically significant.

3 CHAPTER 3

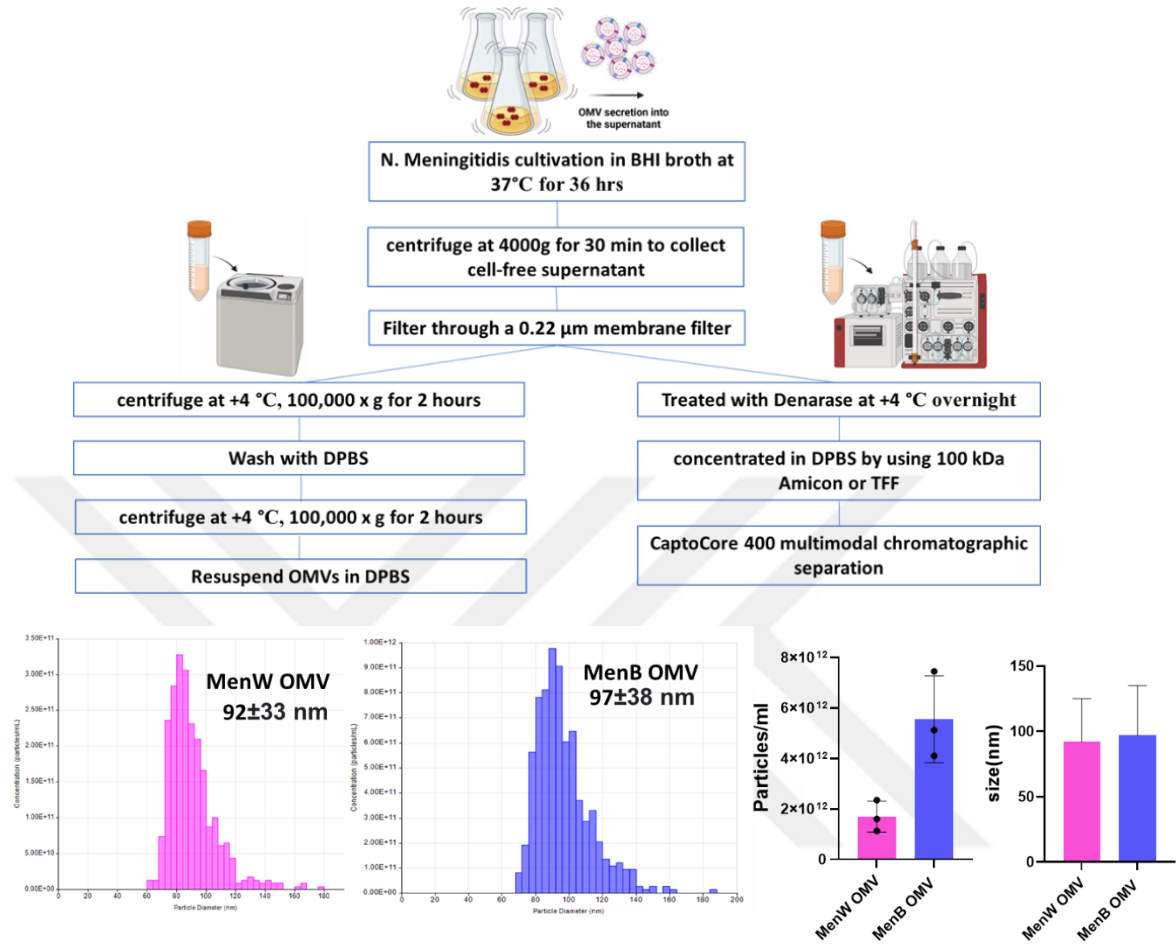
RESULTS

3.1 PART I: The Development of a Combined Vesicle-Vaccine against SARS-CoV-2 and *Neisseria meningitidis*

3.1.1 Production and Characterization of *Neisseria meningitidis* OMVs

We isolated *N. meningitidis* OMVs by two distinct methods throughout the study for different purposes, as summarized in Figure 1a. Since size-exclusion chromatography provides higher purity, we used this method to isolate all OMVs used in immunization studies. However, setting up the chromatography conditions can sometimes be laborious, so we used ultracentrifugation for some optimization studies. We extracted native OMVs from *N. meningitidis* serogroup W (ST-11) and serogroup B (ST-32). The purification process, depicted in Fig. 3.1a, involved loading Denarase-treated and clarified cell culture supernatant onto a HiScreen CaptoCore 400 multimodal size-exclusion/ion-exchange chromatography column. The resulting flow fraction was passed through tangential flow filtration or Amicon columns. We conducted TRPS (as shown in Fig. 3.1b) and SEM (as depicted in Fig. 3.1c), verifying the vesicular and spherical structure of the *N. meningitidis* OMVs with expected sizes (92 ± 33 nm and 97 ± 38 nm for the MenW and MenB OMVs, respectively).

(a)



(c)

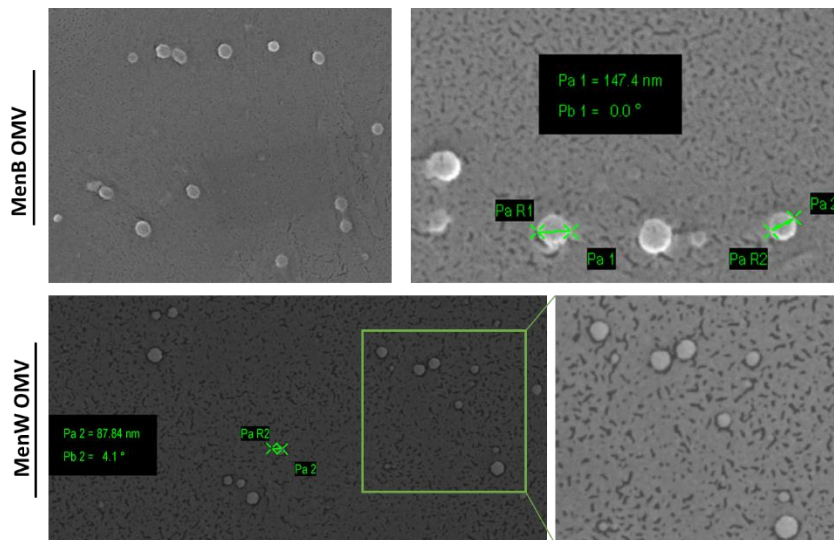


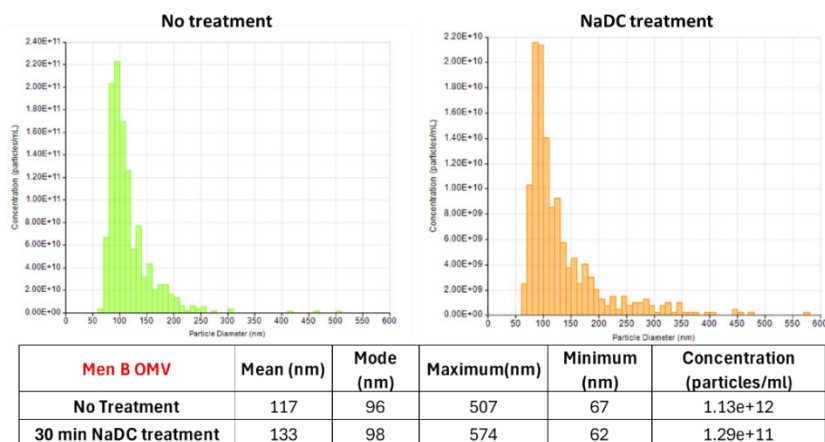
Figure 3.1. Production and characterization of the *N. Meningitidis* OMVs. a) Schematic representation of the steps followed in OMV isolation methods. b) TRPS measurement of OMV

concentration and size distribution. c) scanning electron microscopy (SEM) images of individual *MenW* and *MenB* strain OMVs.

3.1.2 LPS Detoxification in *N. meningitidis* OMVs

The presence of LPS in the OMVs presents challenges related to safety since it is highly endotoxic and capable of causing adverse inflammatory responses. When OMVs containing LPS are administered as vaccines, they can induce fever, inflammation, and, in severe cases, toxic shock. Therefore, its toxic effects need to be minimized to ensure vaccine safety. However, LPS is an essential immune stimulant in OMV vaccines, and it is necessary to maintain balance and not reduce vaccine immunogenicity while performing LPS detoxification. As discussed in introduction 1.3.3, one of the methods used to remove LPS found on OMV surfaces during meningitidis vaccine production is to treat the source organism with detergent. We used a sodium deoxycholate treatment protocol adapted from Frasch et al. (198). Then, we evaluated their characterization by TRPS and their toxicity level compared to the Bexsero *MenB* vaccine, including detergent-treated OMVs.

(a)



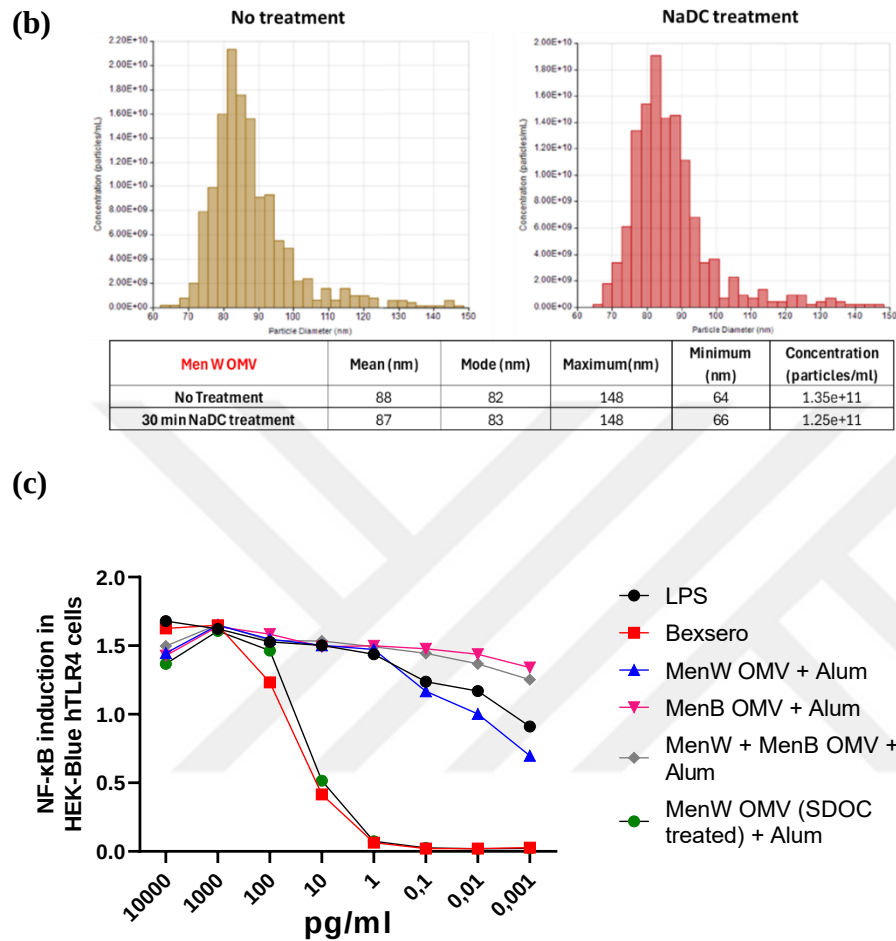


Figure 3.2. OMV characterization after treatment of sodium deoxycholate (a, b) TRPS size distribution measurement (nm) and concentration (particles/ml) of OMVs. (c) HEK-Blue hTLR4 cells were cultured and stimulated for 24 hours with lipopolysaccharide (LPS), the Bexsero vaccine, and meningococcal OMV-vaccine formulations, each containing the same varying doses of OMVs. After 24-hour incubation, the NF- κ B-induced SEAP activity was determined by reading the OD at 620 nm.

As shown in Figure 3.2, the mean size of detergent-treated OMVs and detergent-free OMVs were similar, but the concentration (particles/ml) of MenB OMVs was reduced 10-fold after detergent treatment.

To examine the effect of sodium deoxycholate on LPS content within OMVs, we tested TLR4 activation utilizing HEK-blue reporter cells expressing human TLR4. Following stimulation with

varying concentrations of OMVs adjuvanted with the same dose of Alum for each formulation (Fig. 3.2c), comparable TLR4 activation profiles were observed between LPS and native OMVs. However, MenW OMVs purified after detergent treatment exhibited significantly reduced h-TLR4 response, similar to Bexsero.

3.1.3 Immunogenicity of bivalent meningococcal OMV vaccine

4CMenB (Bexsero, GSK) and MeNZB vaccines are the only licensed vaccines composed of meningococcal serogroup B OMVs. Since serogroup B and W are Türkiye's most common causative agents, we developed a bivalent vaccine containing MenB and MenW OMVs. Then, we conducted a comparative analysis with commercially available vaccines. To investigate the immunogenicity of the MenB + MenW OMV vaccine, 6- to 8-week-old female BALB/c mice were subcutaneously immunized with 2.5 µg OMVs of *N. meningitidis* serogroup W and 2.5 µg OMVs of *N. meningitidis* serogroup B, separated by a 2-week interval. OMVs were either administered as MenB + MenW OMVs or combined with K3 CpG ODN (30 µg/mouse) or Alum (20 µg/mouse). A conjugate vaccine ACYW (NimenrixTM, Pfizer) and serogroup B vaccine Bexsero were also administered to BALB/c mice as comparator vaccines (1/5 of the human dose containing 5 µg of OMVs for Bexsero, 2/5 of the human dose for Nimenrix).

After booster injections, mice immunized with MenB + W OMV or adjuvanted counterparts had higher anti-MenW and MenB-specific IgG, IgG1, and IgG2a levels (Fig. 3.3). As expected, the MenB + W OMV vaccine, whether with or without adjuvants, induced a significantly higher antibody response against MenW OMV compared to Nimenrix. Recently, bactericidal activity and model estimations constructed in children immunized with the 4CMenB vaccine showed the direct protection of 4CMenB against MenW:cc11 disease (199). Consistent with these findings, we have shown that sera from mice immunized with Bexsero are cross-reactive against MenW OMV, containing a higher anti-MenW OMV IgG response than Nimenrix (at least 100-fold or more for all OMV groups over Nimenrix, Fig. 3b) Mice vaccinated with W + B OMV and/or adjuvanted formulations exhibited similar antibody responses to that of Bexsero response (Fig. 3.3c).

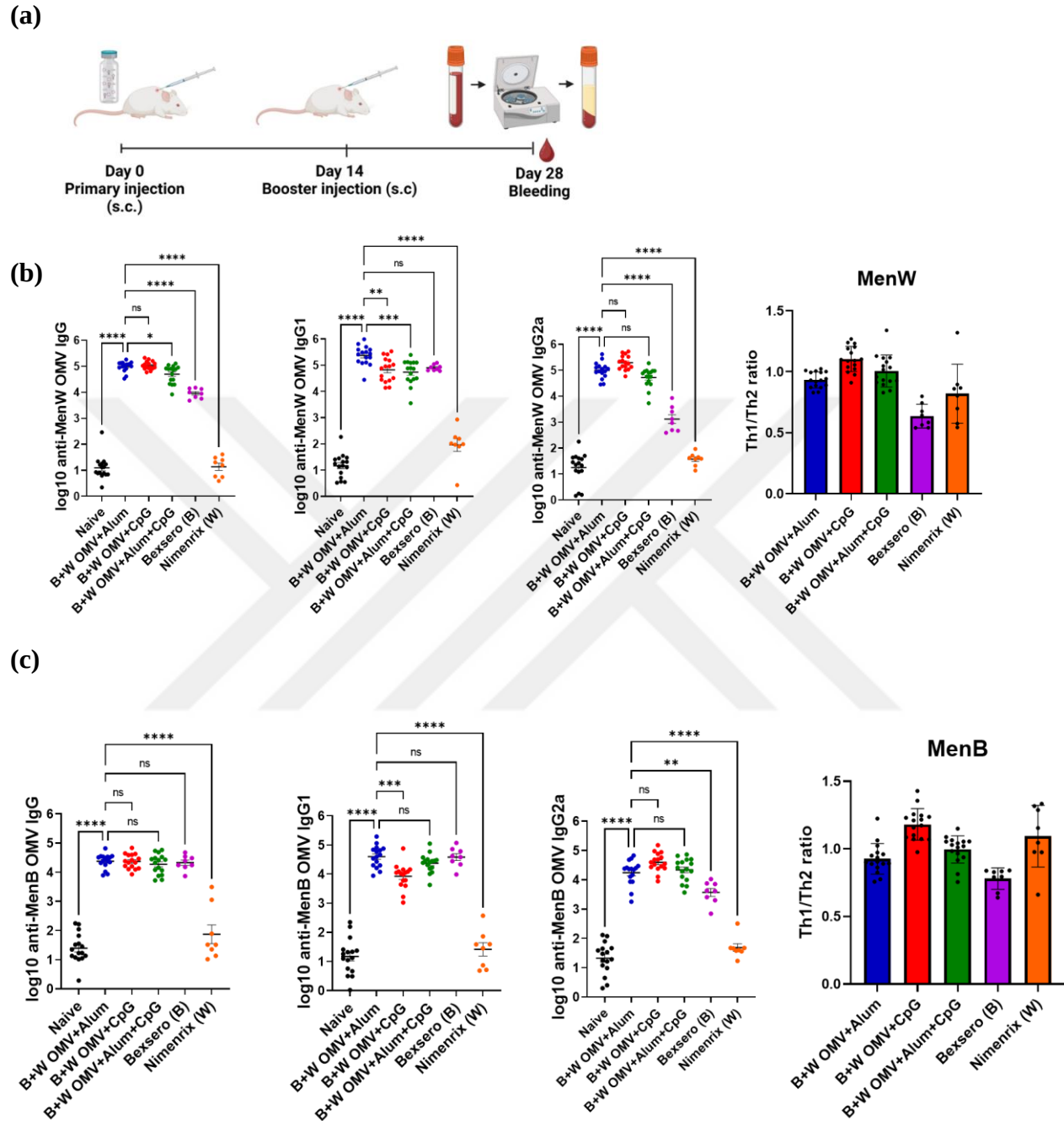


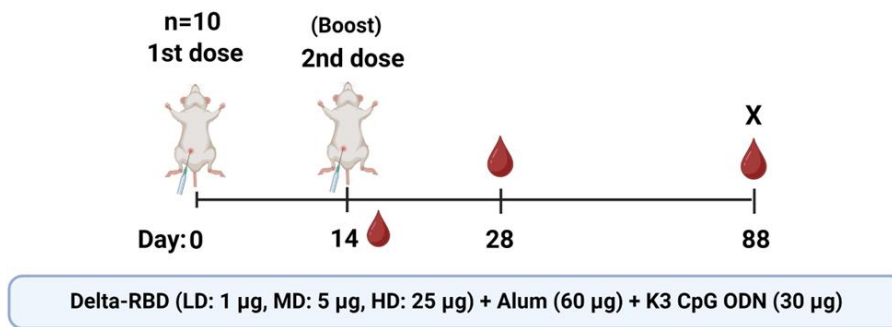
Figure 3.3. W+B OMV vaccine elicits robust antibody responses in mice. BALB/c mice ($n=16/\text{group}$) were immunized (s.c.) on days 0 and 14 with $2.5\ \mu\text{g}$ MenW OMVs plus $2.5\ \mu\text{g}$ MenB OMVs with or without Alum ($20\ \mu\text{g}/\text{mouse}$), without or with K3 CpG ODN ($30\ \mu\text{g}/\text{mouse}$) or with Alum + CpG ODN. Comparator BALB/c mice ($n=8/\text{group}$) were injected on (a) days 0 and 14 with $100\ \mu\text{l}$ of 4CMenB vaccine (Bexsero®, GSK, includes $25\ \mu\text{g}$ MenB OMV in $0.5\ \text{ml}$ for human dose) or $200\ \mu\text{l}$ of MenACYW conjugate vaccine (Nimenrix™, Pfizer). Sera were collected 2

weeks post-boost and assessed for (b) MenW-OMV and (c) MenB-OMV-specific IgG, IgG1 and IgG2a by ELISA. Vaccinated groups were compared by one-way ANOVA with Dunnett's multiple comparisons test. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$. Data are presented as Mean $\pm$ SEM.

3.1.4 Immunogenicity of SARS-CoV-2-RBD vaccine

Kalyoncu et al. developed an effective vaccine candidate for the SARS-CoV-2 Delta Plus variant (B.1.617.2.1). In this study, they produced and purified the recombinant RBD of the Delta Plus strain utilizing yeast *Pichia pastoris* expression system (200). Alum-adsorbed Delta-Plus RBD (DP-RBD) was adjuvanted with K3 CpG ODN, and we tested the efficacy of this formulation in vivo. Three different formulations, including low dose (1 μ g), medium dose (5 μ g), and high dose (25 μ g) DP-RBD with adjuvants, were administered twice into two different types of mouse strains (BALB/c and hACE2-BL6) at 2-week intervals. Humoral responses were evaluated from the sera obtained at various times after the immunizations by ELISA (Fig. 3.4).

