DEVELOPMENT AND PRECLINICAL CHARACTERIZATION OF MENINGOCOCCAL OUTER MEMBRANE VESICLE VACCINE

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March 2024

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

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To my family

ABSTRACT

DEVELOPMENT AND PRECLINICAL CHARACTERIZATION OF MENINGOCOCCAL OUTER MEMBRANE VESICLE VACCINE

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Invasive meningococcal disease (IMD) is caused by *Neisseria meningitidis*, with the main serogroups responsible for the disease being A, B, C, W, X and Y. To date, several vaccines targeting *N.meningitidis* have been developed albeit with a short-lived protection. Given that MenW and MenB are the most common causes of IMD in Europe, Turkey, and Middle East, we aimed to develop an outer membrane vesicle (OMV) based bivalent vaccine as the heterologous antigen source. Herein, we compared the immunogenicity, and breadth of serum bactericidal assays (SBA) based protective coverage of OMV vaccine to X serotype with existing commercial meningococcal conjugate and polysaccharide (PS) vaccines in a murine model. BALB/c mice were immunized with preclinical batches of the W+B OMV vaccine, either adjuvanted with Alum, CpG ODN or their combinations and compared with a MenACYW conjugate vaccine (NimenrixTM, Pfizer) and a MenB OMV-based vaccine (Bexsero®, GSK). The immune responses were assessed through ELISA and SBA. Antibody responses and SBA titers were significantly higher in the W+B OMV vaccine when adjuvanted with Alum or CpG ODN, as compared to the control groups. Moreover, the SBA titers were not only significantly higher than those achieved with available conjugated ACYW vaccines but also on par with the 4CMenB vaccines. In conclusion, the W+B OMV vaccine demonstrated the capacity to elicit robust antibody responses, surpassing or matching the levels induced by licensed meningococcal vaccines. Consequently, the W+B OMV vaccine could potentially serve as a viable alternative or supplement to existing meningococcal vaccines.

Key words: Vaccination; adjuvant; meningitis; outer membrane vesicle; *N. meningitidis; Men B; Men W serotypes*

ÖZET

MENİNGOKOKKAL DIŞ MEMBRAN VEZİKÜL AŞISININ GELİŞTİRİLMESİ VE PREKLİNİK KARAKTERİZASYONU

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İnvazif meningokokal hastalığa (IMD) *Neisseria meningitidis* neden olurken hastalıktan sorumlu ana serogruplar A, B, C, W, X ve Y'dir. Bugüne kadar kısa süreli koruma özelliği de olsa *N.meningitidis'i* hedef alan çeşitli aşılar geliştirilmiştir. Avrupa, Türkiye ve Orta Doğu'da MenW ve MenB'nin IMD'nin en yaygın nedenleri olduğu göz önüne alındığında, heterolog antijen kaynağı olarak dış membran vezikül (OMV) bazlı iki değerlikli bir aşı geliştirmeyi amaçladık. Bu çalışmada bir fare modelinde mevcut ticari meningokokal konjuge ve polisakkarit (PS) aşıları ile geliştirdiğimiz OMV aşısının X serotipine yönelik koruyuculuğunu temel alan serum bakterisidal analizlerin (SBA) immünojenliğini ve kapsamını da karşılaştırdık. BALB/c fareleri, Alum, CpG ODN veya bunların kombinasyonları ile adjuvanlanmış W+B OMV aşısının klinik öncesi serileri ile immünize edildi ve MenACYW konjuge aşısı (NimenrixTM, Pfizer) ve MenB OMV bazlı (Bexsero®, GSK) aşılar ile karşılaştırıldı. Bağışıklık yanıtları ELISA ve SBA yoluyla değerlendirildi. Antikor yanıtları ve SBA titreleri, Alum veya CpG ODN ile adjuvanlandığında W+B OMV aşısında kontrol grupları ile karşılaştırıldığında anlamlı derecede yüksekti. Üstelik SBA titreleri yalnızca mevcut konjuge ACYW aşılarıyla elde edilenlerden önemli ölçüde yüksek olmakla kalmadı, aynı zamanda 4CMenB aşılarıyla da aynı seviyedeydi. Sonuç olarak, W+B OMV aşısı, lisanslı meningokok aşılarının indüklediği seviyeleri aşarak veya bunlarla eşleşerek güçlü antikor yanıtları ortaya çıkarma kapasitesini gösterdi. Özetle, W+B OMV aşısı mevcut meningokok aşılarına poatnsiyel bir alternatif veya varolan aşılara tamamlayıcı olarak kullanılabilir.

Anahtar Kelimeler: Aşılama; adjuvan; menenjit; dış membran vezikülü; *N. meningitidis*; Men B; Men W serotipleri

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ABBREVIATIONS

Invasive meningococcal disease	IMD
Outer membrane vesicle	OMV
Serum bactericidal assay	SBA
Polysaccharide vaccine	PS
Neisseria meningitidis type B	MenB
Neisseria meningitidis type W	MenW
Neisseria meningitidis type X	MenX
Neisseria meningitidis type A	MenA
Neisseria meningitidis type C	MenC
Neisseria meningitidis type Y	MenY
Cytosine-phosphate-guanine	CpG
Deoxyribonucleic acid	DNA
Multilocus sequence type	MLST
Oligodeoxynucleotide	ODN
Antigen presenting cell	APC
Anthrax vaccine adsorbed	AVA
Pathogen-associated molecular patterns	PAMP
Pattern recognition receptors	PRR
Cytotoxic T cells	CTL
Diphteria-tetanus-pertussis	DTP
Wild-type outer membrane vesicle	wtOMV
Haemophilus influenzae type b	Hib
Microorganism-associated molecular pattern	MAMP
Tunable-resistive pulse sensing	TRPS

Brain Heart Infusion broth	BHI
Colony forming unit	CFU
Interleukin	IL
IL-1 receptor associated kinase 4	IRAK4
Nuclear factor (NF)-kB	NFKB
Tumor necrosis receptor-associated factor	TRAF
Myeloid differentiation marker 88	MyD88
Natural killer	NK
Trapped ion mobility spectrometry	TIMS
T helper	Th
Cluster of differentiation	CD
Interleukin	IL
Lipopolysaccharide	LPS
Toll-like receptor 4	TLR4
Extracellular vesicles	EVs
Enzyme-linked Immunosorbent Assay	ELISA
Room temperature	RT
Phosphate-Buffered Saline	PBS
Interferon	IFN
Ultracentrifugation	UC
Scanning electron microscopy	SEM
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis	SDS–PAGE

1.CHAPTER 1

INTRODUCTION

1. 1. *Neisseria meningitidis,* Serotypes and Epidemiology

Invasive meningococcal disease (IMD) is a serious disease caused by *Neisseria meningitidis* which is a gram-negative diplococcus and can rapidly be fatal despite of the treatment [1-3]. In a surveillance study, the average annual incidence of the disease between 1992 and 1996 was 1.1 per 100,000, and this rate remained the same in 2002. The highest age-specific incidence was observed in children under 1 year of age (9.2 per 100,000 cases), while the peak incidence was reported in children aged 4 and 5 months (15.9 per 100,000 cases) [4]. Between 1990 and 2002 in the United States, the mortality rate for meningococcal disease was 0.1 per 100,000, with 58% of these deaths occurring in those younger than 25 years. It has also been reported that mortality in infants is higher than in other age groups [5]. Despite the introduction of effective antibiotics in the treatment, mortality has decreased to 10%, but has not disappeared. In survivors, significant sequalae such as neurological disorders, limb losses and hearing disorders may occur up to 20% [6]. Figure 1.1 shows us some affected cases in our center. Although *N. meningitidis* currently has 13 defined serogroups, the majority of invasive diseases are associated with 6 meningococcal serogroups, namely A, B, C, W, X and Y and the distribution of serogroups that cause IMD in many parts of the world varies both by region and over time [1, 7, 8].

Figure 1.1. Two patients with meningitis caused by Neisseria meningitides. A. Petechial lesions on trunk at the 4th hour after the beginning of the symptoms. B. Disseminated intravascular coagulation (DIC) at the 48th hour despite of the treatment (1A and 1B are belonged from the same patient). 1C and 1D. Extremity amputations of another case.