(a)



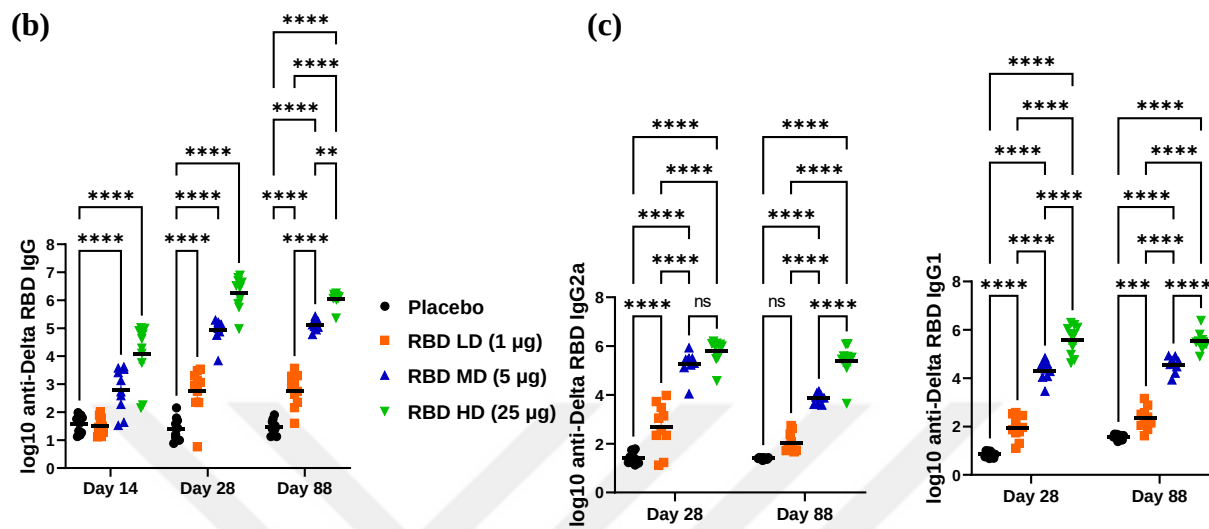


Figure 3.4. Humoral response induced by Delta-plus SARS-CoV-2 RBD vaccine in BALB/c mice. (a) Study design for mice immunizations. BALB/c mice ($n=10/\text{group}$) were immunized on days 0 and 14 with Delta Plus-RBD (low dose: $1\ \mu\text{g}$, medium dose: $5\ \mu\text{g}$, high dose: $25\ \mu\text{g}$), Alum ($60\ \mu\text{g}/\text{mouse}$), and K3 CpG ODN ($30\ \mu\text{g}/\text{mouse}$). Sera were collected on days 14, 28, and 88 post-immunization and assessed for (b) Delta RBD-specific IgG, (c) IgG1, and IgG2a by ELISA. Two-way ANOVA compared vaccinated groups with Sidak's multiple comparisons test. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$. Data are presented as Mean $\pm$ SEM.

In BALB/c mice, low-dose DP-RBD was insufficient to induce primary antibody responses at day 14. (Fig 3.4.b). Mice administered with medium and high-dose formulations had higher IgG titers than ones with low-dose (between 10^5 and 10^6 , approximately 10^2 respectively) 2 weeks after booster injection, and they still had stable IgG titers 2 months after 2nd injection (Fig 3.4.b). When we determined the IgG subtype titers, we observed a high induction of both IgG1 and IgG2a, along with a balanced IgG2a/IgG1 ratio (Fig. 3.4.c).

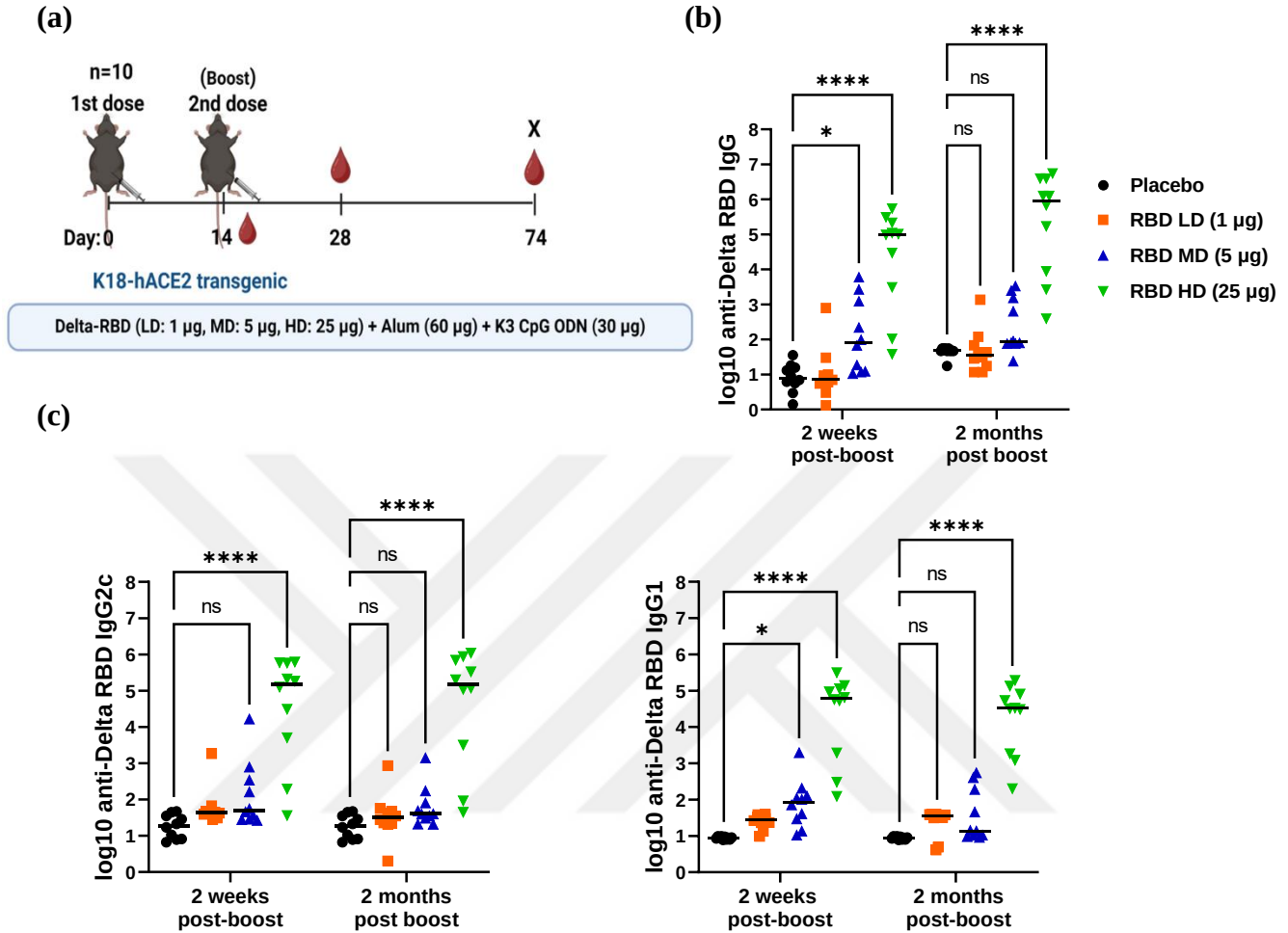


Figure 3.5. Humoral response induced by Delta-plus SARS-CoV-2 RBD vaccine in K18-hACE2 transgenic mice. (a) Study design for mice immunizations. hACE2-BL6 mice (n=10/group) were immunized on days 0 and 14 with Delta-RBD (low dose: 1 µg, medium dose: 5 µg, high dose: 25 µg), Alum (60 µg/mouse), and K3 CpG ODN (30 µg/mouse). Sera were collected at two weeks and two months post-boost and assessed for (b) IgG, (c) IgG1, and IgG2c by ELISA. Two-way ANOVA compared vaccinated groups with Sidak's multiple comparisons test. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$. Data are presented as Mean $\pm$ SEM.

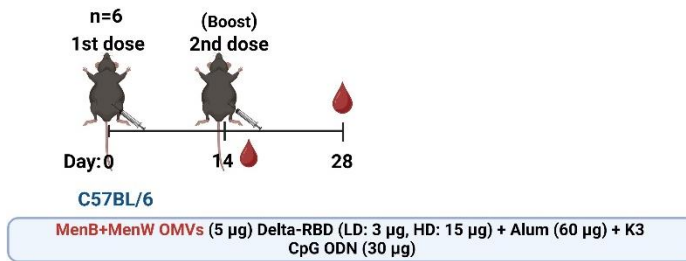
In hACE2 BL6 transgenic mice, only the high-dose DP-RBD group had strong IgG titers after booster injections (Fig. 3.5).

3.1.5 Combination Vaccines against SARS-CoV-2 and *Neisseria meningitidis*

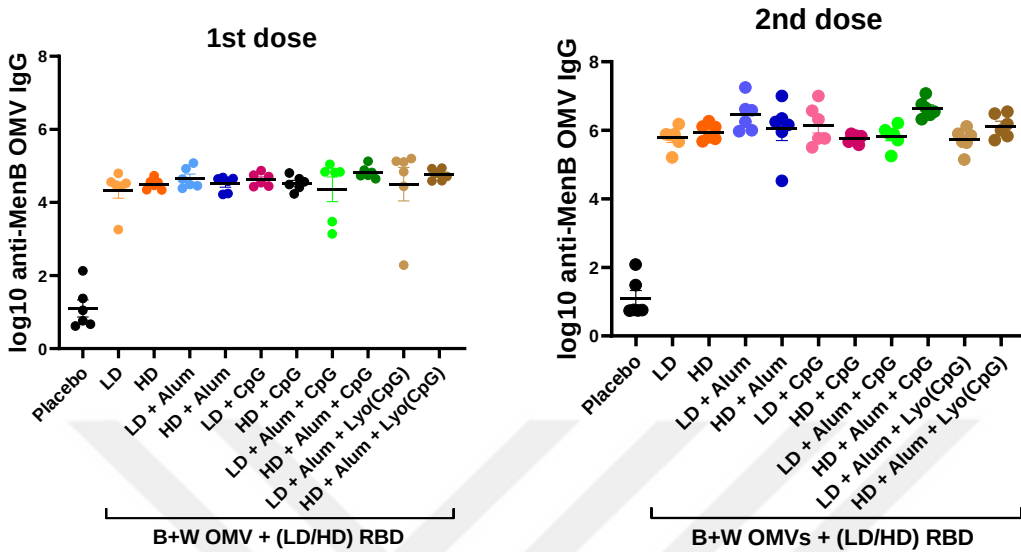
Organizing and managing the administration of various vaccines requiring multiple injections is a critical obstacle to successfully immunizing a population. The availability of combined vaccines against diseases requiring global immunization in childhood would simplify the implementation, decrease the cost of vaccination schedules, increase the prevalence of use, and make disease eradication possible. Considering the desirability of combination vaccines after growing infectious diseases, we tested the compatibility of a combined vaccine, including both viral and bacterial antigens. We investigated the immune efficacy of 2 different combined vaccines against SARS-CoV-2 and *Neisseria meningitidis* W (ST-11) and B (ST-32) strains, the leading cause of childhood meningitis in Türkiye.

3.1.5.1 A combination vaccine formulated with recombinant Delta-Plus SARS-CoV-2 RBDs and *Neisseria meningitidis* outer membrane vesicles (OMVs)

(a)



(d)



(e)

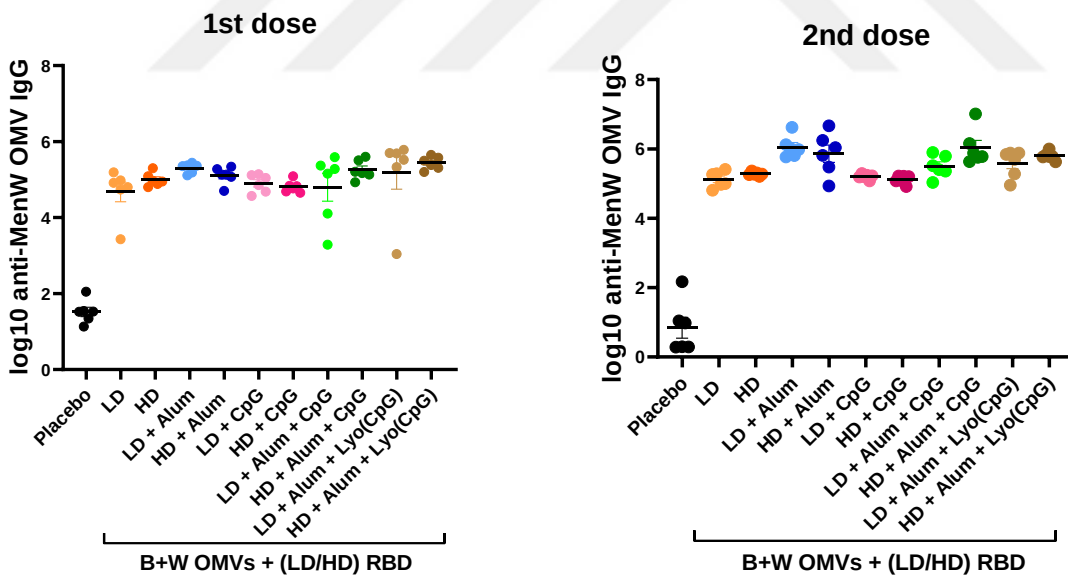


Figure 3.6. Humoral response induced by the combination vaccine formulated with recombinant Delta-Plus SARS-CoV-2 RBDs and *N. meningitidis* OMVs (a) Study design. C57BL/6 mice ($n=6/\text{group}$) were immunized on days 0 and 14 with a combined Delta-Plus SARS-CoV-2 RBD (LD: 3 μg RBD, HD: 15 μg RBD) and bivalent meningococcal vaccine (2.5 μg MenB

OMV and 2.5 μg MenW OMV) adjuvanted with Alum (60 $\mu\text{g}/\text{mouse}$) and K3 CpG ODN (30 $\mu\text{g}/\text{mouse}$). After two weeks of first and second injections, sera were collected and assessed for Delta RBD-specific (b) IgG, (c) IgG1 and IgG2c, (d) MenB OMV, and (e) MenW OMV-specific IgG by ELISA. One-way ANOVA compared vaccinated groups with Tukey's multiple comparisons test. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$. Data are presented as Mean $\pm$ SEM.

3.1.5.2 A combination vaccine formulated with SARS-CoV-2 virus-like particles (VLPs) and *Neisseria meningitidis* outer membrane vesicles (OMVs)

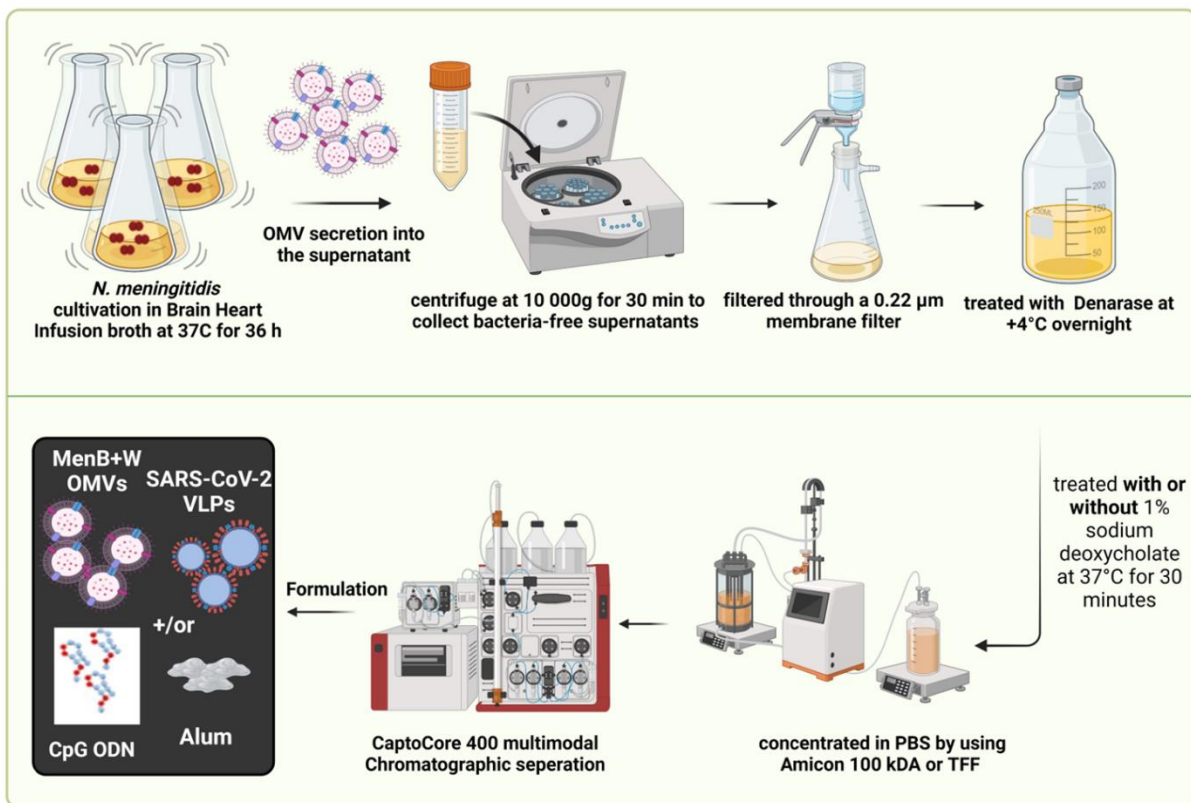


Figure 3.7. Formulation process of the combination vaccine formulated with SARS-CoV-2 VLPs and *N. meningitidis* OMVs. *Neisseria meningitidis* (serogroups B and W) OMVs were reproducibly produced from the Brain Heart Infusion supernatant, where bacteria were cultured for 36 hours at 37 C. The supernatant was centrifuged at 10000 g for 30 min to remove bacteria and filtered through a 0.22 μm membrane filter. This harvest constituted the secreted OMVs. The

harvest was treated with 200 U/ml Denarase (c-LEcta) at +4 C to eliminate cell-derived nucleic acids. For in vitro toxicology assays, the harvest was treated with 1% sodium deoxycholate (DOC) at 37 C for 30 minutes and concentrated in PBS using Amicon 100 kDa column. OMVs were purified on a Hi-Screen Capto Core 400 (Cytiva) column using an AKTA-GO fast protein liquid chromatography system. SARS-CoV-2 VLPs that incorporate four viral structural proteins: 6P-prefusion stabilized Spike (6P-S), membrane (M), envelope (E), and nucleocapsid (N) were produced in suspension-adapted HEK293 cells by applying the same protocol followed for the OMVs (VLPs produced by Ismail et al., 2021). The combination vaccine is formulated by mixing SARS-CoV-2 VLPs and *N. meningitidis* OMVs and adjuvanted with Alum or K3 CpG ODN. This figure was created with BioRender, Canada.

LPS and other PAMPs found in OMVs constitute a significant concern for vaccine developers due to the risk of systemic reactogenicity. Some studies show Alum adsorption reduces reactogenicity in vaccine formulations containing OMV (163,201). Therefore, Alum was used to increase immunogenicity while simultaneously decreasing reactogenicity. Alum is known to shift the immune response towards Th2. Although this type of response supports the production of IgG1 antibodies and is beneficial for bacterial infections, it is necessary to induce Th1 responses, especially against viral infections like SARS-CoV-2. In light of this information, Alum, CpG ODN, and the combination of the two were used as adjuvants, and the aim was to determine their effect on humoral immune responses.

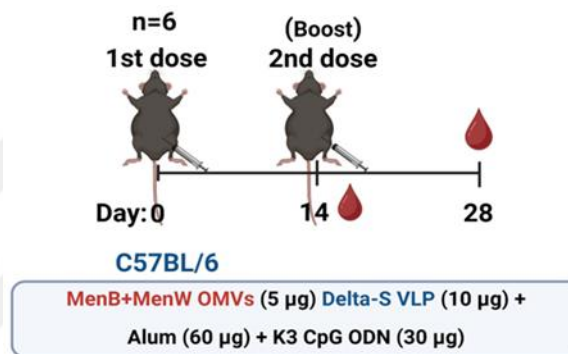
3.1.5.2.1 Immunization of mice with VLPs expressing Delta (B.1.617.2) SARS-CoV-2 Spike and Meningococcal OMVs

Table 4. (a) Groups of vaccinated mice (n=6). (b) The immunization timeline and vaccine formulations prepared with 6P prefusion-stabilized **Delta SARS-CoV-2 Spike**-containing VLPs and OMVs of *N. meningitidis* B and W strains.

(a)

Groups	
1.	PBS
2.	MenB+MenW OMV + VLP + Alum
3.	MenB+MenW OMV + VLP + CpG
4.	MenB+MenW OMV + VLP + Alum + CpG
5.	CpG loaded MenB+MenW OMV and VLP + Alum

(b)



Mice (8-10 weeks old, female C57BL/6, n=6 mice/group) were injected intraperitoneally (i.p.) twice, 14 days apart. Weight, feed, and water consumption were monitored every day. No adverse effects were observed in mice. In vaccination, 10 µg SARS-CoV-2 VLP, 2.5 µg MenB OMV, 2.5 µg MenW OMV, 30 µg K3 CpG ODN, and 60 µg Alum were used for all groups. One day before the booster vaccine, the mice were bled from their tails, and their serum was obtained to determine primary immune responses. 14 days after booster vaccination, all mice were bled again, euthanized, and spleen cells were collected. Table 4 demonstrates groups, vaccination, and bleeding protocol. In the 5th group, we integrated CpG ODN into the mixture of OMV and VLP by lyophilization method. Both primary and secondary immune responses (total IgG, IgG1, and IgG2a) from mice sera were studied by ELISA against anti-Delta RBD, anti-Spike, anti-Nucleocapsid, anti-MenW OMV, and anti-MenB OMV antigens.

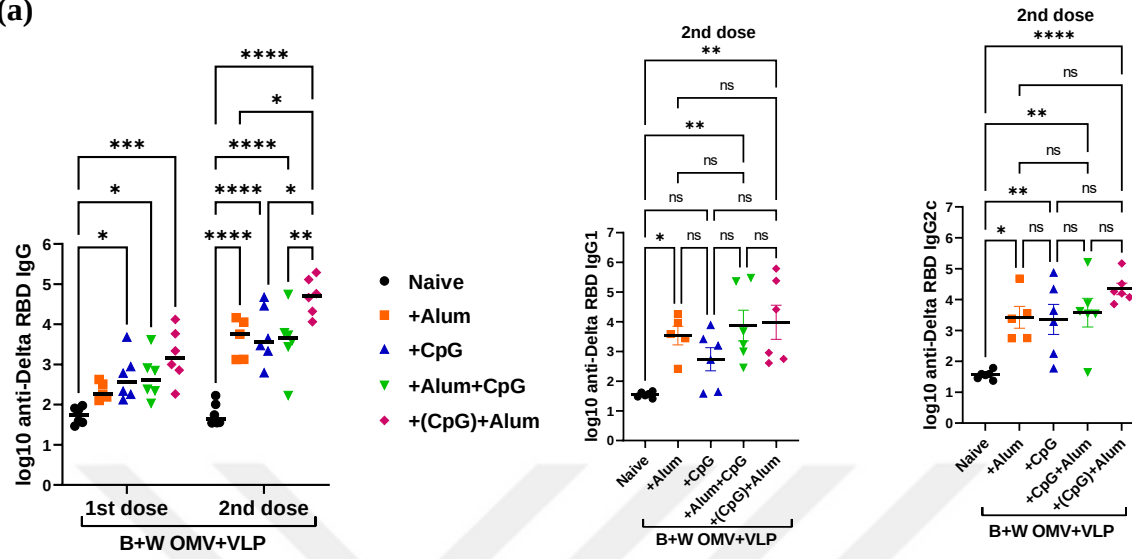
Figure 3.8a (left graph) shows total IgG antibody responses against Delta SARS-CoV-2 RBD 2 weeks after the first injection. It is well known that primary immune responses become very low or do not occur in recombinant protein-based vaccines. However, when a potent adjuvant is used,

obtaining a certain level of antibody response is possible after the first vaccination. Unlike Alum, vaccine groups containing CpG ODN were able to induce a primary immune response (total IgG) against anti-Delta RBD, albeit to a lesser extent (Fig. 3.8a). After the booster vaccine, we observed that antibody responses against all SARS-CoV-2 antigens studied, including Delta-RBD, recombinant spike (recS) and recombinant Nucleocapsid (recN), increased in all groups, and the most effective formulation was observed in the group in which CpG ODN was integrated into vesicles. When anti-RBD IgG1 and IgG2c responses were examined, it was observed that IgG2c responses were higher in the groups containing CpG ODN, as expected, and IgG1 responses were statistically significant only in the groups containing Alum. The highest anti-recS IgG2c response occurred in the Alum adsorbed B+W OMV+VLP(CpG) group (Fig. 3.8b).

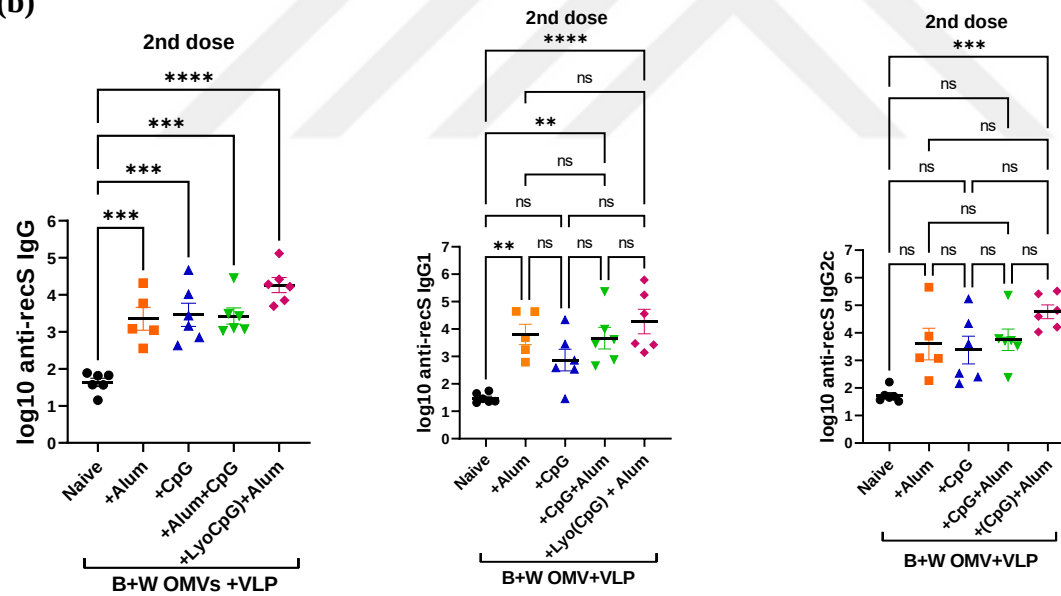
Cellular immune responses are as necessary as antibody responses to produce an effective vaccine. While the antibody subtype that provides neutralization is considered IgG1, IgG2c is the antibody subtype that plays a role in the differentiation of Th1-type CD4⁺ T cells. As presented in Figure 3.8, we observed that the vaccine group that creates the most robust response to both IgG1 and IgG2c is the animals vaccinated with B+W OMV+VLP(CpG)+Alum. In addition, statistically significant IgG1 responses were observed only in the Alum groups and were very low in the group adjuvanted only with CpG ODN.

Total IgG titers against anti-MenB OMV and anti-MenW OMV were very high (higher than 10^4) after the first vaccination and increased further after the booster vaccination. IgG1 and IgG2c titers against both antigens were strikingly high (approximately 10^6) in all groups (Fig. 3.9).

(a)



(b)



(c)

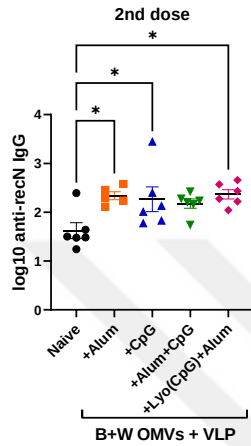


Figure 3.8. Delta SARS-CoV-2 VLP and bivalent meningococcal OMV combination elicits robust antibody response against SARS-CoV-2 specific antigens in mice. Total IgG, IgG1, and IgG2a responses (log10 values) in mouse sera on day 13 post-first immunization against (a) Delta RBD (left) and on day 14 post-second immunization against (a) Delta RBD (right), (b) recS, and (c) recN. Vaccinated groups were compared using one-way ANOVA with Dunnett's multiple comparisons test. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$

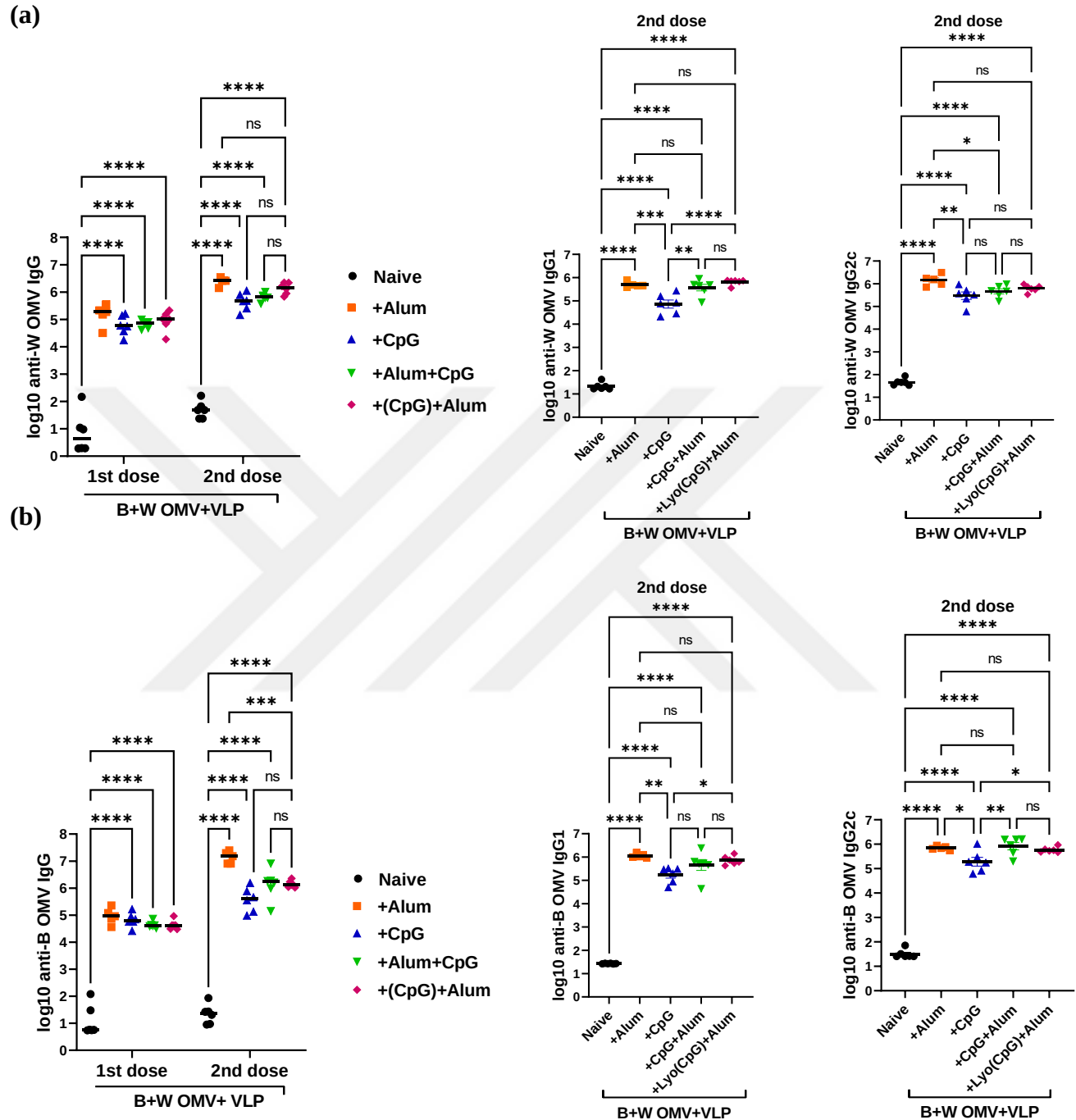


Figure 3.9. Delta SARS-CoV-2 VLP and bivalent meningococcal OMV combination elicit robust antibody response against MenB and MenW OMV-specific antigens in mice. Total IgG, IgG1, and IgG2a responses (log₁₀ values) in mouse sera on day 13 post-first immunization against (a) MenW OMV (left) and on day 14 post-second immunization against (a) MenW OMV (right) and (b) MenB OMV. Vaccinated groups were compared using one-way ANOVA with Dunnett's multiple comparisons test. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$.

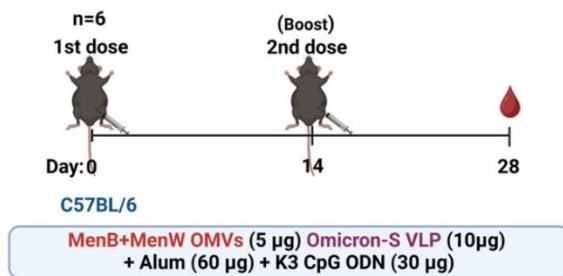
3.1.5.2.2 Immunization of mice with VLPs expressing Omicron (B.1.1.529) SARS-CoV-2 Spike and Meningococcal OMVs

As in the first study, mice (8-10 weeks old, female C57BL/6, n=6 mice/group) were vaccinated intraperitoneally (i.p.) twice at 14-day intervals with vaccine formulations prepared by mixing antigens and adjuvants at the doses shown in scheme II. Then, total IgG responses against Omicron RBD, recS, MenB OMV, and MenW OMV antigens from the mouse sera, obtained 2 weeks after the booster injection, were examined by ELISA.

We obtained strong IgG responses against SARS-CoV-2 antigens and meningococcal OMVs in all relevant groups. The highest response to SARS-CoV-2 antigens was given by the VLP+CpG+Alum (“our clinically approved vaccine for Phase II”) and MenB+MenW OMV+VLP+Alum groups.

Table 5. (a) Groups of vaccinated mice (n=6). (b) The immunization timeline and vaccine formulations prepared with 6P prefusion-stabilized **Omicron SARS-CoV-2 Spike**-containing VLPs and OMVs of *N. meningitidis* B and W strains.

(a)



(b)

	Groups
1	Naive
2	MenB+MenW OMV
3	MenB+MenW OMV + Alum
4	MenB+MenW OMV
5	MenB+MenW OMV
6	MenB+MenW OMV + VLP + Alum
7	MenB+MenW OMV + VLP + CpG
8	MenB+MenW OMV + VLP + CpG +
9	VLP
10	VLP + CpG + Alum

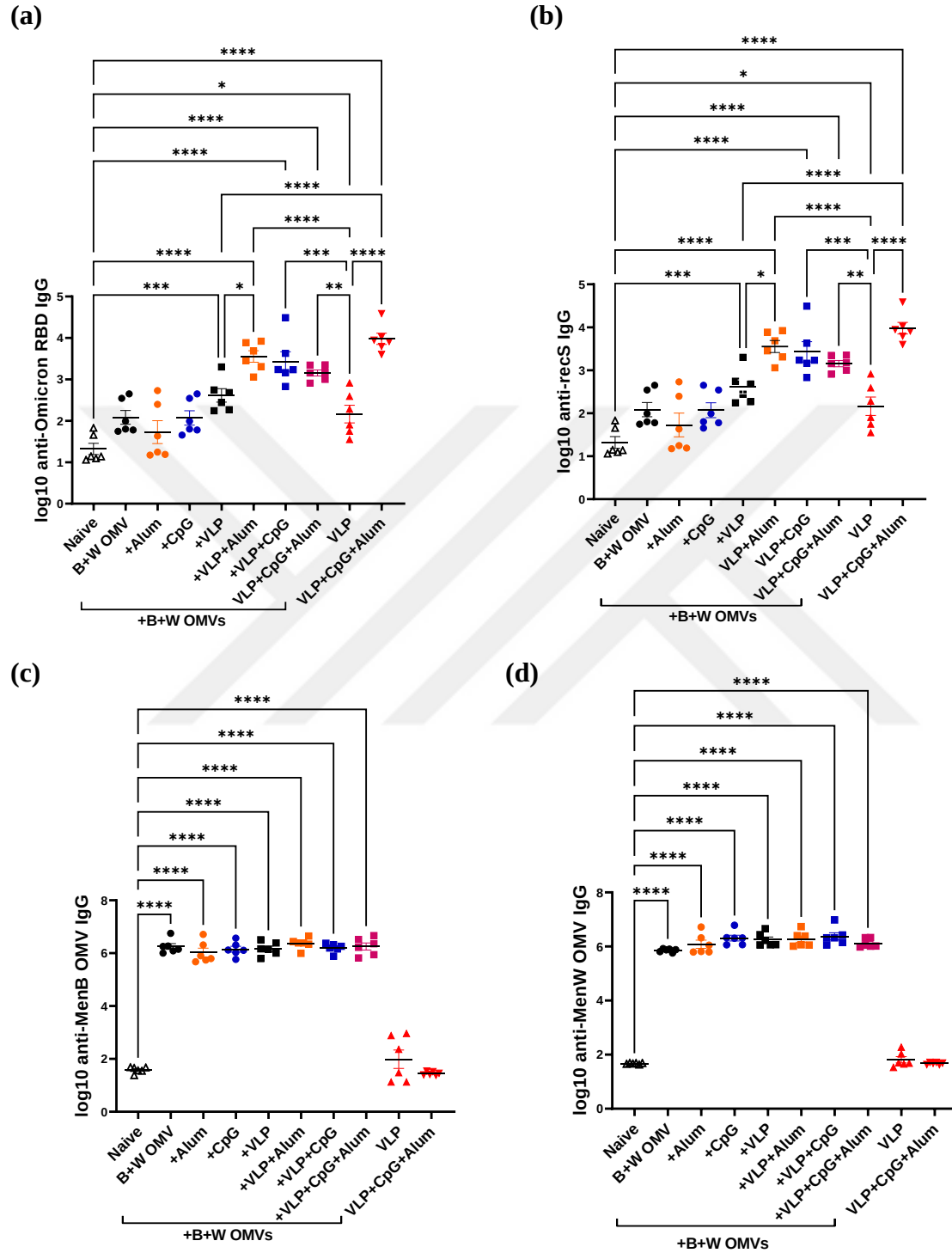


Figure 3.10. SARS-CoV-2 Omicron-S VLP and bivalent meningococcal OMV combination elicits robust antibody response in mice. C57BL/6 mice ($n=6/\text{group}$) were immunized on days 0 and 14 with 2.5 μg of MenB and MenW OMVs, 10 μg of SARS-CoV-2 6p-Omicron-S VLPs with

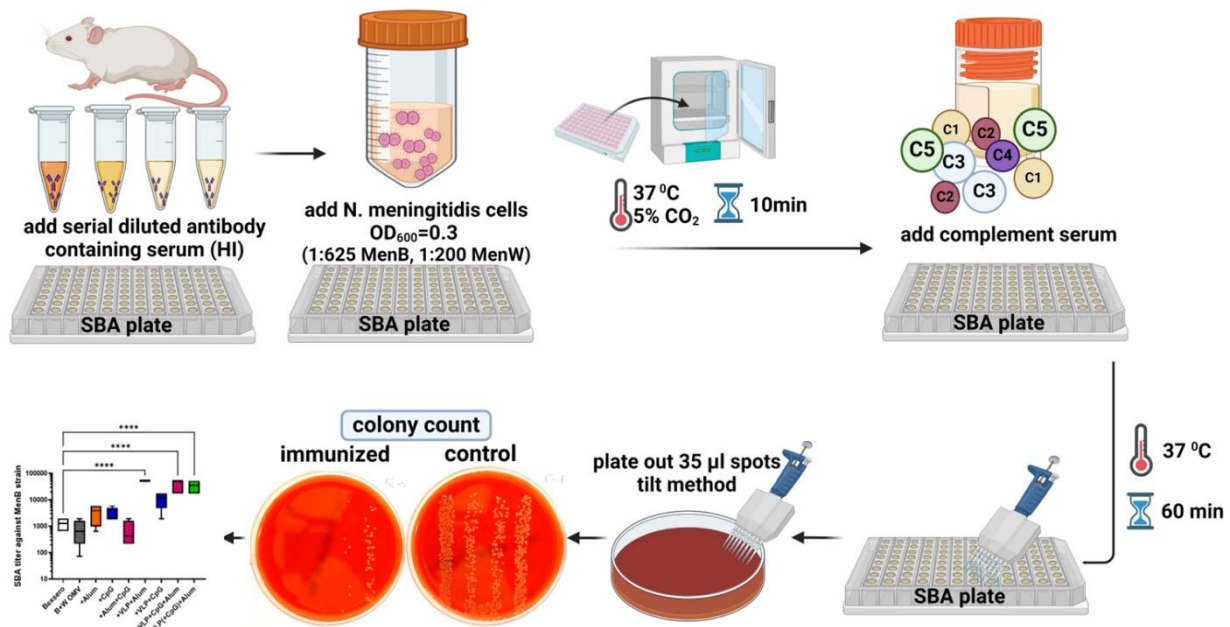
*Alum (60 µg/mouse), K3 CpG ODN, Alum + K3 CpG ODN, or an encapsulated-CpG formulation. Post-boost sera were assessed for SARS-CoV-2 (A) Omicron-RBD, (B) spike, and (C) MenB and D) MenW OMV-specific IgG by ELISA. Vaccinated groups were compared using one-way ANOVA with Dunnett's multiple comparisons test. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$."*

3.1.6 Complement-mediated Serum Bactericidal Activity (SBA) of Antibodies Elicited by the SARS-CoV-2 and Meningococcal Combine Vaccine

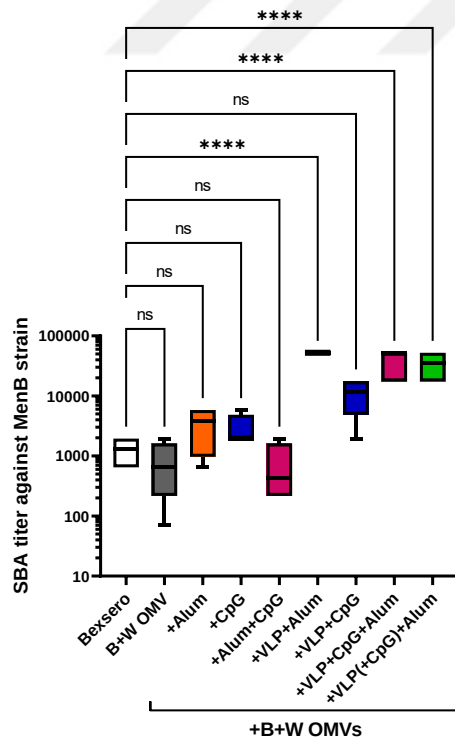
Serum bactericidal activity (SBA) assay is the “gold standard” for measuring the ability of vaccine-induced antibodies to kill *Neisseria meningitidis*. Licensing meningococcal vaccines requires confirmed immune responses measured by the SBA assay. Therefore, we conducted an SBA to evaluate the antibodies' complement-mediated killing activity in vaccinated mice's sera.

Mice immunized with the bivalent (MenB+MenW) OMV+VLP vaccine and its adjuvanted counterparts developed bactericidal antibodies against the MenW vaccine strain clonal complex ST-11 and MenB clonal complex ST-32 2 weeks after the booster injection. “For *N. meningitidis* serogroup C, a serum bactericidal assay (SBA) titer of ≥ 4 with human complement and ≥ 8 with rabbit complement was established as the correlate of protection, based on comparative assay results.” (15) Interestingly, we obtained dramatically higher SBA responses in groups with VLP than in other groups. Correlated with the antibody responses, the highest SBA titers were given by the MenB+MenW OMV+VLP+Alum (Fig. 3.11b). Notably, all groups that received the (MenB+MenW) OMV+VLP vaccine exhibited higher efficacy in eliciting SBA titers against the MenW strain than the Nimenrix vaccine (Fig. 7c). All vaccine groups lacking VLP and the (MenB+MenW) OMV+VLP+CpG induced similar SBA titers against the MenB strain. In contrast, others containing the VLP+Alum combination, regardless of the CpG presence, had significantly higher SBA titers than Bexsero (Fig. 3.11b).

(a)



(b)



(c)

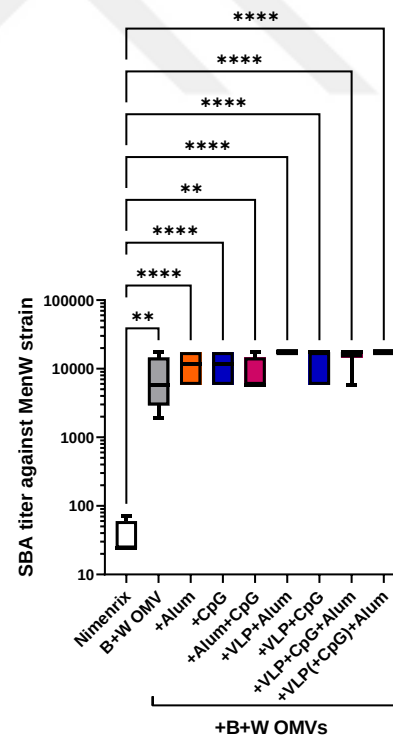


Figure 3.11. Serum bactericidal activity (SBA) titers in sera from MenB+MenW OMV+SARS CoV-2 VLP vaccine immunized mice (n=8). (a) Schematic representation of the assay procedure.