1.2. Importance of Invasive Meningococcal Diseases in the Worldwide

The first epidemic of meningococcal meningitis was described by Vieusseux in Geneva in 1805 [9]. Disease-causing serogroups can vary between regions and over time [10]. Epidemics of meningococcal meningitis are known in the sub-Saharan meningitis zone in the last century and major epidemics occur every 5-10 years in this region [11]. Meningococcal serogroup A (MenA) is the most dominant serogroup in the sub-Saharan meningitis belt of Africa and continues to be an important factor in Asian countries such as India and China [12, 13]. Meningococcal serogroup C (MenC) and Meningococcal serogroup B (MenB) are a major problem in industrialized countries such as North America, Europe and the Americas, while serogroup W (MenW) causes major epidemics in the Middle East and Africa [14, 15].

In the United States, serogroups B, C, and Y are the most common serogroups that cause meningococcal disease. More than 50% of meningococcal diseases caused by serogroup B are seen in children younger

than one year old. Group Y was reported to be more common in older patients in the 1990s and generally cause pneumonia. Another important change in the epidemiology of meningococcal disease in the United States is the increasing reporting of outbreaks with serogroup C. Apart from that, epidemics related to serogroup C were observed in North America and England, especially in university dormitories and schools, after 1985 [10, 14-17]. About 2 million pilgrims from all over the world travel to Mecca each year, and this single area plays a role as an ideal setting for the transmission of meningococcal carriage. Because of these returning pilgrims, outbreaks of meningococcal disease have spread worldwide [18]. A major epidemic of serogroup A meningococcal disease occurred in the 1980s. The disease, which initially affected Muslim pilgrims, later spread to other communities in the Middle East and African countries. It continued to be an important agent in parts of the Asian continent, including India [11,12]. After this epidemic, pilgrims were vaccinated with bivalent (A and C) meningococcal polysaccharide vaccine before entering Saudi Arabia. Later, meningococcal diseases caused by serogroup W were seen in pilgrims. Serogroup W meningococcal infections, which were first known as the causative agent of sporadic cases, first appeared in the form of epidemics during pilgrimage in Saudi Arabia in 2000 and 2001. A clonal (ET-37ST-11 clonal complex) outbreak with serogroup W occurred among pilgrims returning to Europe from Saudi Arabia in 2000. Then, in 2001, the epidemic with group W occurred in Nigeria and Burkino Faso [15]. Later, serogroup W became the dominant strain in the etiology of IMD, especially in some countries in Europe, South America and Australia, as well as in the African meningitis belt [19]. The localized increase in MenW strains in South America was first seen in Brazil and then this increase in incidence included many countries such as Chile, Uruguay and Paraguay [20,21]. In Figure1.2 we see the distribution of meningococcal serogroups in the world. Shifts from one serogroup to another within a community or country have been reported. Although the introduction of vaccines has changed this serogroup distribution, the underlying mechanisms of this pattern of change in disease-causing meningococcal serogroups are still unknown [10].

Figure 1.2. Distribution of meningococcal serogroups in the world [Adapted from Ref 10]

1.3. Importance of Invasive Meningococcal Diseases in Turkey

Every year more than 100,000 pilgrims and Ummah go to Saudi Arabia from Turkey. Although epidemiological studies are limited, cases of invasive meningococcal disease have started to be reported in Turkey after the 2000s [22]. In particular, this rapid increase was attributed to Hajj and Umrah visits, and after 2002, polysaccharide vaccines consisting of ACWY started to be given to all pilgrims. However, despite these vaccines, which had no effect on nasopharyngeal carriage, W strains continued to be seen in our country. In an epidemiological study conducted from our center in Turkey, *N.meningitidis* was reported as the most common agent in polymerase chain reaction (PCR) positive bacterial meningitis cases with a rate of 56.5% between 2005 and 2006. Among the serogroups, W was the most dominant serogroup with a rate of 42.7%. In our ongoing studies, *N.meningitidis* was the most common agent in 2007-2008, while serogroup B was the most dominant serogroup with 35.1% in these years. When the whole study period was evaluated, it was seen that the most common bacterial meningitis agent was N.meningitidis and the most common serogroups were W and B, although it changed over the years,

according to the data of 2005-2012. In addition, while the annual incidence of invasive meningococcal disease was 1.9/100000 in 2005-2006, this rate decreased to 0.9/100000 in 2014 [23,24].

In another study we conducted in our unit, it was determined that the most dominant serogroup was serogroup B in recent years. Especially after 2014, the rapidly decreasing MenW strains were replaced by MenB strains (Figure 1.3). With these epidemiological follow-ups carried out as in the whole world, both the region-by-region distribution of the causative serogroups can be monitored and the change can be followed over the years [25].