*This figure was created with BioRender, Canada. SBA responses against (b) MenB and (c) MenB strains after two doses of the Delta SARS-CoV-2 VLP combined OMV vaccine, Nimenrix™, and Bexsero®. Data in (b) and (c) are shown as box-plot; whiskers show min to max data points. The bactericidal antibody titer was expressed as the reciprocal of the final serum dilution, yielding $\geq 90\%$ killing after 1 hour compared to the average for controls. Vaccinated groups were compared by one-way ANOVA with Dunnett's multiple comparisons test. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$.*

3.2 PART II: Antibody Responses and Cytokine Profiling among Children with Multisystem Inflammatory Syndrome (MIS-C) versus COVID-19 Infection and the Contribution of Circulating Proinflammatory Extracellular Vesicles to MIS-C Pathogenesis

3.2.1 SARS-CoV-2 Humoral Response in MIS-C and Pediatric COVID-19

SARS-CoV-2-specific anti-recombinant RBD, anti-6p-Spike (S) protein and anti-nucleocapsid (N) IgM, IgG, and IgA antibody titers of different cohorts (control $n=14$, COVID-19 $n=65$, and MIS-C $n=19$) were measured by indirect ELISA on admission (Figure 1a).

Anti-RBD IgG (Fig. 1b), IgA (Fig. 1e), and IgM (Fig. 1h) antibody titers were significantly higher in severe COVID-19 and MIS-C patients compared to healthy controls, asymptomatic, and mild-to-moderate COVID-19 cases ($p < 0.05$). Consistent with previous studies (202), most severe COVID-19 patients had low anti-S and anti-N IgG titers indistinguishable from asymptomatic and mild-moderate cases (Figure 1c, d). In contrast, MIS-C patients exhibited significantly higher anti-S and anti-N IgG levels than all cohorts, indicating a strong and sustained immune response to the Spike and Nucleocapsid proteins. Considering that adults with severe COVID-19 have higher antibody titers than adults with mild disease, pediatric COVID-19 appears to manifest a distinct disease course compared to adults (203–205).

COVID-19-positive children also had detectable levels of anti-S and anti-RBD IgA (Fig. 1f), with significantly higher titers observed in the MIS-C cohort when compared to asymptomatic and mild-moderate cases ($p < 0.0001$ and $p < 0.0001$ respectively). Notably, significant levels of anti-S IgM (Fig 1i) were evident exclusively in MIS-C patients.

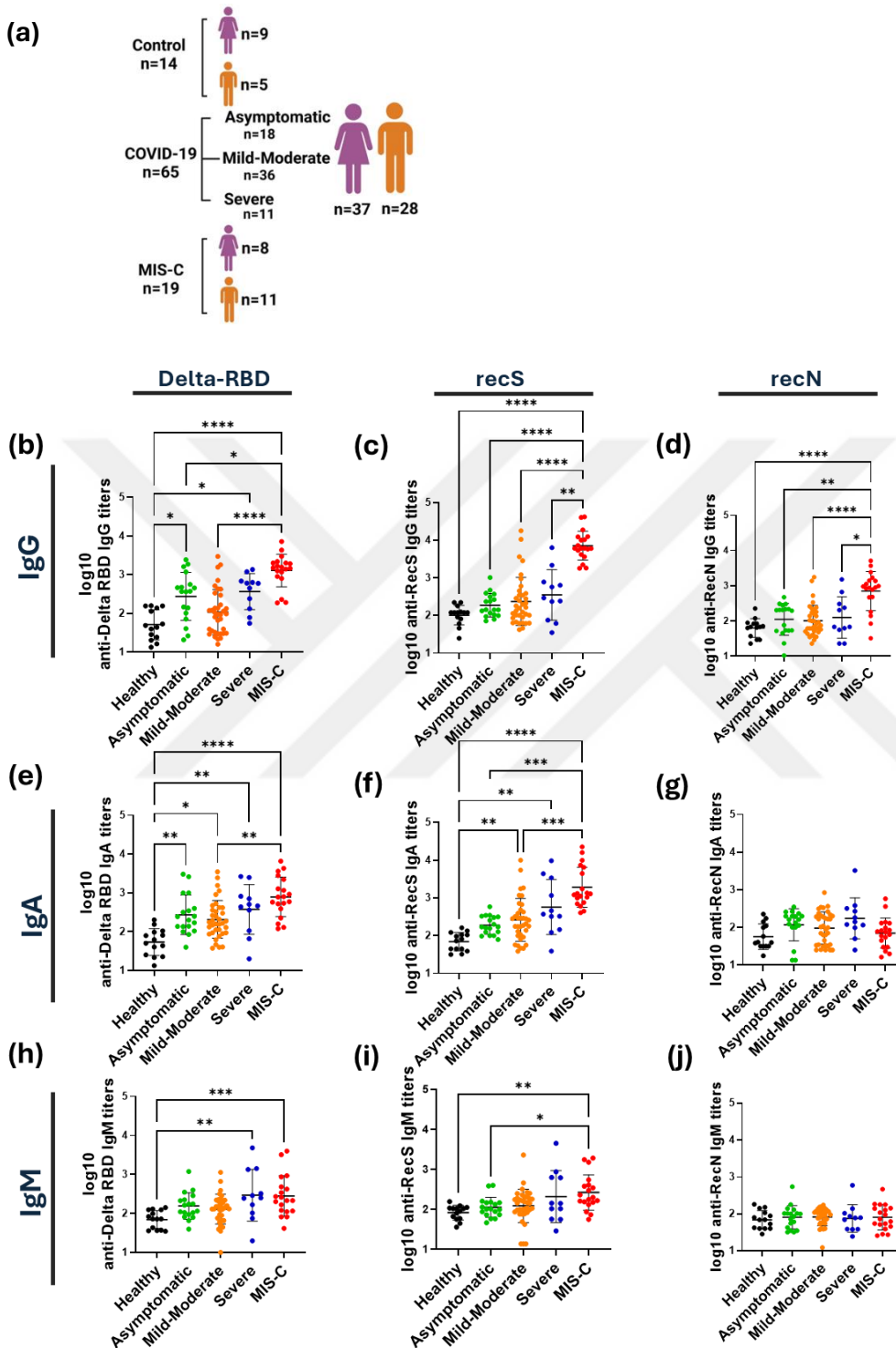


Figure 3.12. Antibody responses against SARS-CoV-2 antigens; Delta-RBD, 6p-prefusion stabilized Spike, and nucleocapsid in the pediatric patients with acute COVID-19 or MIS-C. a) overview of the pediatric cohort, including SARS-CoV-2 negative control (n=14) and children with

*asymptomatic (n=18), mild/moderate (n=36), or severe COVID-19 (n=11) or MIS-C (n=19) (a). Titers of IgG (b-d), IgA (e-g), and IgM (h-j) directed against Delta-RBD (left panel), recS (middle panel), and recN (right panel) in serum of healthy controls and patients were determined by ELISA. Each dot represents one sample. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Kruskal Wallis test and Dunn's multiple comparisons test).*

Longitudinal Analysis of Antibody Responses

To gain further insight into the dynamics of antibody responses, Anti-RBD, -S and -N IgG, IgA, and IgM titers of severe COVID-19 and MIS-C patients were followed longitudinally for 50 days (Figure 2a). RBD-, S- and N- specific IgG titers gradually increased and peaked around 20-30 days (Fig1b, Fig1c and Fig1d). In contrast, the corresponding titers, which were already significantly elevated on admission, were sustained but did not increase over time in MIS-C (Fig1b, Fig1c, and Fig1d), suggesting prolonged exposure to viral antigens.

Anti-RBD and S-specific IgA titers peaked around day 20 in both cohorts, followed by a gradual level decline after this time point (Fig1e, Fig1f, and Fig1g), although the magnitude of the IgA response was lower than that of IgG. IgM titers that were already low on admission did change significantly over time (Fig1h, Fig1i, and Fig1gj). In summary, the higher RBD-, S- and N-specific IgG titers in MIS-C patients on admission reflect the delayed and post-infectious nature of the syndrome, coupled with the hyperinflammatory immune response characteristic of MIS-C, resulting in a robust and sustained antibody response compared to the acute-phase immune response seen in severe COVID-19.

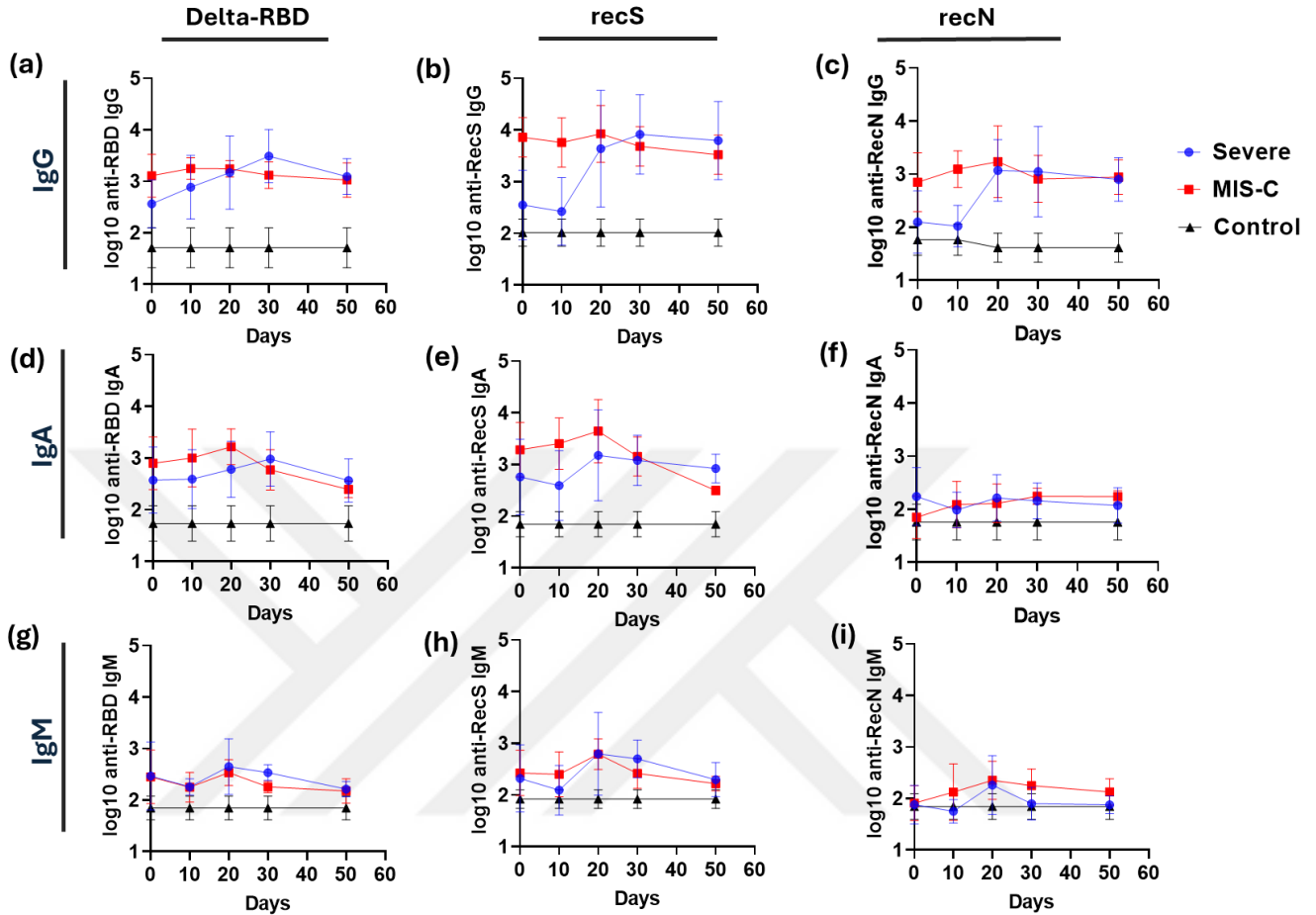


Figure 3.13. Longitudinal analysis of antibody dynamics in MIS-C and Severe COVID-19 pediatric patients. Mean (SD) anti-Delta spike RBD (left panel), anti-recS (middle panel), and anti-recN (right panel) IgG (a-c), IgA (d-f), and IgM (g-i) titers of children with severe COVID-19 and with MIS-C followed up and taken blood samples. The samples were divided into five time points: on admission, 10, 20, 30, and 50 days after symptoms onset.

3.2.2 Cytokine Profiling of MIS-C and Pediatric COVID-19

Elevated and uncontrolled circulating levels of pro-inflammatory cytokines, referred to as “cytokine storm,” have been reported in adults with COVID-19, particularly those with severe symptoms and complications (206–208). This hyperinflammation is dominated by an array of cytokines, including IL-6, TNF α , IL-1 β , IL-8, IL-2, GM-CSF, and IFN γ (209). However, there is limited data on pediatric patients, particularly children with MIS-C. We, therefore, compared 18

cytokine levels related to COVID-19 in the serum of children with MIS-C and other cohorts with COVID-19. We observed that specific cytokines, including IL-6, TNF α , IL-8, IFN γ , G-CSF, and IL-2, were significantly elevated to a greater extent in MIS-C patients (Fig 3a). Notably, IL-10 was highly elevated in MIS-C, potentially reflecting an attempt to counteract the exacerbated inflammation. IL-17A and IL-22 cytokines act synergistically to maintain the function and integrity of epithelial tissues, particularly in the skin, lungs, and gastrointestinal tract (210). Interestingly, both cytokines were drastically higher in children with MIS-C compared to healthy controls (IL-17A $p=0.01$ and IL-22 $p<0.0001$), suggesting a compensatory mechanism to counteract tissue damage and inflammation in affected organs, such as the gastrointestinal tract and the lungs. Of note, IL-22 levels were upregulated in both severe COVID-19 and MIS-C cohorts, whereas IL-17A was significantly increased only in MIS-C patients (Fig 3a), highlighting a potential role for IL-17, distinguishing it from severe COVID-19. Concentrations of IP-10/CXCL10, MCP-1/CCL2, IL-13, IL-5, IL-4, and IL-1RA were also significantly elevated in MIS-C patients than healthy controls, in support of a highly dysregulated response in MIS-C patients (Fig 3a).

Principal Component Analysis (PCA) of cytokine profiles across patient cohorts (Figure 3b) demonstrates the distribution of cohorts based on the first two principal components (PC1 and PC2), which together account for 63.98% of the total variance (PC1: 49.74%, PC2: 14.24%). MIS-C and severe COVID-19 patients clustered separately from healthy controls, and specifically, MIS-C patients were distinctly separated from other cohorts along PC1, highlighting the unique cytokine signature associated with this syndrome. The main contributing features explaining this difference included IL-6, MCP-1, G-CSF, IL-1RA, IL-8, TNF- α , and IP-10, strongly associated with PC1, suggesting that these pro-inflammatory cytokines are key drivers of the variance observed between cohorts. IL-17A and IL-22, which were demonstrated to be elevated in MIS-C patients (Figure 3a), also contributed significantly to PC1, further supporting their role in distinguishing MIS-C from other conditions. These findings suggest a role for IL-17A, an inflammatory cytokine that promotes neutrophil recruitment, which collectively could contribute to the multisystem involvement observed in MIS-C.

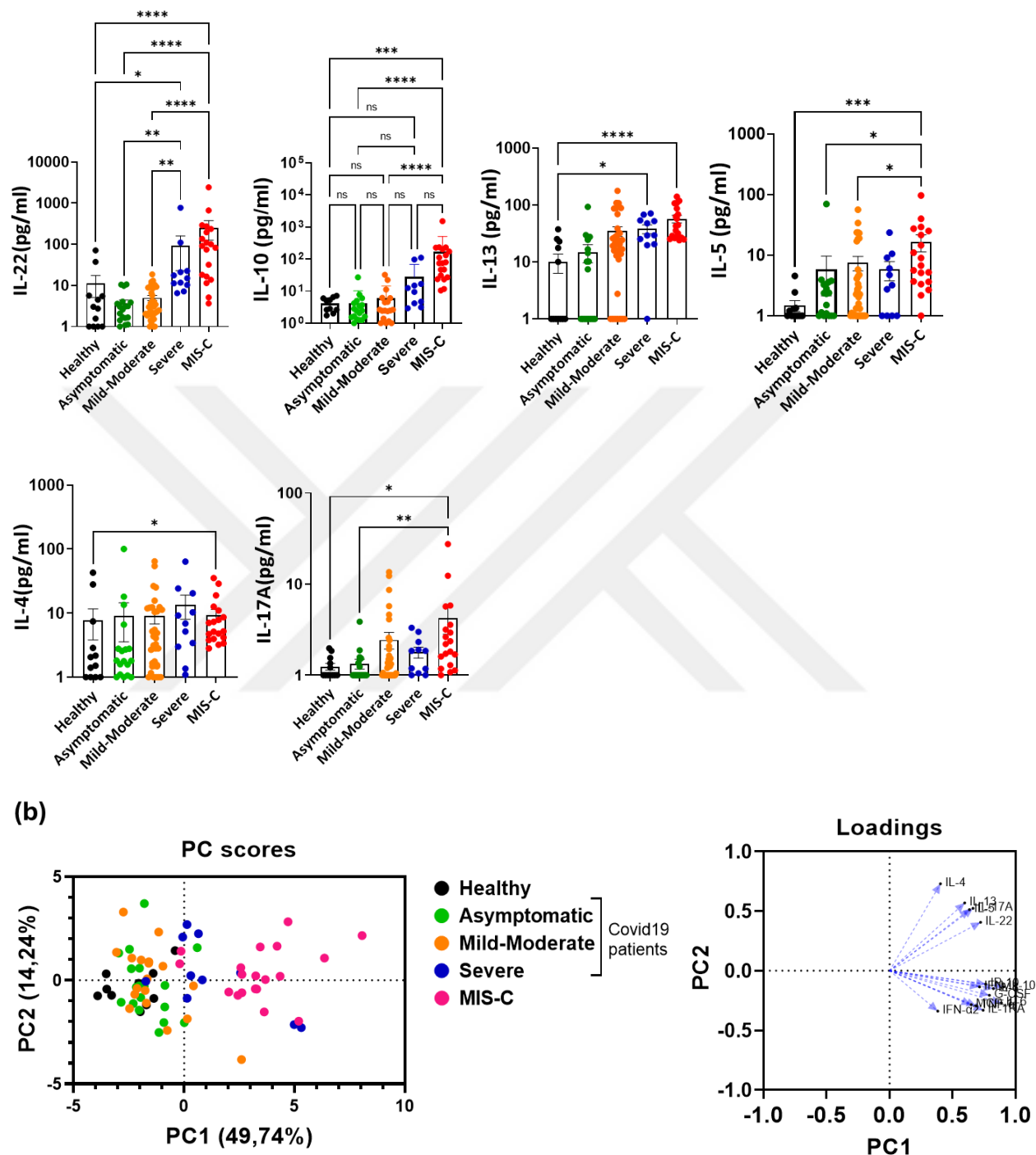


Figure 3.14. Cytokine profiling of MIS-C. Cytokine concentrations in the serum samples obtained at admission were determined using a COVID-19 Cytokine Storm fluorometric bead panel. (a) The concentrations in each group were compared by one-way ANOVA with Dunn's multiple comparisons test (mean $\pm$ SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$). Sample sizes for groups are as follows: control $n=10$, asymptomatic $n=18$, mild/moderate $n=18$, severe

$n=10$, MIS-C $n=19$ (b) Principal Component Analysis of cytokine expression in pediatric patients with COVID-19 and MIS-C. A PCA score plot shows the separation of patients based on first and second principal component scores. Each dot represents a sample projected in the two main principal components (PC1 and PC2); the dots are colored according to the group they belong to. PCA loading plot shows the cytokines responsible for the differences between the groups.

3.2.3 Plasma-derived extracellular vesicles as a potential immunostimulatory activity in MIS-C

It is well-established that extracellular vehicles (EVs) play a critical role in viral transmission, facilitating the spread of viral components without direct cell-to-cell contact. However, studies regarding EV function in MIS-C pathology still need to be completed (211). We purified EVs from the plasma of COVID-19 and MIS-C patients using size exclusion chromatography. Tunable resistive pulse sensing (TRPS) analysis confirmed the spherical, vesicular nature of the EVs, measuring 84 ± 20 nm, 87 ± 21 nm, and 88 ± 24 in diameter for control, severe COVID-19 and MIS-C EVs, respectively (Fig. 3.15a). Furthermore, EVs were verified by detecting the expression of CD81, a specific surface marker for EVs (Fig. 3.15b) (212).