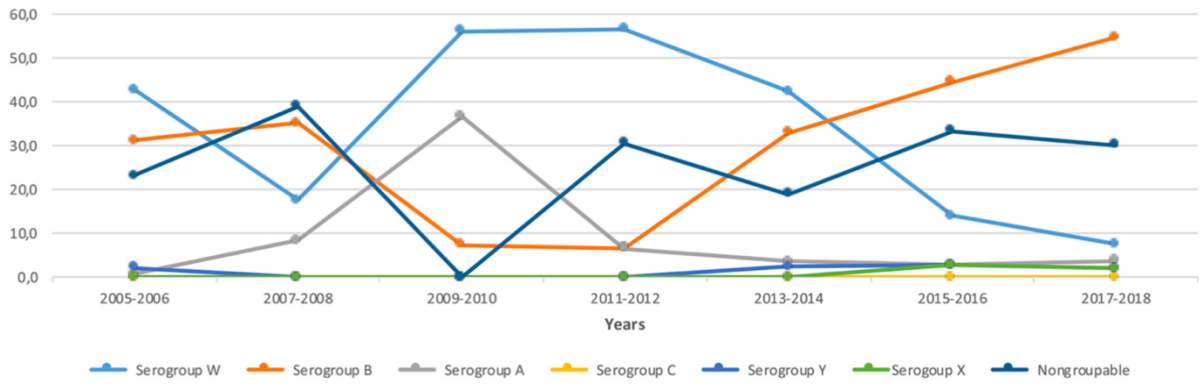


Figure 1.3. The serotype distribution of *N.meningitidis* over the years. An increase in MenB serogroups is observed between 2015-2018 in Turkey [25].

1.4. Prevention and Vaccination from Invasive Meningococcal Diseases

One of the most important ways to prevent invasive meningococcal diseases is vaccination. Polysaccharide capsule-based meningococcal vaccines for *N.meningitidis* were developed in 1968, and the first modern polysaccharide vaccine was used in the US military. A tetravalent vaccine including A, C, Y, and W serogroups was licensed in 1978 in US [26,27]. Bivalent (A and C), trivalent (A, C and W) or tetravalent (A, C, Y and W) vaccines have been used for years, but the effectiveness of these vaccines has been found to be low, especially in childhood. Therefore, it is not recommended for children under 2 years

of age. For this reason, it has been shown that in conjugated vaccines made by conjugation of bacterial polysaccharide to a carrier protein, T-cell dependent immunity is stimulated, increasing immunogenicity in infants, prolonging the protection period and decreasing meningococcal nasopharyngeal carriage [28]. Following these developments, protein-conjugated meningococcal vaccines were introduced to increase the protection period of the vaccine and to protect young children. Meningococcal ACWY protein-conjugated vaccine was licensed in 2005 in USA [26]. Conjugated vaccines consisting of monovalent serogroups A and C, bivalent serogroups A and C, and tetravalent A, C, Y, and W have been reported to be highly immunogenic (>90%) [28].

After the introduction of monovalent or tetravalent conjugate polysaccharide vaccines, MenB has become the prominent cause of IMD in Europe and the USA [29,30]. However, development of vaccines to protect against MenB has been a very complex problem due to the similarity of the poly-a-2,8-sialic acid that is a capsular polysaccharide to the polysaccharide component of human neural glycoproteins [31,32]. Vaccine development efforts have focused on proteins include factor H binding protein (FHbp), neisserial adhesin A (NadA), and neisserial heparin binding antigen (NhbA) and they have been formulated for MenB vaccines. As an alternative way, outer membrane vesicle-based vaccines have been used to combat serogroup B epidemics. These vaccines directly induce antibodies against the multivariate surface-exposed protein por A (PorA). These outer membrane vesicle-based vaccines generally provide effective protection against serogroup B strains carrying PorA structures homologous to the vaccine [33,34]. Meningococcal fHBP, also known as lipoprotein 2086 or LP2086, is a surface lipoprotein that is suppressed by complement-mediated bacterial lysis by binding to human factor H. The gene encoding LP2086 has been identified in all serogroup B strains and LP2086 has been shown to elicit bactericidal antibodies against a wide range of serogroup B strains. Epidemiological studies show that this antigen is divided into 2 subfamilies (A and B). A vaccine containing the A and B subfamilies of the LP2086 protein is predicted to provide broad protection against diseases caused by serogroup B [35, 36]. MenB-FHbp (Trumenba, Pfizer) [37] and 4CMenB in combination with outer membrane vesicles (OMVs) (Bexero, GSK) [38] are currently approved for use in several countries worldwide [39].