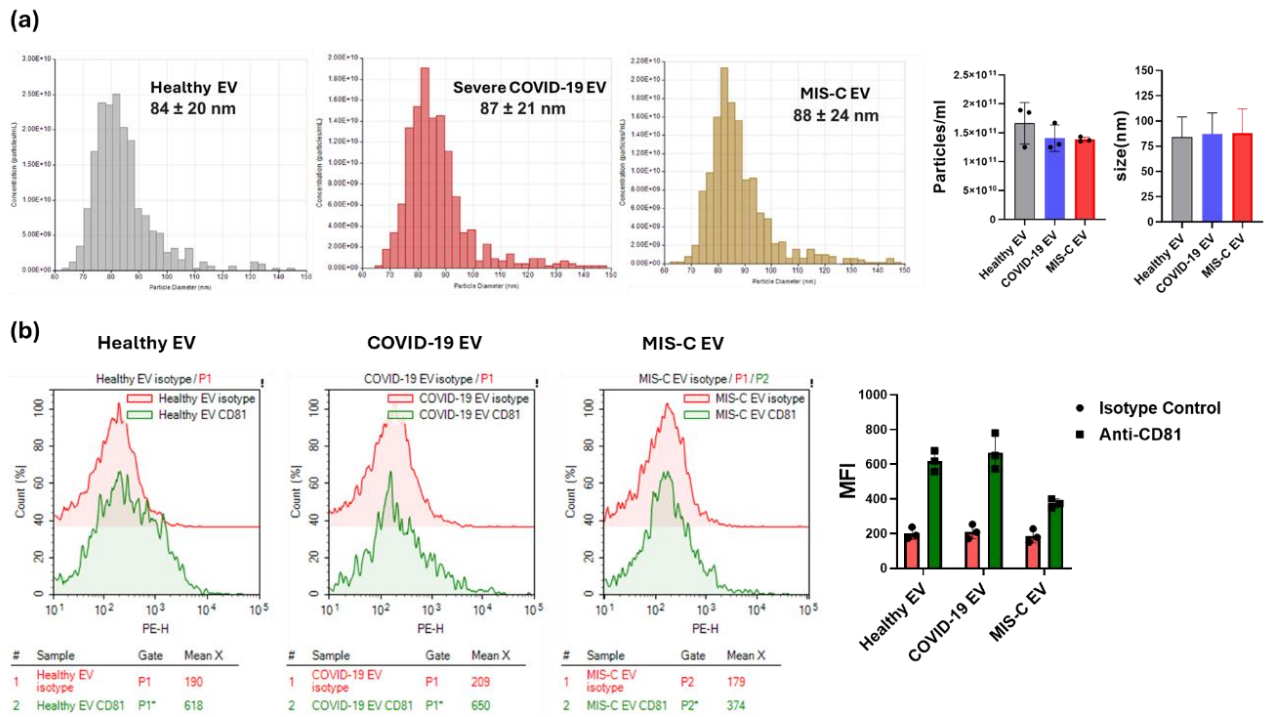


Figure 3.15. Characterization of patient plasma-derived EVs. (a) TRPS size distribution measurement (nm) and particle number of EVs. (b) EVs derived from pediatric patients were

captured using anti-hCD63-conjugated beads, stained with anti-hCD81 antibodies, and analyzed via flow cytometry. Bar graphs display the average MFI values of specific antibodies relative to isotype control staining in EVs from three patients per cohort.

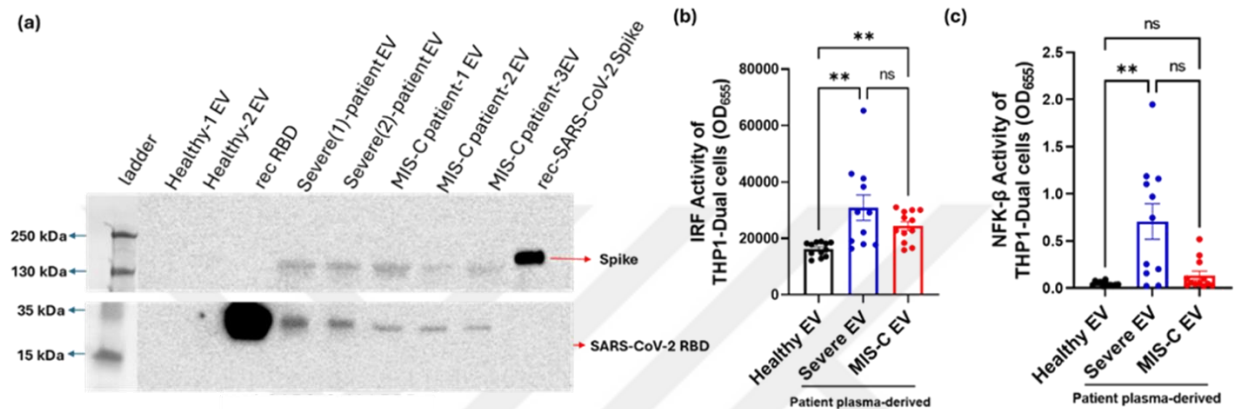


Figure 3.16. Circulating EVs of children with severe COVID-19 and MIS-C carry SARS-CoV-2 RBD—western blot showing anti-SARS-CoV-2-S-RBD immunoblotting in EVs of MIS-C, COVID-19 patients, or healthy donors. Blots are representative of three independent experiments.

It was shown before that adult COVID-19 plasma-derived EVs carry SARS-CoV-2 Spike-derived peptides, having a role in enhancing immune response (213). To our knowledge, the presence of SARS-CoV-2-related components in plasma-derived EVs of MIS-C patients has yet to be evaluated. Therefore, we analyzed the presence of SARS-CoV-2 Spike in the EVs of both COVID-19 and MIS-C patients. We detected Spike protein and RBD in MIS-C EVs, suggesting that viral components can persist in the body and contribute to ongoing inflammation even after the acute phase of infection.

Next, we evaluated the functional impact of patient-derived EVs on immune activation using THP-1 dual reporter cells. Severe COVID-19 but not healthy EVs induced significant activation of both NF-κB (214,215) and IRF (216) pathways ($p < 0.01$), indicating the presence of immune-stimulating molecules capable of triggering pro-inflammatory and antiviral responses (Fig. 3.16 b,

c). In contrast, MIS-C EVs preferentially induced IRF but not NF- κ B activation ($p < 0.01$), suggesting that severe COVID-19 versus MIS-C EV cargoes may substantially differ.

3.2.4 Proteomic Profiling of MIS-C EVs

Proteomic analysis of EVs isolated from healthy versus MIS-C patient plasmas revealed distinct protein signatures in MIS-C EVs. Within group reproducibility of proteomics, results were found to vary between 27% and 82% in healthy (Figure 3.17a) and 32% and 62% in MIS-C sample groups (Figure 3.17b), given the extreme sensitivity of the bottom-up LC-MS/MS experiments. Group-intersection analysis of the proteomics data identified a total of 168 proteins exclusive for MIS-C EVs (Figure 3.17c). While only 29 proteins were unique to healthy EVs, 142 were shared between the two groups, indicating some overlap in EV cargo but also significant ($p\text{-value} < 1.703\text{e-}255$). Gene Ontology (GO) biological processes and p pathway enrichment analyses of MIS-C-specific proteins demonstrated enrichment in pathways associated with the innate immune system and neutrophil degranulation (Figure 3.17d-e), suggesting that MIS-C EVs potentially contribute to hyperinflammation. Cell marker analysis (2024) indicated that MIS-C EVs likely originate from myeloid cells in the bone marrow (Figure 3.17f), suggesting that hematopoietic cells contribute to the release of EVs into the circulation of MIS-C patients, reflecting the ongoing systemic immune dysregulation. At the molecular level, MIS-C EVs were found to be enriched in β -integrins (Figure 3.17g), which are considered key mediators of focal adhesion and cellular communication. KEGG pathway enrichment analysis was consistent with findings from BioPlanet (Figure 3.17h). Finally, STRING network analysis showed strong protein-protein interaction (PPI) clusters associated with immune system regulation, especially with respect to proteins linked to myeloid cell activation and inflammatory signaling cascades (Figure 3.18). Collectively, these findings indicate that the unique cargo of MIS-C EVs reflects the ongoing dysregulated innate immune activity and hyperinflammation, suggesting a potential role in MIS-C pathogenesis.

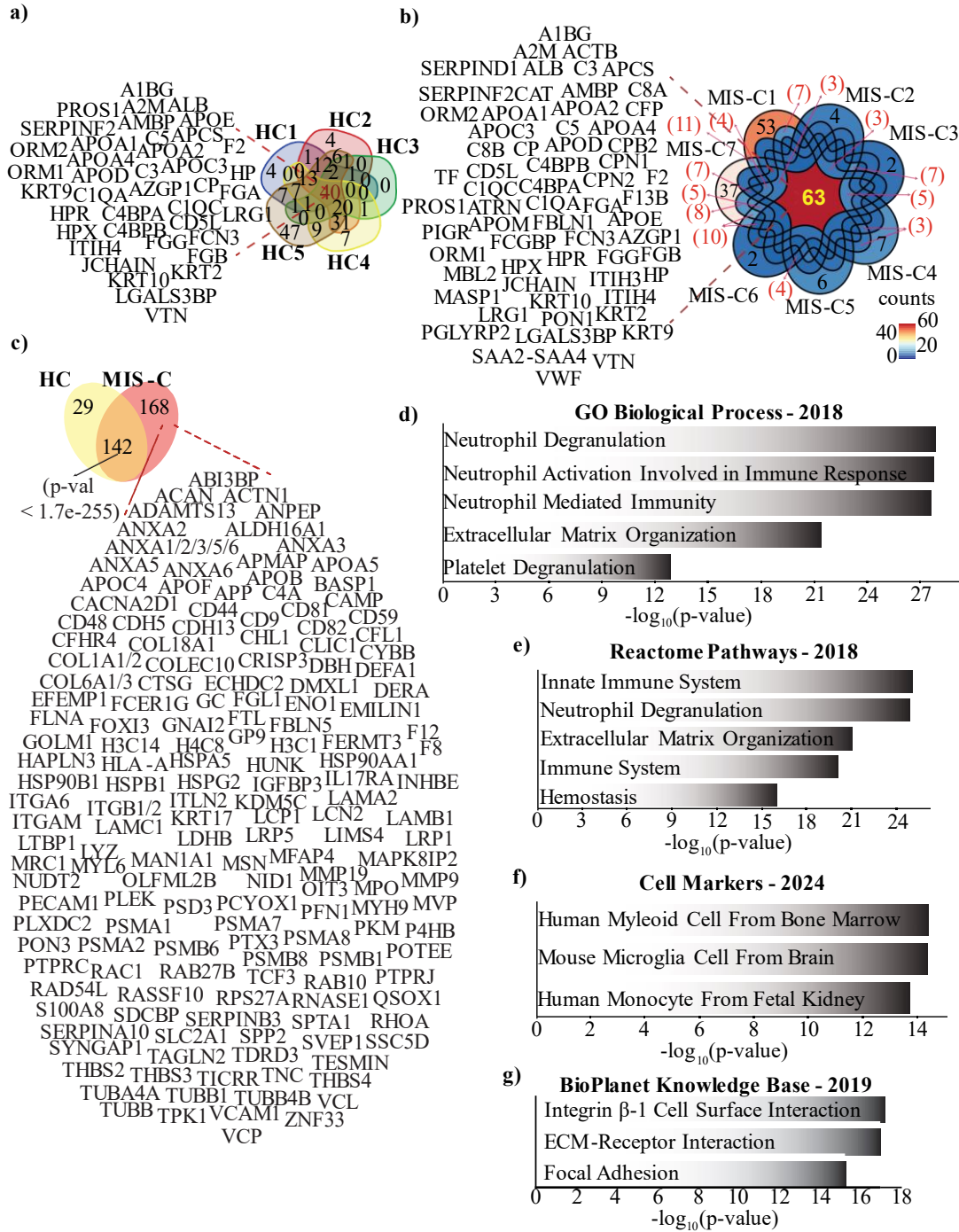


Figure 3.17. Proteomics profiling of MIS-C EVs. Within group reproducibility of LC-MS/MS read-outs across biological replicates per sample group is within range (between 27% and 82% in healthy controls (a) while 32% and 62% in MIS-C sample group (b). Sectors in the multi-set Venn diagram in (b) that have protein counts less than two were not numerically labeled. (c) Between group comparison made with a whole set of non-redundant proteins across all biological

replicates in a sample group identified 142 shared proteins, 168 proteins unique to MIS-C, and 29 proteins exclusively found in healthy controls. The significance of the overlap between two groups of proteins (given in parentheses) was computed using a hypergeometric test. (d-g) The key functional terms enriched in 168 proteins unique to MIS-C EVs using various ontology databases: (d) GO Biological Process (2018), (e) Reactome Pathways (2018), (f) Cell Markers (2024), and BioPlanet Knowledge Base (2019).

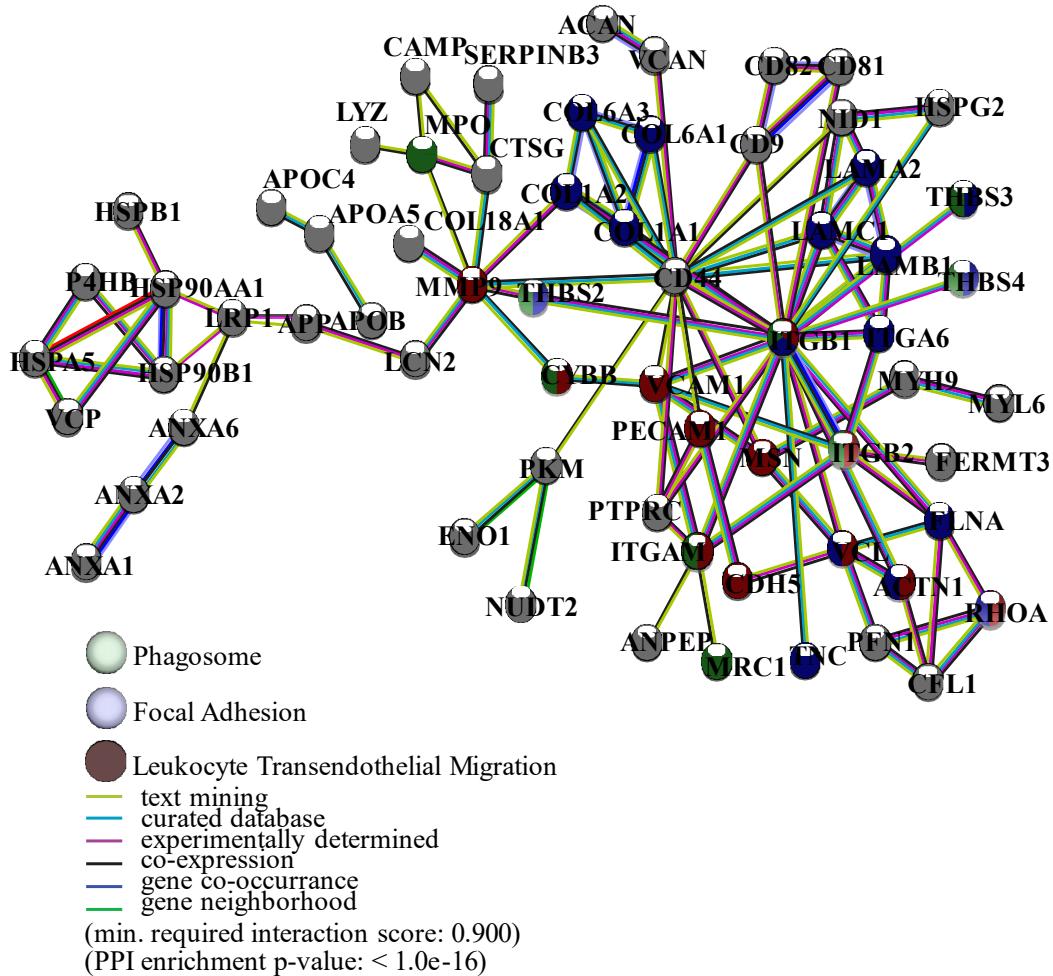


Figure 3.18. A PPI analysis of 168 proteins unique to MIS-C. STRING results indicate significant enrichment ($p\text{-value} < 1.0e-16$) of elements of focal adhesion, phagosome components, and leukocyte transendothelial migration in the EV cargo of MIS-C. Nodes: proteins. Edges; interactions. Edge thickness correlates with the interaction strength between two proteins. Edges are color-coded, indicating various lines of evidence for interaction—minimum required interaction score: 0.900.

4 CHAPTER 4

DISCUSSION

4.1 PART I: The Development of a Combined Vesicle-Vaccine against SARS-CoV-2 and *Neisseria meningitidis*

Meningococcal vaccines in human use have some limitations, and developing a universal vaccine encompassing all serogroups of *Neisseria meningitidis* is challenging for the following reasons: i) current conjugate polysaccharide vaccines target specific serogroups and often fail to provide cross-protection against non-targeted strains ii) structural and antigenic variability among the capsules make a single vaccine formidable, iii) capsule-deficient strains causing invasive disease renders capsule-based approaches ineffective and complicates vaccine design, and iv) developing a vaccine that is not only efficient but also accessible and affordable for low-income countries is challenging. Therefore, developing a meningococcal vaccine comprising OMVs from several serogroups is an innovative and favorable approach to overcome these limitations.

In our study published in *International Immunology*, “Immunogenicity and protective capacity of a CpG ODN adjuvanted alum adsorbed bivalent meningococcal outer membrane vesicle vaccine,” we evaluated the safety, immunogenicity and protective efficacy of a bivalent MenW + MenB OMV vaccine using serum bactericidal antibody (SBA) assays, a benchmark for meningococcal disease protection (Fig 3.11). All strains used to prepare the OMVs were isolated from patients and are considered representative of recent Türkiye epidemic strains. The bivalent MenW + MenB OMV vaccine induced MenB-specific antibody titers comparable to those of 4CMenB (Bexsero®), a licensed MenB vaccine, and produced much higher MenW-specific antibody than that produced by Nimenrix, a polysaccharide conjugate vaccine. Combining two OMVs in a single formulation did not negatively impact the immune response to either strain. This research led to the development of an immunogenic and potentially protective bivalent OMV vaccine against MenW and MenB, targeting the most prevalent serogroups in Türkiye and Europe.

OMV-based vaccines have multiple advantages, including their capacity to present several antigens that can stimulate broad immune responses. This provides cross-protection between different serogroups as our vaccine also produced bactericidal antibodies against *N. meningitidis*

serogroup X (197). Although containing only MenB OMVs, Bexsero also had drastically higher antibody titers against MenW (Fig. 3.3b), indicating that the conserved proteomic profile of OMVs among serogroups contributes to cross-protection. However, it is still limited by antigen variability and strain-specific efficacy. Expanding this concept to include OMVs from multiple meningococcal serogroups could solve strain-specific coverage issues and improve vaccine efficiency in diverse populations. Such formulations could diminish the drawbacks of single-serogroup vaccines, especially in regions where multiple serogroups co-circulate.

Furthermore, they may indirectly reduce the carriage of related pathogens in individuals not vaccinated with a vaccine specific to that species. For instance, like Bexsero (4CMenB), other OMV-based vaccines provide cross-protection against *Neisseria gonorrhoeae*, emphasizing their wider potential (217,218). Therefore, we will further study the cross-protection capacity of our native MenB OMV preparation against other serogroups and even other *Neisseria*-related pathogens.

The source organism and the method of isolation for OMVs are also as important as the choice of antigen. In the case of the current meningococcal vaccine, OMVs are isolated from wild-type *N. meningitidis* extract disrupted by a detergent due to the highly toxic nature of LPS found in the outer membrane surface. The LPS amount in OMVs used for the vaccine is critical. It is a potent adjuvant stimulating TLR4 on macrophages, but a high level of LPS could cause septic shock. The detergent treatment mostly eliminates LPS, but it comes with essential drawbacks. Detergent disrupts the structural integrity of the membrane proteins; even destroying particular proteins results in reduced and altered antigen repertoire in OMVs. This weakens the immune response and requires the addition of recombinant proteins into vaccine formulations externally. For example, fHbp is an essential antigen that induces bactericidal antibodies to protect against meningococcus; however, it is lost during detergent treatment and later incorporated into next-generation vaccines like Bexsero (219). Therefore, we used detergent-free meningococcal OMVs to protect the broad spectrum of antigens.

Combination vaccines offer noteworthy advantages from an immunological and practical standpoint. Combined vaccines comprising viral and bacterial antigens could improve the breadth of protection against multiple pathogens while reducing the required injections. This is particularly critical in children, as it minimizes the discomfort of numerous vaccine administrations, thereby

improving compliance with vaccination schedules. The co-immunization of antigens not only simplifies immunization logistics by reducing costs and storage requirements but may also induce a more synergistic and diverse immune response without compromising the effectiveness of individual components. Given these benefits, we developed a combination vaccine incorporating SARS-CoV-2 VLPs or Spike RBD and *Neisseria meningitidis* serogroup B and W OMVs to promote worldwide immunization coverage and protection against these infectious diseases. We observed that *N. meningitidis* OMVs (Nm OMVs) were promising adjuvants for SARS-CoV-2 RBD protein as 3 µg of rec RBD adjuvanted with Alum and *N. meningitidis* OMVs produced higher IgG response even 14 days after 1st injection (***P < .001 compared to placebo, Fig. 3.6) than the IgG response of 5 µg of rec RBD adjuvanted with CpG ODN and Alum 2 weeks after booster injection (*P < .05 compared to placebo, Fig. 3.5). However, this observation could not go beyond an interpretation based on the results of two different experiments, as our experimental setup lacks a group where RBD is adjuvanted solely with the OMVs. Therefore, the contribution of Alum to the observed difference cannot be disregarded. Nevertheless, Omicron SARS-CoV-2 VLP + Nm OMVs elicited potent antibody titers both against rec RBD and recS (***P < .001, Fig.3.10) without Alum, agreeing with other studies that used OMVs from different Gram-negative bacteria as adjuvants for SARS-CoV-2 (115,116).

The right choice of adjuvants is important in vaccine design, as it significantly affects the immunogenicity of vaccines. Adjuvants are necessary, primarily for subunits or OMV vaccines where antigens are insufficient to induce immune responses. They could direct the immune system toward Th1 or Th2-dominant responses and enhance robust and long-lasting immune reactions. Alum is a reliable adjuvant for OMV vaccines due to its safety, especially for pediatric vaccines. Alum-absorbed OMVs can easily be taken up by DCs to boost humoral immunity (220). This results in the massive production of IgG1, which is important in opsonization and complement-mediated killing of *Neisseria meningitidis*. As high-dose RBD antigen with meningococcal OMVs in our Delta-Plus SARS-CoV-2 RBD + MenB+MenW OMV vaccine study could not give a sufficient primary antibody response, Alum-absorbed formulation with low-dose RBD elicited a strong IgG response against DP-RBD only after 1 dose injection. (***P < .001) (Fig. 3.6). In our immunization studies with the combination vaccine, total antibody responses specific to meningococcal antigens did not differ significantly when using Alum or CpG ODN alone or in combination. However, for SARS-CoV-2-specific antibody responses, groups adjuvanted with

Alum alone showed higher antibody levels, while CpG ODN-containing groups exhibited a Th1-biased response. Notably, the highest levels of bactericidal activity, known to be predominantly IgG1-dependent, were observed in the Alum-containing groups.