1.5 What is a Bacterial Outer Membrane Vesicle (OMV)?

Membrane vesicles derived from bacteria as a source of PRR ligands.

1.5.1. Membrane Vesicle Formation

It is known that all eukaryotes and prokaryotes produce vesicles and release them into the environment. Such vesicles are called exosomes or microparticles in eukaryotes and outer membrane vesicle in prokaryotes. Outer membrane vesicles (OMV) produced by pathogenic and gram-negative bacteria have been demonstrated decades ago [40,41]. The outer membrane vesicles are double-layer lipid membrane vesicles and their sizes range from 20 to 250 nm. It is secreted at all stages of growth. Proteomic and biochemical analyzes show that membrane vesicles from gram-negative bacteria are composed of outer membrane and periplasmic components and contain lipopolysaccharide (LPS), DNA, RNA, periplasmic and membrane-bound proteins, enzymes, toxins, and peptidoglycan. Molecules such as LPS, DNA and RNA are considered as microorganism-associated molecular pattern (MAMP). OMVs contain multiple PRR ligands and initiate proinflammatory immune response by stimulating cytokine, chemokine and antimicrobial peptide production [42].

Microvesicle secretion in Gram-positive bacteria has only recently been described. Microvesicles obtained from Staphylococcus aureus were the first microvesicles obtained from gram-positives in the literature. Since gram-positive bacteria do not have an outer membrane, microvesicles from gram-positive bacteria contain cytoplasmic membrane and cytoplasmic components [43,44].

1.5.2. Membrane Vesicle Function

Bacterial microvesicles have many different functions, such as molecular transport of toxins and virulence factors, biofilm formation, and modulation of immune and stress response. Current information on microvesicles from bacteria is generally based on pathogenic gram-negative bacteria. Microvesicles also play an important role in quorum sensing and intraspecies cell-cell communication [45]. Microvesicles

have been shown to transfer resistance patterns for antibiotic resistance between the same or different species [46]. Microvesicles have also been reported to modify polysaccharide secretion and virulence factor secretion into the environment. Bacillus anthracis, one of the Gram-negative bacteria, is known to produce microvesicles containing anthrax toxin [47]. In the presence of environmental stress, microvesicle production is of vital importance for the life of the bacteria, and they also have an important place in biofilm formation to ensure the continuation of the bacteria's viability [48].

1.6. OMV-based Vaccines

It has been shown that a significant proportion of serum antibodies target non-capsular antigens with bactericidal activity against meningococcus in normal subjects. Therefore, meningococcal outer membrane proteins are alternative vaccine antigen candidates to capsular polysaccharides [49]. Meningococcal outer membrane proteins have been used in more than 80 million doses for over 20 years, either as carrier protein in a *Haemophilus influenzae* type b (Hib) polysaccharide conjugate vaccine or as vesicle vaccine formulations against meningococcal disease. Traditional wild-type outer membrane vesicle (wtOMV) vaccines are the only formulations that show activity against serogroup B meningococcal disease. Outer membrane proteins induce protective immunity in clonal outbreaks of meningococcal disease, as previously shown in MenB OMV vaccines [50]. The efficacy, tolerability and safety of OMV vaccines have been proven in many studies conducted in different countries. OMV vaccines have been shown to protect against MenB epidemics by stimulating protective immunity, as seen in New Zealand and France, where most cases are caused by the homologous strain [50-53]. There are many ongoing OMV-based vaccine studies in countries such as sub-Saharan Africa where current vaccines cannot be added to the national immunization schedule for economic reasons. In some of these studies, it has been shown that OMV-based vaccine candidates extracted from Men A and Men W, when formulated with aluminum hydroxide adjuvant, are highly immunogenic in mice and induce bactericidal antibodies against both serogroups [49, 54]. A preliminary result from a Phase 1 study in Cuba reported that the A+W OMV vaccine is safe and highly immunogenic [50].

1.7. Adjuvants

Aluminum salts have been used as adjuvants since 1926. Since then, it continues to be a licensed vaccine adjuvant in the United States. Aluminum is a widely used adjuvant and childhood vaccines containing aluminum-based adjuvant are DTP (diphtheria-tetanus-pertussis), Pediatrix (DTP-HBV-Polio combination), Pentacel (DTP-H.influenza type B-Polio combination), Hepatitis A, HBV, HPV, and HIB [55]. Aluminum hydroxide has also been an adjuvant option for MenB OMV vaccines in Norway, New Zealand and Cuba [56, 57]. However, aluminum-containing adjuvants have limitations such as local reactions, increased IgE-mediated response, ineffectiveness of some antigens, and inability to enhance cell-mediated immune response, especially cytotoxic T cell response [55]. Therefore, there are ongoing studies with many new vaccine adjuvants. Therefore, we preferred to use a new adjuvant in our vaccine formulation.

The innate immune system responds to the presence of pathogens by detecting "pathogen-associated molecular patterns (PAMP)" released from infectious microorganisms. Recognition of PAMPs is provided by pattern recognition receptors (PRR), such as Toll-like receptors (TLR), Nod-like receptors, and RIG-I-like receptors. TLRs are the most widely and most active pattern recognition receptors. The innate immune response mediated by TLR activation is primarily characterized by the production of proinflammatory cytokines, chemokines, type 1 interferon (IFN) and antimicrobial peptides. This recognition initiates a strong innate immune response, allowing the host to control the spread of the initial infection, followed by an adaptive immune response. Innate immunity is the first step in active defense against infectious pathogens. Then, adaptive immunity, characterized by proliferation of antigen-specific T and B cells, comes into play and causes the production of high-affinity antibodies and cytotoxic T cells (CTL), which provide scavenging immunity and protective long-term memory against subsequent infections [58].

Bacterial deoxyribonucleic acid (DNA) is an important example of innate immune system activating PAMPs. One of these pathogenic nucleic acid types, which constitute a critical group of PAMPs, is

unmethylated cytosine-phosphate-guanine (CpG) motifs, which are found in high frequency in bacterial DNA. Unmethylated CpG DNA is recognized by TLR-9 found on B lymphocytes, dendritic cells (DC) and macrophages. Unmethylated CpG motifs containing synthetic oligodeoxynucleotide (ODN) double the ability of bacterial DNA to stimulate the TLR-9-mediated immune system [59]. In other words, unmethylated CpG motifs (CpG ODN) containing single-chain synthetic ODN mimic the immune stimulatory effect of bacterial DNA [60]. Multimerized CpG ODN is retained in the early endosome, crosslinking with TLR9, causing IFN-alpha secretion from pDCs and triggering the MyD88/IRF-7-mediated intracellular signaling pathway [61].

Four classes of CpG ODN have been defined based on their structural and biological properties. A or D-type CpG, B or K-type CpG, C-type CpG and P-type CpG, in general, K-type ODNs stimulate the differentiation of pDCs and cause TNF-alpha secretion; conversely, D-, C-, and P-type ODNs are capable of stimulating different amounts of IFN-alpha secretion from pDCs. D-type ODN interacts with the MyD88/IRF-7 complex, triggering the signaling pathway to produce IFN-alpha. CpG DNA directly activates pDC and B cells, stimulating both innate and adaptive immune responses. pDC also responds to CpG DNA with the production of Type 1 IFN, thus preventing the spread of many viral and bacterial infections. Because of all these effects, CpG ODNs are used as vaccine adjuvants [58, 60].

Direct conjugation of the antigen with CpG ODN or incorporation into lipid emulsions or vesicles elicits a 10-1000-fold greater IgG response than that induced by antigen alone [62, 63].

ODN and antigen-containing conjugates are preferentially uptake by professional antigen presenting cells (APC), resulting in a significantly higher CTL response. In addition, the duration of the immune response can be improved with CpG-based adjuvants. For example, CpG ODNs dramatically extended the protection induced by the licensed human anthrax vaccine, Anthrax Vaccine Adsorbed (AVA). Moreover, protective antibody titers persisted for more than one year compared to AVA alone [64].