4.2 PART II: Antibody Responses and Cytokine Profiling among Children with MIS-C versus COVID-19 Infection and the Contribution of Circulating Proinflammatory Extracellular Vesicles to MIS-C Pathogenesis

Pathophysiological mechanisms contributing to MIS-C development in a fraction of SARS-CoV-2 infected children are not fully defined. To further our understanding of this condition, we longitudinally analyzed the humoral and plasma cytokine responses of MIS-C patients in comparison to asymptomatic, mild, and severe COVID-19 pediatric patients and performed proteomic analysis of plasma-derived EVs from MIS-C patients

SARS-CoV-2-specific antibody responses in MIS-C patients were found to differ from those with severe COVID-19, as evidenced by elevated anti-Spike and anti-RBD IgG titers, suggestive of exaggerated humoral immune response that is characteristic of MIS-C (221,222).

The sustained nature of this response possibly indicates prolonged antigenic stimulation associated with persistent viral antigens in MIS-C. Furthermore, similar to previous findings, we found that MIS-C patients had higher titers of RBD- and S- but not N-specific IgA (223), which might be related to gastrointestinal inflammation frequently observed in MIS-C patients (224). Our findings on serum cytokine levels indicate a role of sustained Th17-mediated inflammation in MIS-C, with significantly elevated levels of IL-17A and IL-22 compared to severe COVID-19. IL-17A promotes neutrophil recruitment and tissue inflammation (225,226), which likely contribute to multisystem organ involvement in MIS-C. The delayed increase in IL-17F during the first interval, which corresponds to the subacute phase of infection, favors a sustained and evolving Th17 response, wherein IL-17A dominates the acute, whereas IL-17F contributes to prolonged inflammation. These findings are consistent with previous data showing Th17 involvement in hyperinflammation underlying the immunopathology of Kawasaki disease (227,228). Collectively, these findings suggest that targeting the Th17 pathway with IL-17 inhibitors like

secukinumab or brodalumab could be a viable therapeutic strategy to manage hyperinflammation associated with MIS-C.

Emerging evidence suggests a critical role for EVs in the pathophysiology of viral infections (229,230). In this context, consistent with previous work [193], our data showed that EVs isolated from both severe- and MIS-C patients were positive for RBD and S, suggesting the involvement of EVs in viral dissemination and/or modulation of host immune responses. These findings are in support for persistence of SARS-CoV-2 proteins in MIS-C EVs, consistent with previous studies in adult COVID-19 patients, where circulating EVs were shown to harbor viral nucleic acids and proteins contributing to the hyperinflammatory state (213,231,232).

Our work with MIS-C versus severe COVID-19 EVs further demonstrated a preferential activation of the IRF but not the NF- κ B pathway by MIS-C EVs, whereas severe COVID-19 EVs activated both pathways. This preferential activation of the IRF pathway by MIS-C EVs suggests that COVID-19 versus MIS-C EV cargoes may substantially differ. We undertook the proteomic analysis of plasma-derived EVs from MIS-C patients to address this. EVs isolated from MIS-C patients exhibited 168 proteins uniquely identified in MIS-C samples, compared to 29 proteins unique to healthy controls. MIS-C-specific proteins were enriched in inflammation, innate immune activation, and neutrophil degranulation pathways, implying that MIS-C EVs could contribute to the hyperinflammatory state. Moreover, β -integrins were found to be enriched in MIS-C EVs, pointing to the role of EVs in promoting immune cell adhesion and migration to inflamed tissues. Considering these findings, targeting integrin signaling (233–235) through integrin inhibitors, such as natalizumab or vedolizumab, could offer an alternative treatment strategy to manage hyperinflammation in MIS-C.

Collectively, our data indicate that MIS-C may differ from pediatric COVID-19 cohorts through the involvement of exacerbated Th17 responses and an altered EV proteome, both of which likely contribute to hyperinflammation and multisystem involvement.

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6 APPENDICES

6.1 Appendix A

Table 6. Clinical and laboratory findings in pediatric COVID-19 patients with severe outcomes.

Children with COVID-19	
Characteristics	N = 10 ¹
Age (year)	9.0 (5.0 - 15.0)
Male:female (n%)	6 (60.0%) : 4 (40.0%)
Underlying Medical Conditions	
Healthy	1 (10.0%)
Obese	3 (30.0%)
Asthma	1 (10.0%)
Prematurity	1 (10.0%)
Floppy infant	1 (10.0%)
I-cell	1 (10.0%)
Down Syndrome	2 (20.0%)
Laboratory Findings	
Hemoglobin (102-127 g/L)	122.5 (117 - 134)
White blood cell (4.0-13.8 x 10 ⁹ /L)	6.0 (4.2 - 8.6)
Absolute Neutrophil Count (1.6-8.3 x 10 ⁹ /L)	4.3 (3.6 - 6.3)
Absolute Lymphocyte Count (1.3-5.8 x 10 ⁹ /L)	0.62 (0.32 - 1.59)
Platelet count (189-394 x 10 ⁹ /L)	236 (168 - 252)
C-reactive protein (<0.5 mg/dL)	4.2 (1.0 – 11.7)
Ferritin (5.3-99.9 ug/L)	203 (110 - 332)
ESR (mm/h)	29 (23 - 41)
Procalcitonin (<0.1 ng/mL)	0.16 (0.05 – 0.18)
¹ Median (Q1 - Q3); n (%)	

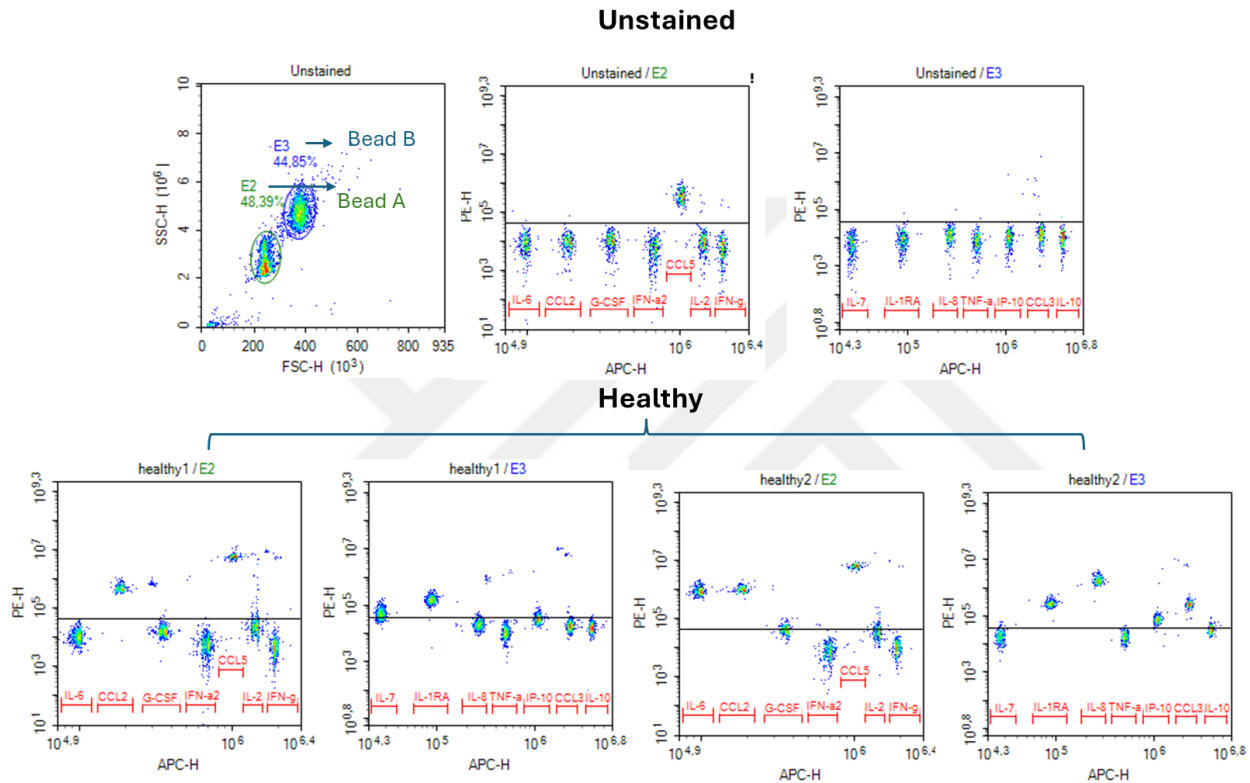
Table 7. Clinical and laboratory findings in pediatric patients with MIS-C.

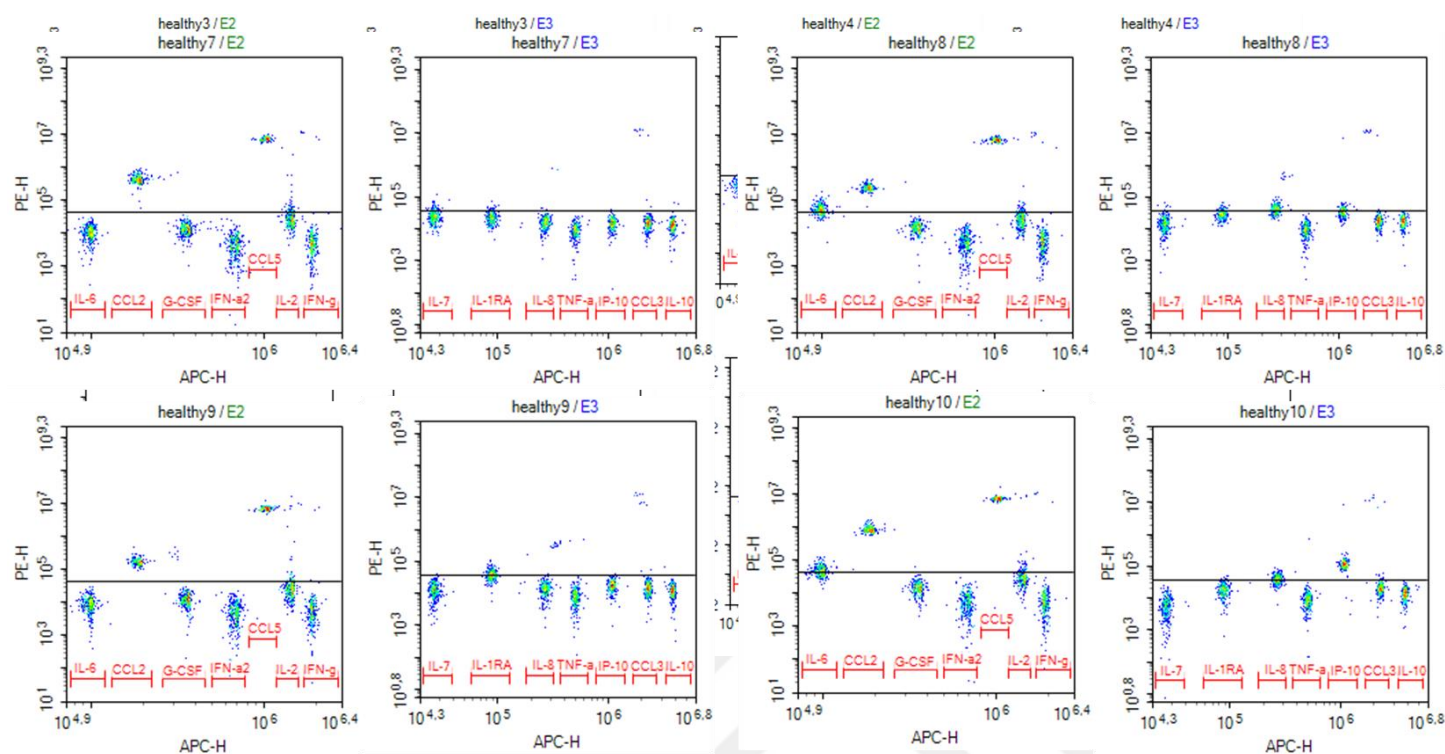
Children with MIS-C			
Characteristic	N = 19 ¹		
Age (year)	8.0 (6.0 - 11.0)		
Male:Female	11 (57.89%) : 8 (42.11%)		
Number of KD criteria	3.00 (2.00 - 4.00)		
Medical Conditions		Laboratory Findings	
Conjunctivitis		Hemoglobin (102-127 g/L)	113 (106 - 120)
No	2 (10.53%)	White blood cell (4.0-13.8 x 10 ⁹ /L)	10.9 (6.9 - 13.4)
Yes	17 (89.47%)	Absolute Neutrophil Count (1.6-8.3 x 10 ⁹ /L)	8.3 (4.6 - 12.0)
Rash		Absolute Lymphocyte Count (1.3-5.8 x 10 ⁹ /L)	1.14 (0.72 - 1.58)
No	7 (36.84%)	Platelet count (189-394 x 10 ⁹ /L)	164 (139 - 265)
Yes	12 (63.16%)	C-reactive protein (<0.5 mg/dL)	22 (11 - 29)
Mucocutaneous		Ferritin (5.3-99.9 ug/L)	400 (270 - 921)
No	2 (10.53%)	ESR (mm/h)	49 (38 - 69)
Yes	17 (89.47%)	IL-6 (<6.4 pg/mL)	73 (15 - 207)
Extremity change		Procalcitonin (<0.1 ng/mL)	5 (2 - 9)
No	17 (89.47%)	Sodium (136-146 meq/L)	134.0 (131.0 - 136.0)
Yes	2 (10.53%)	Albumin (35-47 g/L)	30.2 (25.2 - 35.2)
Lymphadenopathy		Creatinine (0.26-0.77 mg/dL)	0.43 (0.32 - 0.70)
No	10 (52.63%)	ALT (<39 U/L)	49 (20 - 78)
Yes	9 (47.37%)	AST (<56 U/L)	37 (25 - 60)
GI involvement		LDH (110-295 U/L)	342 (257 - 399)
Yes	16 (84.21%)	Triglycerides (<150 mg/dL)	189 (143 - 255)
No	3 (15.79%)	INR (0.8-1.2)	1.16 (1.12 - 1.30)
Abdominal pain		PTT (24-36 s)	25.4 (21.9 - 27.7)
Yes	12 (63.16%)	Fibrinogen (180-350 mg/dL)	601 (465 - 704)
No	7 (36.84%)	D-dimer (<0.55 mg/L)	3.1 (2.0 - 6.8)
Diarrhea			
Yes	8 (42.11%)		
No	11 (57.89%)		
Altered mental status			
No	17 (89.47%)		
Yes	2 (10.53%)		
Renal involvement			
No	8 (50.00%)		
Yes	8 (50.00%)		
¹ Median (Q1 - Q3); n (%)			

ALT, Alanine transaminase; AST, Aspartate aminotransferase; LDH, lactate dehydrogenase; INR, international normalized ratio; PTT, partial thromboplastin time; BNP, B-Type Natriuretic Peptide.

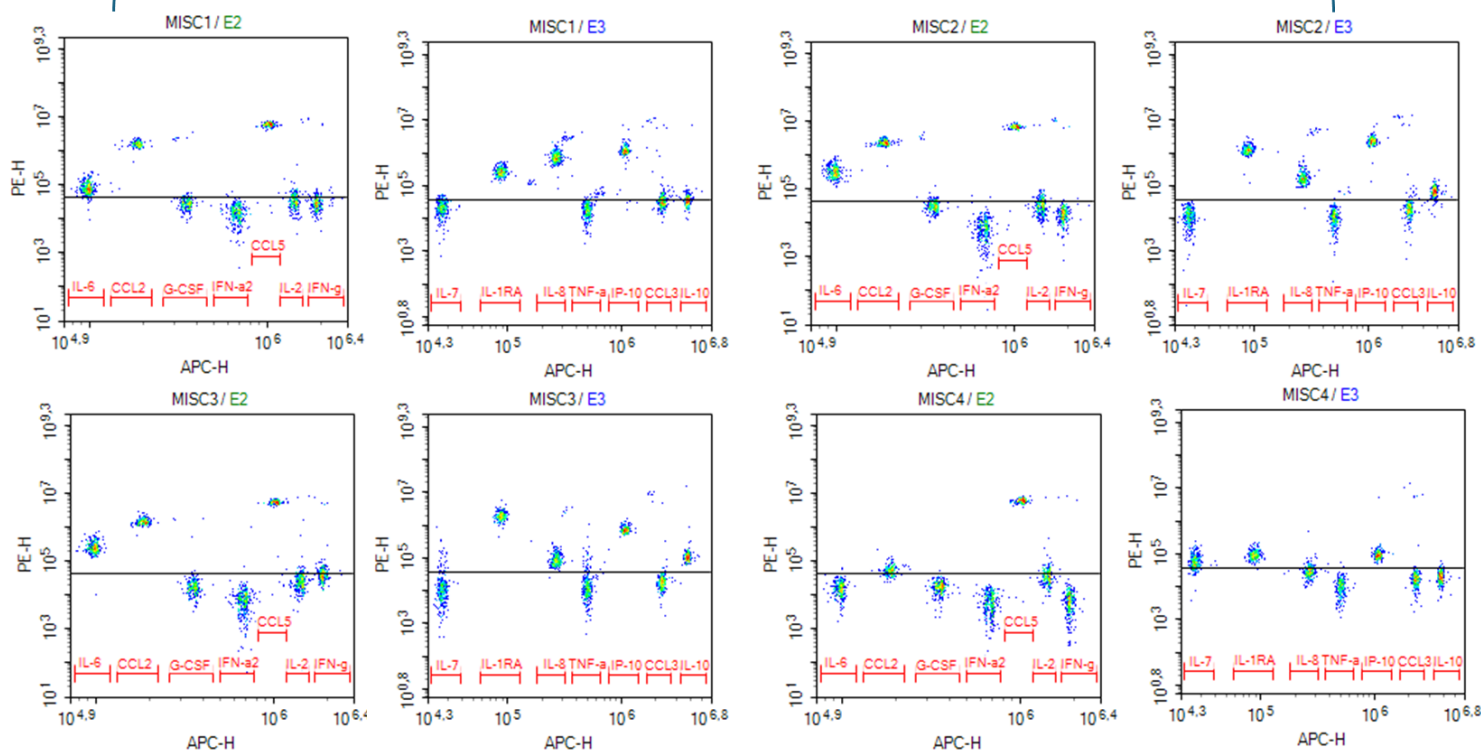
6.2 Appendix B

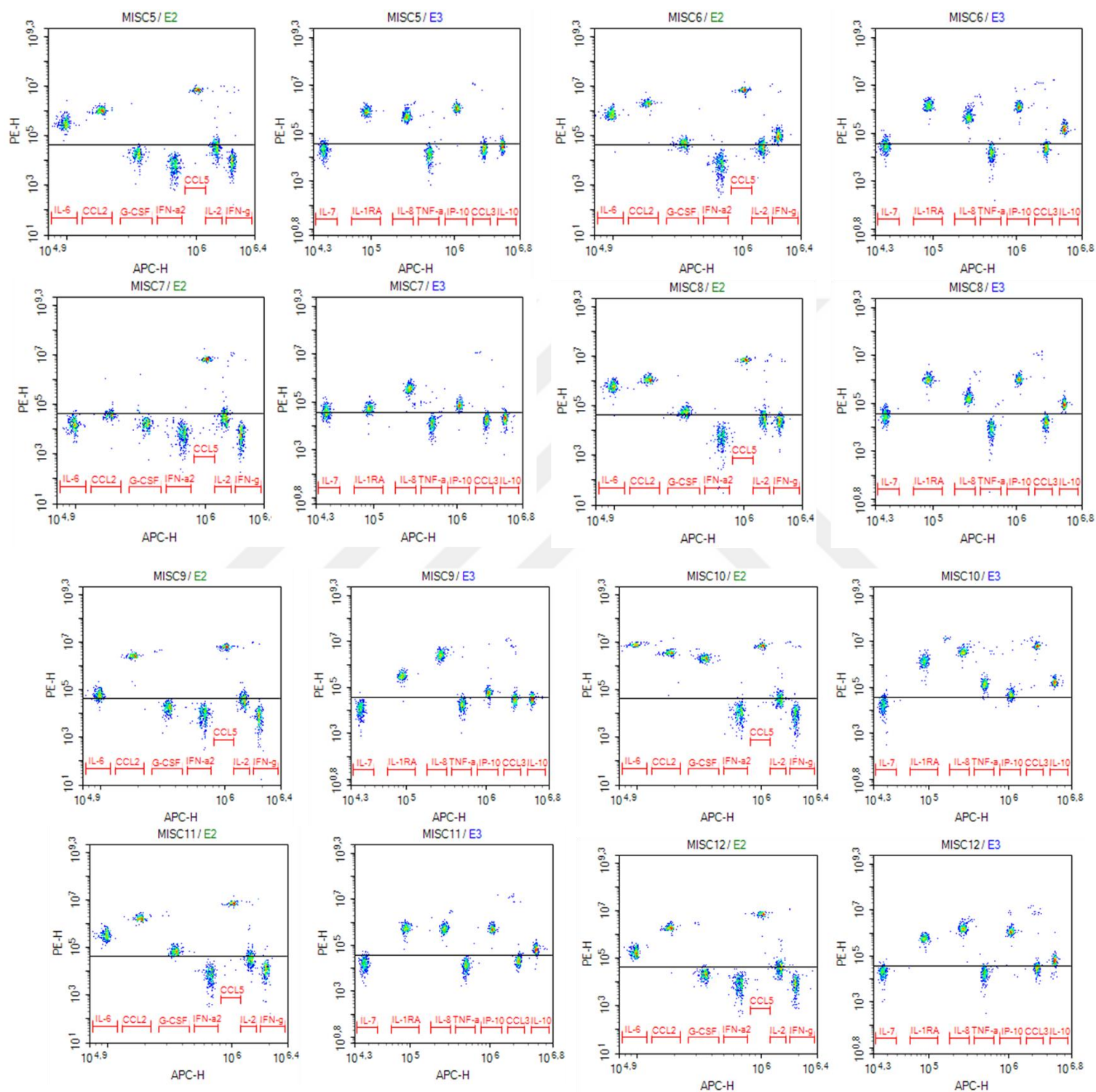
NovoCyte flow cytometer analysis of serum cytokine levels of pediatric patients assessed by cytometric bead-based multiplex assay panels; LEGENDplex™ HU Th Cytokine Panel (12-plex) and COVID-19 Cytokine Storm Panel 1 (LEGENDplex™, Biolegend).