CpG-based adjuvants also affect the cellular immune response. In one study, it was reported that the number and lifespan of CD8+ T cells was significantly increased by administration of CpG ODN two days before peptide vaccination [65]. More than 600 preclinical studies have been published examining CpG-

adjuvanted vaccine immunity. Studies have been carried out with vaccines against a wide range of pathogens such as anthrax, leishmania, influenza and measles viruses, hepatitis B, and it has been shown that humoral immune response is stimulated. This makes CpG ODN-based adjuvants more advantageous [58]. Also, Heplisav-B (HepB-CpG), a vaccine with CpG adjuvant against hepatitis B, was approved by the ACIP in February 2018 [66].

Enhancement of an effective vaccine-mediated immune response is closely related to the ability of vaccine adjuvants to enhance and direct an adaptive immune response against antigens. Type 1 IFN plays an important role in regulating innate and adaptive immune responses by participating in the maturation and trafficking of DCs, forming effector and memory T cell responses, and stimulating humoral immunity. Therefore, the development of Type 1 IFN stimulants as vaccine adjuvants is becoming increasingly important in clinical practice. Type 1 IFN-inducing agents elicit potent effector/memory T cell responses and humoral immunity when used as vaccine adjuvants [60]. It was mentioned above that aluminum is used as an adjuvant in meningococcal vaccines. However, there are some publications on the weak effect of the use of aluminum as an adjuvant on immunity in meningococcal vaccines. For example, in a clinical study with the Norwegian MenB OMV vaccine, it was reported that the relatively small difference in immunogenicity between formulations with and without aluminum supports the minimal additive effect of aluminum immunogenicity in humans [67]. However, CpG ODN containing synthetic ODN cause Type 1 IFN production via TLR9-MyD88-IRF7-mediated signaling pathway [60]. Therefore, we planned to use a CpG-based adjuvant for the vaccine formulation we designed in our study and to increase the effectiveness of the vaccine.

1.8. Aims and Outline of the Study

Due to the weak immunogenicity of *N. meningitidis* B serotype, it is a very complex task to develop vaccines and to prepare formulations together with other serotypes to provide protection. There is a group

of OMV and protein-based vaccines developed and patented against serogroup B. One of the rare vaccines used with other serogroups is VA-MENGOC-BC ® (Finlay Institute), and the vaccine consists of capsular polysaccharide OMVs of meningococcal serogroups B and C. The vaccine was used successfully in a meningococcal epidemic in Cuba, and even the vaccine was considered "tailor-made" because the serogroup contained in the vaccine was the strain that was the causative agent of most cases in the country. Different vaccination phases have been implemented in the country, and the vaccine was last included in the national vaccination calendar for children under one year old. It has been shown that the average efficacy of the vaccine, which was started to be administered in Cuba in 1991 as a two-dose scheme (3rd month and 5th month), reaches 84% in children under one year old and 93% in pre-school period. The vaccine has been used with success in many other countries such as Brazil, Uruguay and Colombia [68].

The epidemiology of meningococcal disease varies greatly from country to country and from continent to continent. The epidemiology of invasive meningococcal disease in our country is quite unique and quite different when compared to the world [69], as seen in Figure 1.8.