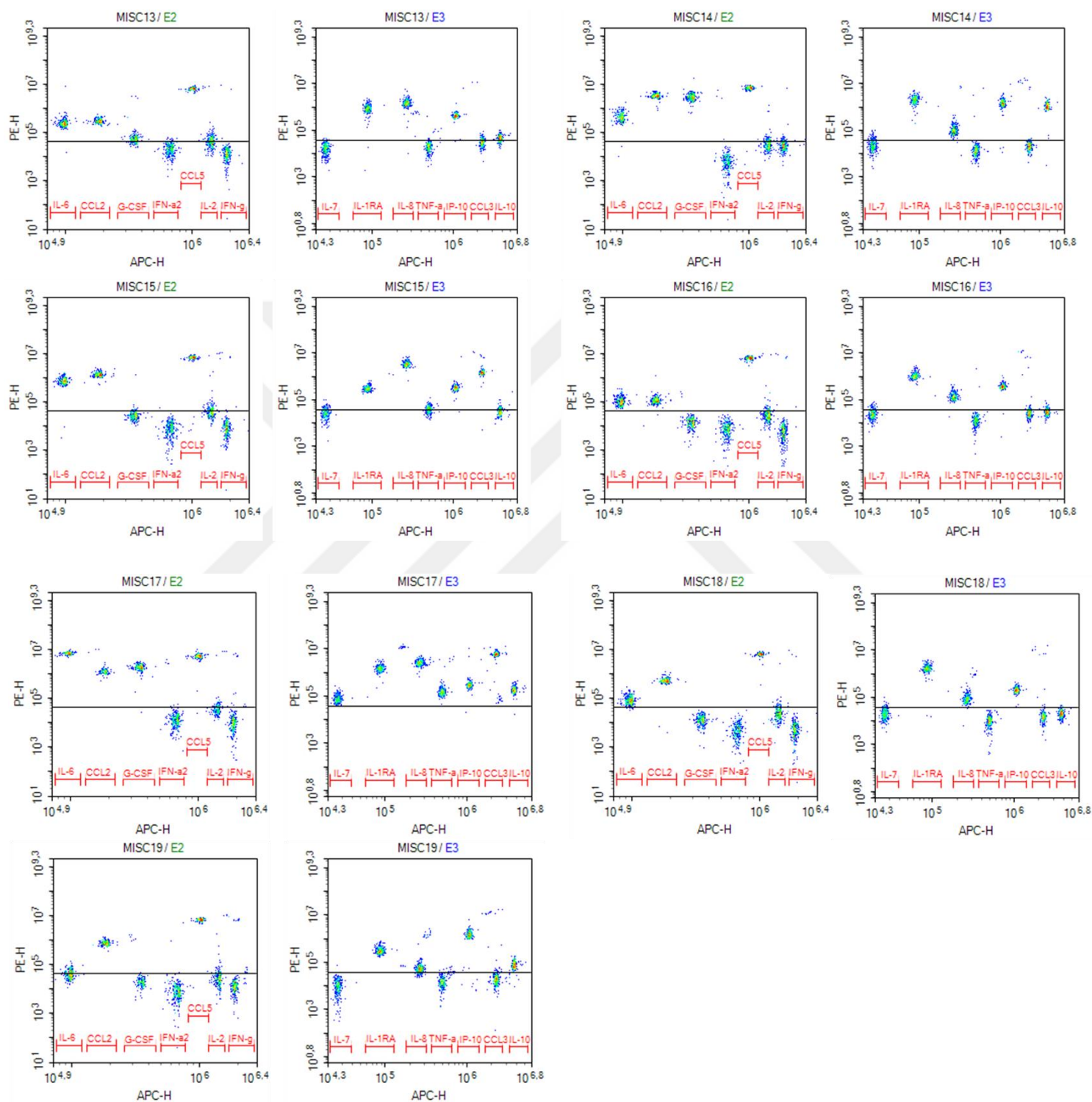




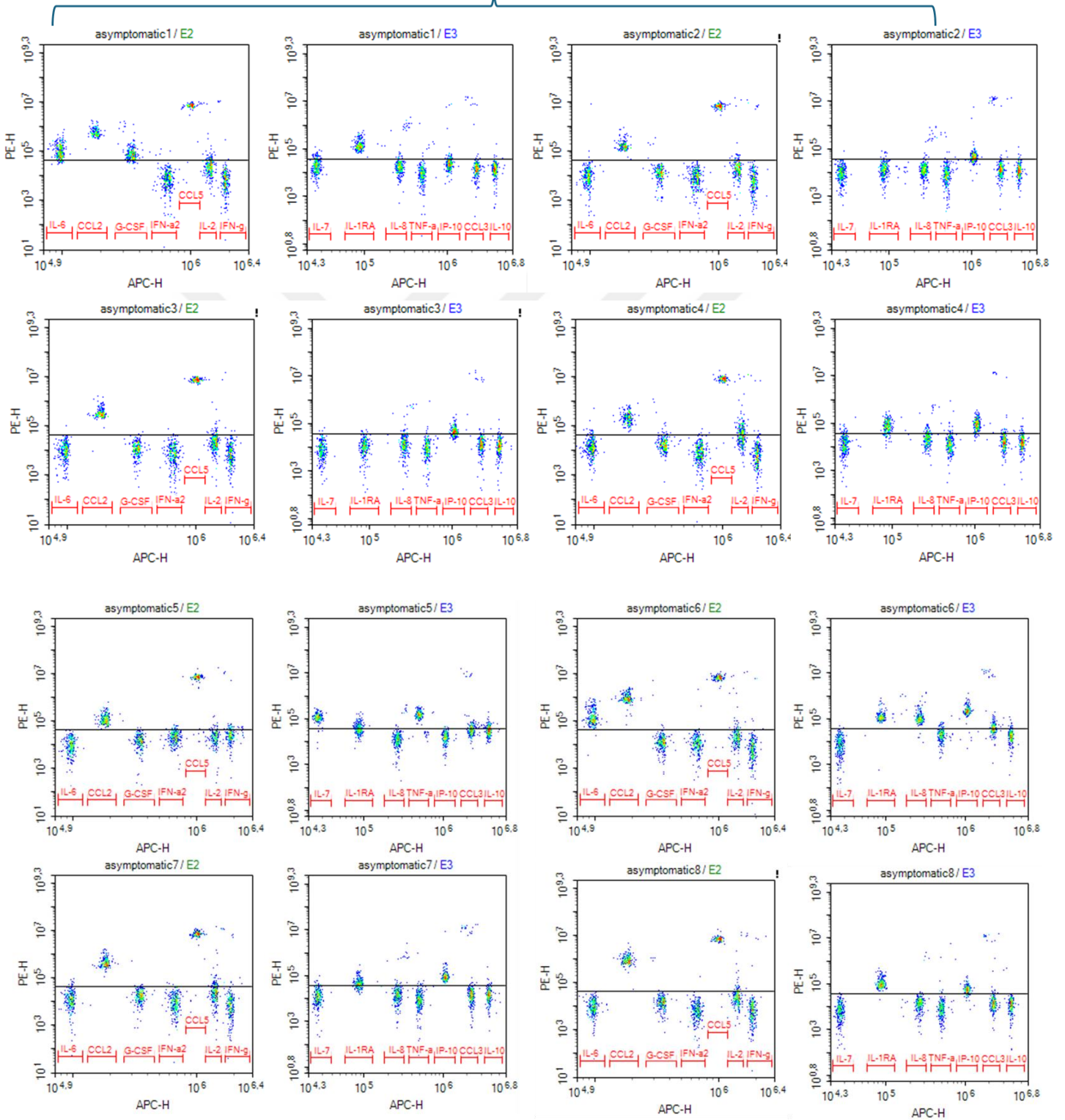
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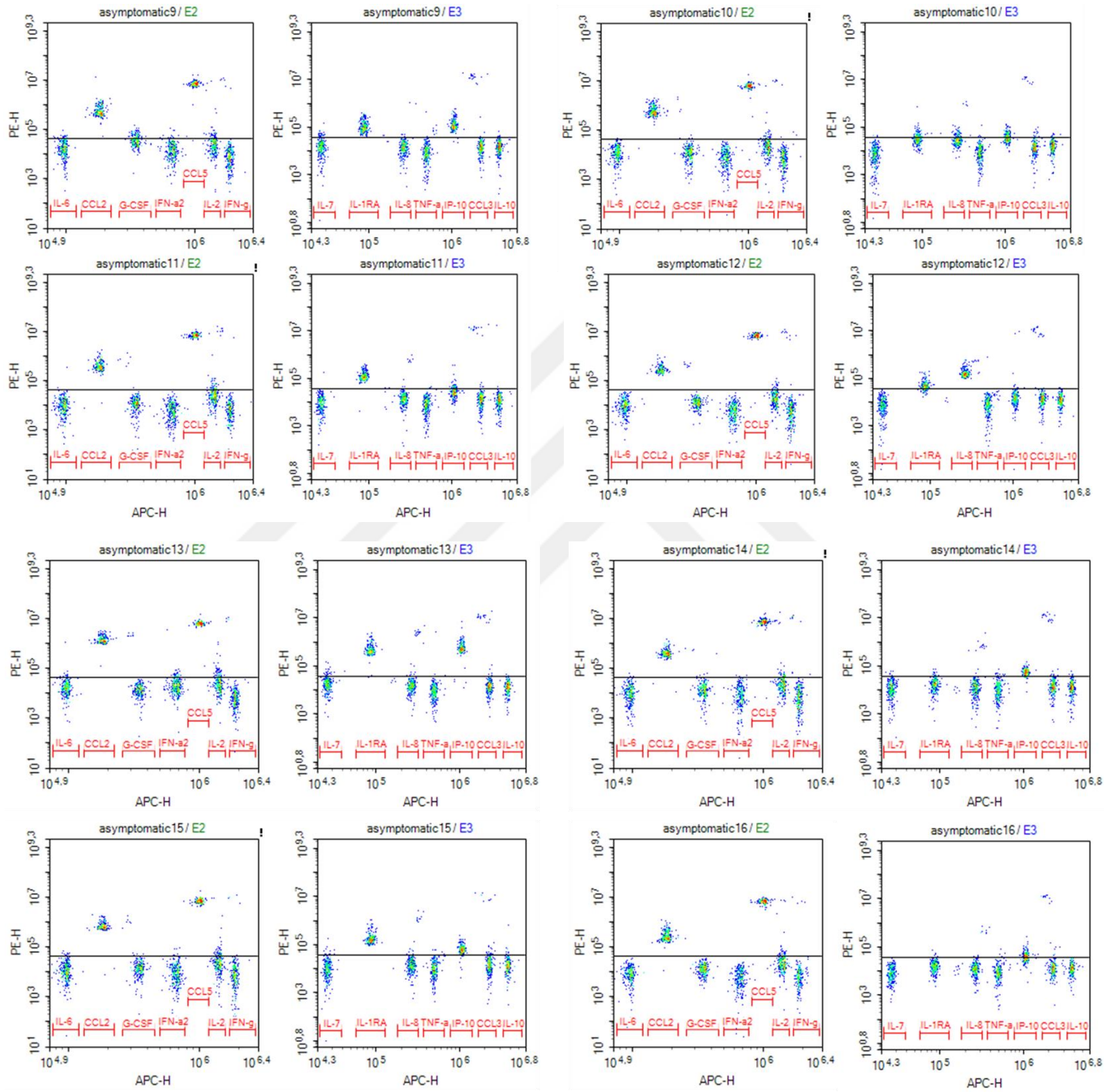


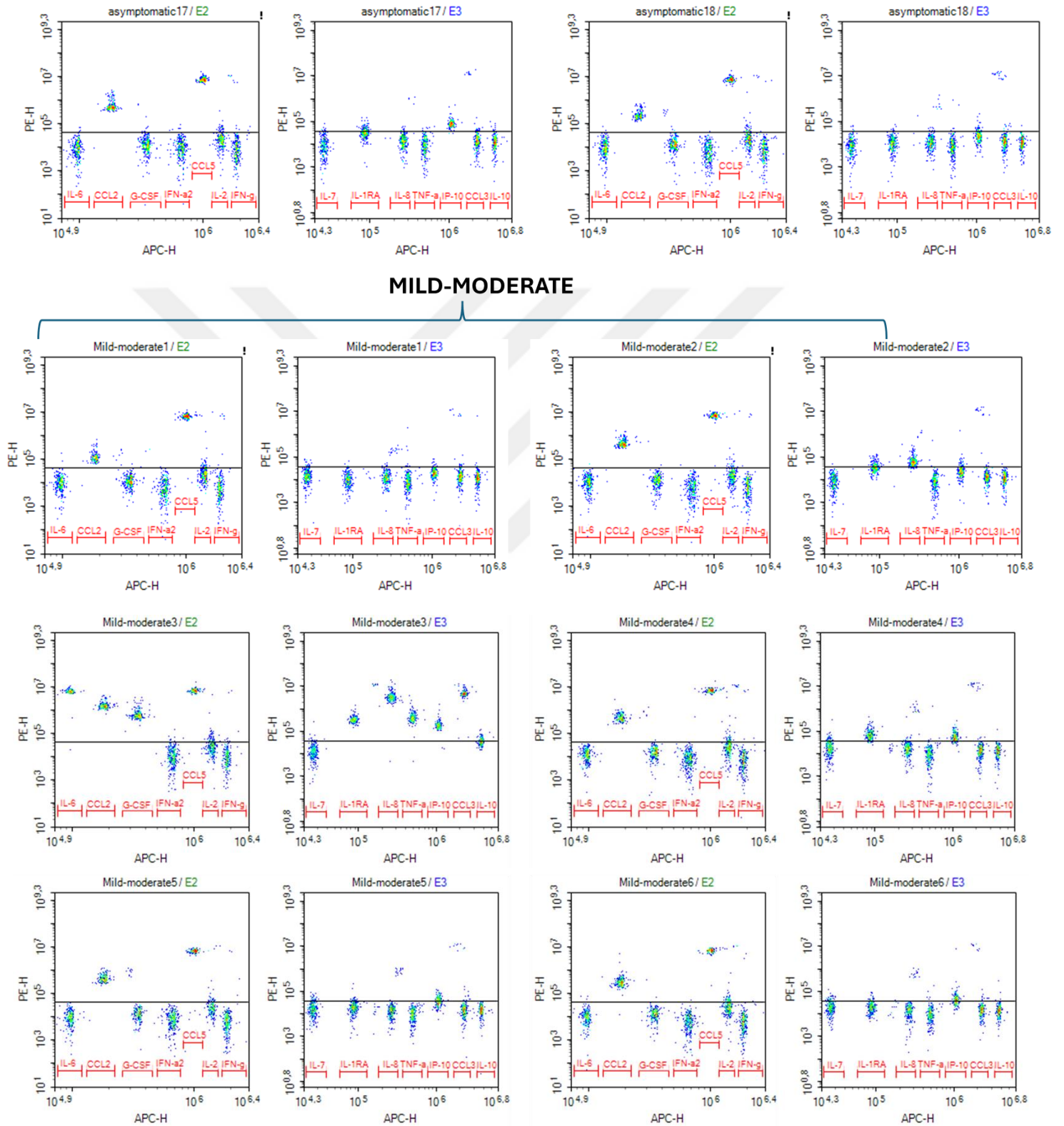


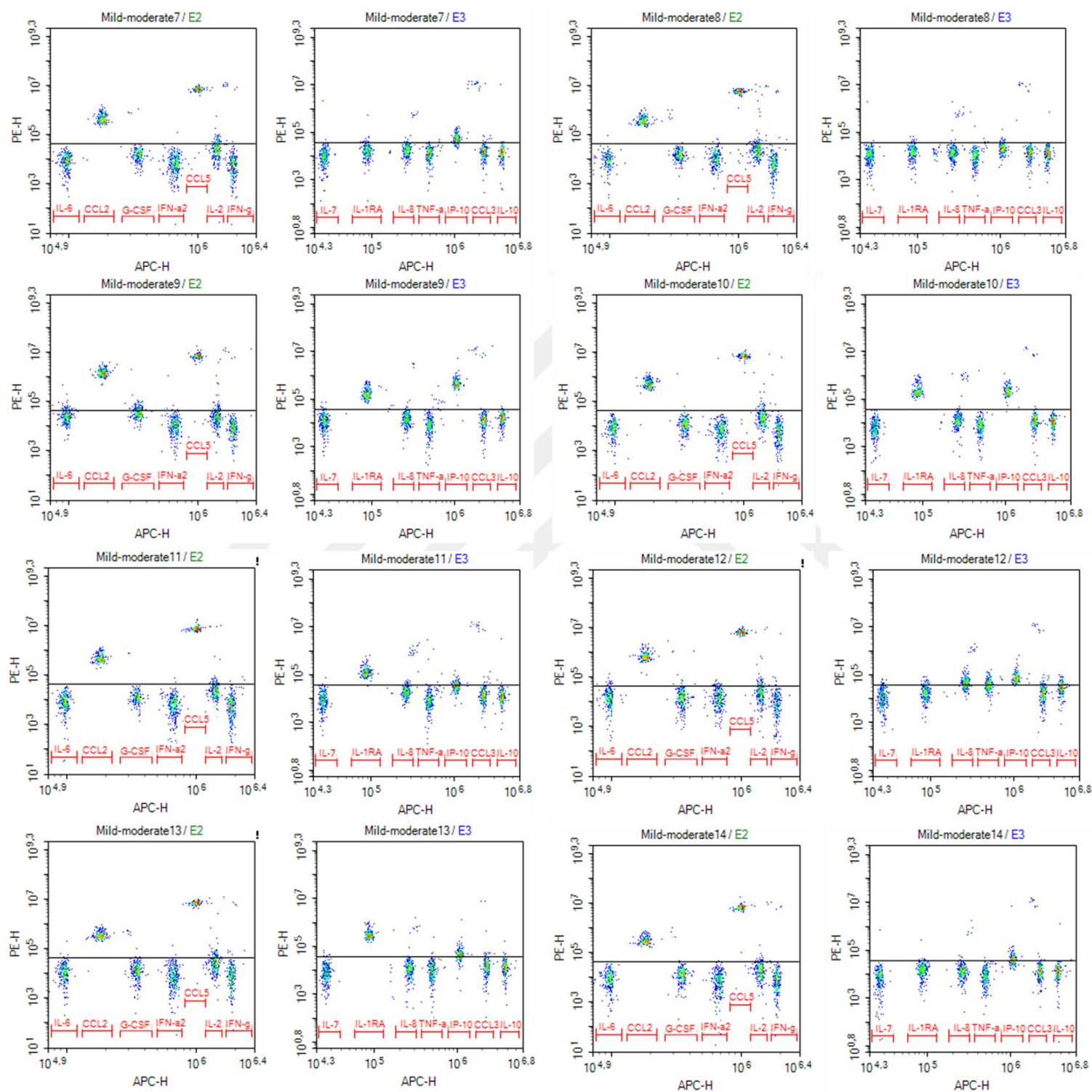


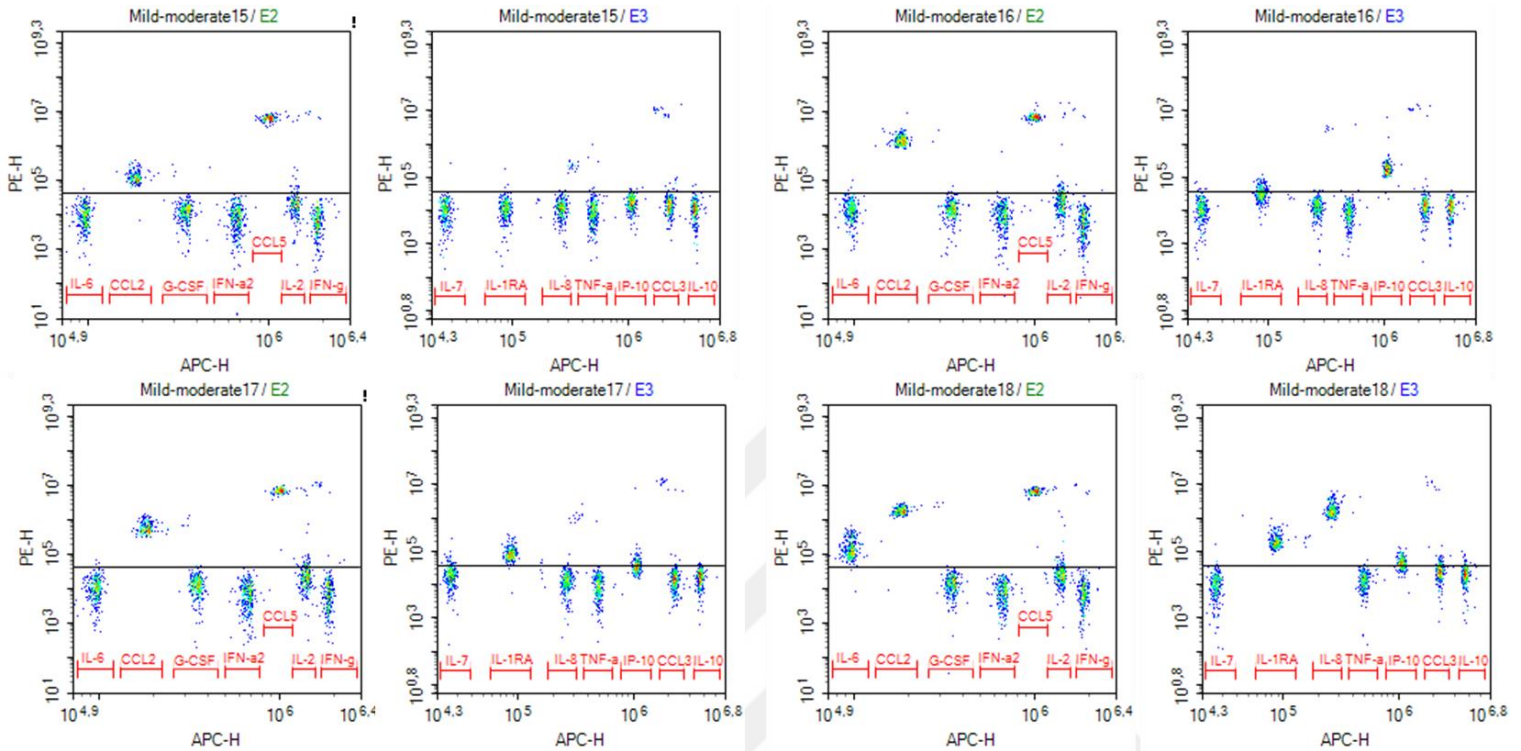
ASYMPTOMATIC











6.3 Appendix C

BUFFER RECIPES

10X Phosphate Buffered Saline (PBS)

- 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)

1X PBS with 5% Bovine Serum Albumin (BSA) and 0.025% Tween®20

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

- 150 mM Sodium chloride
- 1.0% NP-40 or Triton X-100
- 0.5% Sodium deoxycholate
- 0.1% SDS
- 50 mM Tris, pH 8.0

Buffer was prepared freshly, kept at +4 °C, and used immediately.

Laemmli buffer (4x)

- 4% SDS
- 10% 2-mercaptoethanol
- 20% Glycerol

- 0.004% Bromophenol blue
- 0.125 M Tris-HCl

Buffer pH was adjusted to 6.8 and stored at -20 °C.

WESTERN BLOTTING Buffers

SDS-PAGE Running buffer (10x)

- 25 mM Tris base
- 195 mM Glycine
- 0.1% SDS

Its pH was adjusted to 8.3 and stored at RT. It was diluted to 1X before use.

SDS-PAGE Transfer buffer

- 25 mM Tris base
- 195 mM Glycine
- 20% Methanol

Its pH was adjusted to 8.3 and stored at +4 °C.

Blocking and antibody dilution buffer

- 1X PBS-T with 5% BSA

The solution was mixed well and stored at +4 °C.

Resolving gel (12%)

- 2.5 ml 1.5 M Tris (pH 8.8)
- 100 µl 10% SDS
- 3.96 ml 30% Acrylamide
- 100 µl 10% APS

- 10 µl TEMED
- 3.44 ml ddH₂O

The solution was mixed well by vortexing and used immediately.

Resolving gel (8%)

- 2.5 ml 1.5 M Tris (pH 8.8)
- 100 µl 10% SDS
- 2.64 ml 30% Acrylamide
- 100 µl 10% APS
- 10 µl TEMED
- 4.76 ml ddH₂O

The solution was mixed well and used immediately.

Stacking gel (5%)

- 2 ml 0.5 M Tris (pH 6.8)
- 80 µl 10% SDS
- 1.3 ml 30% Acrylamide
- 80 µl 10% APS
- 16 µl TEMED
- 4.5 ml ddH₂O

The solution was mixed well and used immediately.

7 Curriculum Vitae and Publication

ACADEMIC DEGREE:

2018-2024

Ph.D. in Molecular Biology and Genetics, Department of Molecular Biology and Genetics Bilkent University, Ankara, TÜRKİYE

Title of Thesis: Development of a Combination Vaccine Against N. meningitidis and SARS-CoV-2 and Analysis of MIS-C Plasma and Extracellular Vesicles in Relation to Disease Severity in Pediatric Patients

Supervisor: Prof. İhsan Gürsel

2016-2018

M.Sc. in Molecular Biology and Genetics, Department of Molecular Biology and Genetics Bilkent University, Ankara, TÜRKİYE

Title of Thesis: Investigating the Role of Mediator Complex on Estrogen Receptor (ER α)-mediated Transcription

Supervisor: Asst. Prof. Murat Alper Cevher

2012-2016

B.Sc. in Molecular Biology and Genetics, Department of Molecular Biology and Genetics

Bilkent University, Ankara, TÜRKİYE

PUBLICATIONS:

1. **Yildirim TC***, Ozsurekci Y*, Yildirim M, Yazar V, Evcili I, Gungor B, Yilmaz IC, Ozen S, Aykac K, Cengiz AB, Guler U, Salih B, Gursel M, Gursel I. Dynamics of Immune Dysregulation and the Contribution of Circulating Proinflammatory Extracellular Vesicles to MIS-C Pathogenesis. *Journal of Extracellular Vesicles*
#Submitted manuscript
* Equal contribution
2. **Yildirim TC***, Ozsurekci Y*, Yildirim M, Evcili I, Yazar V, Aykac K, Guler U, Salih B, Gursel M, Gursel I. Immunogenicity and protective capacity of a CpG ODN adjuvanted alum adsorbed bivalent meningococcal outer membrane vesicle vaccine. *Int. Immunol.*, Volume 36, Issue 8, August 2024, Pages 393–404, <https://doi.org/10.1093/intimm/dxae016>
*Equal contribution
3. Cetinkaya, P. G., Abras, I. F., Evcili, I., **Yildirim, T.**, Ceylan, Y., Kara Eroglu, F., ... Gursel, I. (2024). Plasma Extracellular Vesicles Derived from Pediatric COVID-19 Patients Modulate Monocyte and T Cell Immune Responses Based on Disease Severity. *Immunol. Inv.*, 1–35. <https://doi.org/10.1080/08820139.2024.2385992>
4. Yildirim M*, **Yildirim TC***, Turay N, Bildik T, Ibibik B, Evcili I, Ersan PG, Tokat UM, Sahin O, Gursel I. TLR ligand loaded exosome-mediated immunotherapy of established mammary Tumor in mice. *Immunol. Letts.* 2021 Nov; 239:32-41. DOI: 10.1016/j.imlet.2021.08.004. Epub 2021 Aug 18. PMID: 34418488. *Equal contribution
5. Kalyoncu S, Yilmaz S, Kuyucu AZ, Sayili D, Mert O, Soy Turk H, Gullu S, Akinturk H, Citak E, Arslan M, Taskinarda MG, Tarman IO, Altun GY, Ozer C, Orkut R, Demirtas A, Tilmensagir I, Keles U, Ulker C, Aralan G, Mercan Y, Ozkan M, Caglar HO, Arik G, Ucar MC, Yildirim M, **Yildirim TC**, Karadag D, Bal E, Erdogan A, Senturk S, Uzar S, Enul H, Adiyay C, Sarac F, Ekiz AT, Abaci I, Aksoy O, Polat UH, Tekin S, Dimitrov S, Ozkul A, Wingender G, Gursel I, Ozturk M, Inan M. Process development for an effective COVID-19 vaccine candidate harboring recombinant SARS-CoV-2 delta plus receptor binding domain produced by *Pichia pastoris*. *Sci. Reports.* 2023 Mar 30;13(1):5224.
6. Yazar V, Kilic G, Bulut O, **Yildirim TC**, Yagci FC, Aykut G, Klinman DM, Gursel M, Gursel I. A suppressive oligodeoxynucleotide expressing TTAGGG motifs modulates cellular energetics through the mTOR signaling pathway. *Int. Immunol.* 2020 Jan 9;32(1):39-48. DOI: 10.1093/intimm/dxz059. PMID: 31633763; PMCID: PMC7185194
7. Yilmaz IC, Ipekoglu EM, Bulbul A, Turay N, Yildirim M, Evcili I, Yilmaz NS, Guvencli N, Aydin Y, Gungor B, Saraydar B, Bartan AG, Ibibik B, Bildik T, Baydemir İ, Sanli HA, Kayaoglu B, Ceylan Y, **Yildirim T**, Abras I, Ayanoglu IC, Cam SB, Ciftci Dede E, Gizer M, Erganis O, Sarac F, Uzar S, Enul H, Adiyay C, Aykut G, Polat H, Yildirim IS, Tekin S, Korukluoglu G, Zeytin HE, Korkusuz P, Gursel I, Gursel M. Development and preclinical

evaluation of virus-like particle vaccine against COVID-19 infection. *Allergy*. 2021 Sep 14. doi: 10.1111/all.15091. Epub ahead of print. PMID: 34519053.

8. Eroglu FK, Yazar V, Guler U, Yıldırım M, **Yildirim T**, Gungor T, Celikkaya E, Karakaya D, Turay N, Ciftci Dede E, Korkusuz P, Salih B, Bulbul M, Gursel I. Circulating extracellular vesicles of steroid-sensitive nephrotic syndrome patients have higher RAC1 and induce recapitulation of nephrotic syndrome phenotype in podocytes. *Am. J. Physiol. Renal Physiol.*, 2021 Sep 27. DOI: 10.1152/ajprenal.00097.2021. Epub ahead of print. PMID: 34569252.
9. Bal SH, Sagdilek E, Karacay M, Kızılmaz MA, Kumas LT, Can FE, Yildirim M, **Yildirim TC**, Gulkaya DK, Ermiş DY, Budak F, Heper Y, Gursel I, Oral HB. The Effect of Exosomes Released from Apheresis Platelet Concentrates Under the Impact of Gamma Irradiation and Storage Time upon the Platelet Aggregation and Hemostasis. *Blood Transfusion*. 2022 Aug. DOI: 10.2450/2022.006-22. PMID: 35969141.
10. Kahraman T, Gucluler G, Yildirim M, Larssen P, Kocabas BB, Yagci FC, Gursel A, Horuluoglu BH, Yazar V, Ayanoglu IC, **Yildirim TC**, Evcili I, Yilmaz IC, Eldh M, Gabriellsson S, Guler U, Salih B, Gursel M, Gursel I. Enhancing preventive and therapeutic cancer vaccine efficacy through biotherapeutic ligand-associated extracellular vesicles. *Journal of Controlled Release* 376 (2024): 618-631.
11. Yozgat Y, Karakoc E, Sahin O, Cimen S, Rabeh WM, Aydin MS, Mardinoglu A, Gursel I, Cakir A, Sensoy O, Ozdemir EM, Bayrak Y, Gunluoglu MZ, Saatci O, Jabbar J, Ferreira JC, Aslan MD, Yildirim M, Mansoor S, Kerman BE, Aladag Z, Kim W, Arif M, Vatandaslar E, Tok OE, Dogru Z, Demir AGO, **Yildirim TC**, Yozgat I, Senturk S, Ozturk G, Cevher MA. Hexokinase 1b is a novel target for metabolic therapy against non-small-cell lung cancer, preprint in *bioRxiv*.
12. "Virüs Benzeri Partikül (VLP) Aşıları" in *Aşı Çalışmaları ve Teknolojisi*, edited by Dr. Mert Döşkaya, Dr. Adnan Yüksel Gürüz, Dr. Ayşe Gülten Kantarcı, Dr. Cemal Ün, Nobel Tıp Kitabevleri, 2024.
13. "Dış Membran Vezikülü (OMV) Aşılar" in *Aşı Çalışmaları ve Teknolojisi*, edited by Dr. Mert Döşkaya, Dr. Adnan Yüksel Gürüz, Dr. Ayşe Gülten Kantarcı, Dr. Cemal Ün, Nobel Tıp Kitabevleri, 2024.

AWARDS AND SCHOLARSHIPS:

- 1004- Preparation of SARS-CoV-2 Vaccine for Clinical PHASE-I, Ph.D. scholarship (2021-2022)
- 2247 - C STAR-Intern Researcher Scholarship Programme (2020-2021)
- Turkish Society of Immunology (TSI) for International Molecular Immunology & Immunogenetics Congress IV, 27-29 April 2019. Bursa, TÜRKİYE. Accommodation Award

- Turkish Society of Immunology (TSI) for Symposium of Molecules in Immunology, 18-21 October 2018, Izmir, TÜRKİYE, Accommodation Award
- Bilkent University, Department of Molecular Biology and Genetics. M.Sc and Ph.D. Program, Full Scholarship.

RESEARCH EXPERIENCE:

2022-present	Researcher in Izmir Biomedicine and Genome Center, Dokuz Eylül University, Izmir, TÜRKİYE
2020- 2022	Research Assistant , Tubitak, 1004- “Preparation of SARS-CoV-2 Vaccine for Clinical PHASE-I” Department of Molecular Biology and Genetics, Bilkent University, Ankara, TÜRKİYE
2016-2018	Research Assistant, EMBO Installation Grants , “Investigating the Role of Mediator Complex on Estrogen Receptor (ER α)-mediated Transcription” Bilkent University, Ankara, TÜRKİYE
2015 (June-September)	Summer Internship at Albert Einstein College of Medicine of Yeshiva University/New York, Department of Genetics <u>Project Title:</u> Revealing the expression mechanism of oscillating clock genes involved in somitogenesis in zebrafish
2014 (June-August)	Summer Internship at Izmir Institute of Technology, Department of Molecular Biology and Genetics <u>Project Title:</u> Cellular distribution of invadopodia is regulated by nanometer-scale surface protein patterns

PROFESSIONAL EXPERIENCE:

2016 - 2022	Teaching/Lab. Assistant, Department of Molecular Biology and Genetics, Bilkent University, Ankara, TÜRKİYE (Lectures: MBG302 Molecular Biology of the Cell; MBG 312 Biochemistry, MBG 223 Molecular Genetics)
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CONFERENCES & WORKSHOPS ATTENDED:

- MIMIC V - International Molecular Immunology and Immunogenetics Congress V, 20-22 October 2022, Izmir, Türkiye.
- 6th European Congress of Immunology (ECI 2021), EFIS-ACTERIA Travel Grant, Belgrad, Serbia
- MIMIC IV - International Molecular Immunology and Immunogenetics Congress IV, 27-29 April 2019, Bursa, Türkiye.
- Symposium of Molecules in Immunology, 18-21 October 2018. Izmir, Türkiye.

POSTER/ORAL PRESENTATIONS:

- **Tugce Canavar Yildirim**, Muzaffer Yildirim, Irem Abras, Yasemin Ceylan, Yasemin Ozsurekci, Mayda Gursel, Ihsan Gursel. ‘Combination of VLP-based SARS-CoV-2 vaccine with OMVs of Neisseria Meningitidis induces a potent immune response in mice’ MIMIC V, 20-22 October 2022, Izmir, TÜRKİYE (**Poster Presentation**)
- Muzaffer Yildirim, **Tugce Canavar Yildirim**, Nilsu Turay, Tugce Bildik, Bilgehan Ibibik, Irem Evcili, Pelin Gulizar Ersan, Ozgur Sahin, Ihsan Gursel. ‘TLR Ligand Loaded Exosome Mediated Immunotherapy Established Mammary Tumor in Mice’ MIMIC V, 20-22 October 2022, Izmir, TÜRKİYE (**Poster Presentation**)
- Irem Abras, Yasemin Ceylan, Muzaffer Yildirim, **Tugce Canavar Yildirim**, Tolunay Atac, Irem Evcili, Ihsan Gursel. ‘Determination of VLP vaccine immune potency in mice when combined with different adjuvants and complexed with liposomes’ MIMIC V, 20-22 October 2022, Izmir, TÜRKİYE (**Poster Presentation**)
- Yasemin Ceylan, Irem Abras, **Tugce Canavar Yildirim**, Muzaffer Yildirim, Tolunay Atac, İrem Evcili, Ihsan Gursel. ‘Humoral immunogenicity of VLP vaccine combined with K3 CpG ODN, p(I: C) and cGAMP’ MIMIC V, 20-22 October 2022, Izmir, TÜRKİYE (**Poster Presentation**)
- Pınar Gur Cetinkaya, Irem Evcili, Irem Abras, Yasemin Ceylan, **Tuğçe Canavar Yıldırım**, Yasemin Ozsurekci, Basak Kayaoglu, Emre Mert Ipekoglu, Aysegul Akarsu, Umit Murat Sahiner, Bulent Enis Sekerel, Mayda Gursel, Ozge Soyer, Ihsan Gursel. ‘Immune responses of children with asthma during and after SARS-CoV-2 infection’ MIMIC V, 20-22 October 2022, Izmir, TÜRKİYE (**Poster Presentation**)
- **Tugce Canavar Yildirim**, Muzaffer Yildirim, Irem Evcili, Bilgehan Ibibik, Yasemin Ceylan, Nilsu Turay, Artun Bülbül, Asli Bartan, Tugce Bildik, Berfu Saraydar, Irem Abras, Naz Yilmaz, Emre Mert Ipekoglu, Ismail Cem Yilmaz, Nese Guvencli, Ilayda Baydemir, Yagmur Aydin, Mayda Gursel, Ihsan Gursel. ‘Virus-Like Particle (VLP) – based SARS- CoV-2 vaccine induces a potent immune response in rats’ Virtual ECI2021- 6th European Congress of Immunology, 1-4 September 2021. (**Poster Presentation**)
- Muzaffer Yildirim, **Tugce Canavar**, Gozde Gucluler, Banu Bayyurt, Tamer Kahraman, Irem Evcili, G.Gokberk Kaya, Naz Bozbeyoglu, Fuat Cem Yagci, Ihsan Gursel “Exosomes Co-encapsulating Antigen and Immunoadjuvants Act as an Effective Therapeutic Cancer Vaccine” Virtual ECI2021- 6th European Congress of Immunology, 1-4 September 2021. (**Poster Presentation**)

- Muzaffer Yildirim, **Tugce Canavar**, Gozde Gucluler, Banu Bayyurt, Tamer Kahraman, Irem Evcili, G. Gokberk Kaya, Naz Bozbeyoglu, Fuat Cem Yagcı, Ihsan Gursel. ‘The use of double-ligand loaded exosomes with cancer antigen as a therapeutic melanoma vaccine’ MIMIC IV, 27-29 April 2019, Bursa, TÜRKİYE (**Poster Presentation**)
- Muzaffer Yildirim, **Tugce Canavar**, Gozde Gucluler, Banu Bayyurt, Tamer Kahraman, Irem Evcili, G. Gokberk Kaya, Naz Bozbeyoglu, Fuat Cem Yagcı, Ihsan Gursel. ‘The use of double-ligand loaded exosomes with cancer antigen as a therapeutic melanoma vaccine’ *Symposium of molecules in immunology*, 18-21 October 2018, Ozdere/Izmir, TÜRKİYE (**Poster Presentation**)
- Ozsurekci Y., Yildirim M., Evcili I., **Yildirim T.**, Ibibik B., Bildik T., Bartan A., Ceyhan M., Gursel M., Gursel I. “Preclinical Immunogenicity of B + W Meningococcal Outer Membrane Vesicles (OMV) Vaccine Adjuvanted with Nucleic Acid-Based TLR Ligands and Comparison with Existing Meningococcal Conjugate- and Polysaccharide Vaccines” 6th European Congress of Immunology, 1-4 September, Virtual, TÜRKİYE. (**Poster Presentation**)
- Bartan A.G., Bildik T., Saraydar B., Evcili I., Ibibik B., Turay N., Bulbul A., Yildirim M., **Yildirim T.**, Ceylan Y., Abras I., Ipekoglu E.M., Baydemir I., Yilmaz N., Aydın Y., Guvencli N., Sanli A., Kayaoglu B., Yilmaz I.C., Güngör B., Gursel M., Gursel I. “Adjuvant Based in vivo Work for VLP Vaccine Technology”, 6th European Congress of Immunology, 1-4 September, Virtual, TÜRKİYE. (**Poster Presentation**)
- Turay N., Yildirim M., **Yildirim T.**, Saraydar B., Bildik T., Evcili I., Bartan A., Ibibik B., Ceylan Y., Abras I., Bulbul A., Yilmaz I., Yilmaz N., Ipekoglu E., Güvencli N., Aydın Y., Baydemir I., Kayaoglu B., Sanli A., Gursel M., Gursel I. “Virus-like particle formation by using four structural proteins of SARS-Cov-2 and investigation of its effectiveness among wild type and prefusion stabilized spike variants”, 6th European Congress of Immunology, 1-4 September, Virtual, TÜRKİYE. (**Poster Presentation**)
- Ipekoglu E., Yildirim M., **Yildirim T.**, Saraydar B., Bildik T., Evcili I., Bartan A., Ibibik B., Ceylan Y., Abras I., Turay N., Bulbul A., Yilmaz I., Yilmaz N., Güvencli N., Aydın Y., Baydemir I., Kayaoglu B., Sanli A., Gursel I., Gursel M. “Ex vivo Th-1 biasing T cell responses in Alum-adsorbed SARS-CoV-2 Inactivated Virus vaccine adjuvanted with K-type CpG ODN”, 6th European Congress of Immunology, 1-4 September, Virtual, TÜRKİYE. (**Poster Presentation**)
- Bulbul A., Yilmaz I.C., Yilmaz N., Turay N., Ipekoglu E.M., Güvencli N., Yildirim M., **Canavar T.**, Evcili I., Bildik T., Bartan A.G., Kasa M.B., Mookala N., Yadav V., Sharma M., Yannabathina T., Jadav V., Gursel M., Gursel I. “Manufacturing of a SARS-CoV-2 Virus-Like Particle Vaccine Adjuvanted with K3 CpG Oligodeoxynucleotide and

Alhydrogel for Phase ½ Clinical Trials”, 6th European Congress of Immunology, 1-4 September, Virtual, TÜRKİYE. **(Poster Presentation)**

- Bildik T., Bartan A.G., Ozsurekci Y., Saraydar B., Evcili I., Ibibik B., Turay N., Bulbul A., Yıldırım M., **Yıldırım T.**, Ceylan Y., Abras I., Gursel I. “Immune Responses of MIS-C and Severe COVID-19 Patients”, 6th European Congress of Immunology, 1-4 September, Virtual, TÜRKİYE. **(Poster Presentation)**
- Saraydar B., Bartan A.G., Bildik T., Evcili I., Turay N., Bulbul A., Yıldırım M., **Yıldırım T.**, Ceylan Y., Ibibik B., Ipekoglu E.M., Baydemir I., Yılmaz N., Yılmaz I.C., Güvencli N., Aydın Y., Gursel I., Gursel M. “Production of Recombinant Proteins Used in ELISA Experiments”, 6th European Congress of Immunology, 1-4 September, Virtual, TÜRKİYE. **(Poster Presentation)**
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