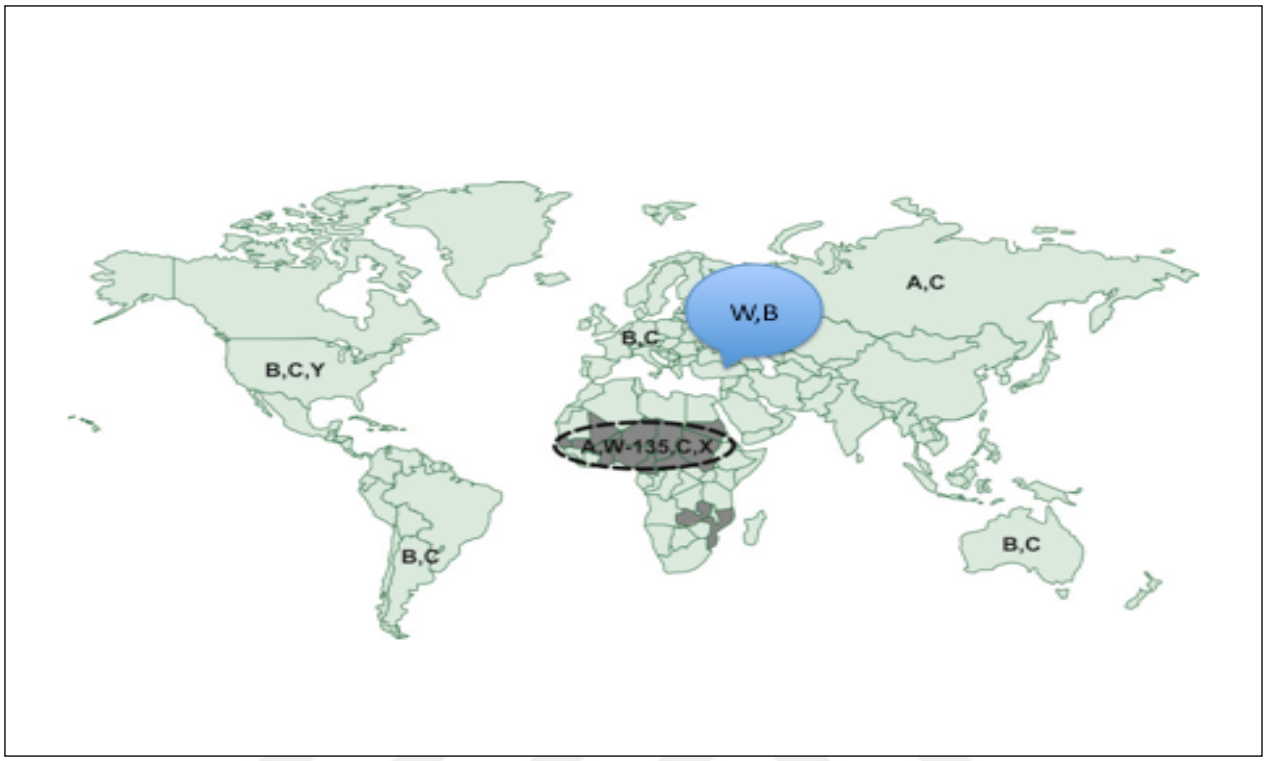


Figure 1.4. Situation in the world and meningococcal epidemiology in Turkey [Adapted from Ref 69 and revised].

Therefore, although not for every disease, it is clear that countries' needs for truly effective vaccines for IMD diseases are different and must be country-specific. With this study, it was aimed to develop a vaccine that is easy, inexpensive, easily implantable in the infrastructure of countries and can be used together with serotype B. Vaccines targeting ACWY and B have not been included in the same formulation up to date because of the poor immunogenic property of MenB. The most common cause of IMD in Turkey are MenW and MenB, and since there is not a single vaccine containing these two serogroups in the same formulation, a child should receive repeated doses of vaccines separately from both vaccines according to the childhood dosage schedule with a high cost. In this study, we prepared OMV-based vaccine from representative Turkish MenB and MenW strains adjuvanted with CpG and/or AlOH and compared the immunogenicity of this vaccine with available vaccines in mice.

2. CHAPTER 2

MATERIALS AND METHODS

2.1. Vaccine strains

The *Neisseria meningitidis* isolates used for the preparation of OMVs were representative patient strains from Turkey: the serogroup W (ST-11), serogroup B (ST-32), and serogroup X (ST-22) (Table 2.1.).

Table 2.1. Characteristics of Neisseria meningitidis vaccine strains used in this study. Results are generated from multilocus sequence type (MLST)

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-11 complex
53401	54	Turkey/2016	X	7-1	1	F4-1	5799	ST-22 complex

2.2. Outer Membrane Vesicles Isolation from *Neisseria meningitidis*

The *Neisseria meningitidis* strains used for preparation of W+B OMV vaccine were the Men W (ST-11) and Men B (ST-32) from Türkiye. Men X (ST-22) was also from Türkiye. The vaccine strains were cultured in Muller-Hilton medium in a shaking incubator (incubated 36 hr until 1.3-1.4 OD was achieved [stationary phase] at 37°C with 5% CO_2). Samples were then centrifugated at 10,000 Xg for 30 minutes and cell-free supernatants were collected. To eliminate the possibility of residual bacterial contamination, supernatants were filtered through a 0.22 μm filter (Corning, USA). Filtered supernatants were then centrifugated (x ultracentrifuge) at 100,000g for 90 minutes, followed by an additional washing with PBS at 100,000g for 90 minutes. This solution constituted secreted OMVs. Finally, the membrane vesicle

pellets were resuspended in PBS (about 300-600 µl) and stored at +4°C and -80°C until further use. Membrane vesicle isolation protocol is shown in Figure 2.1.

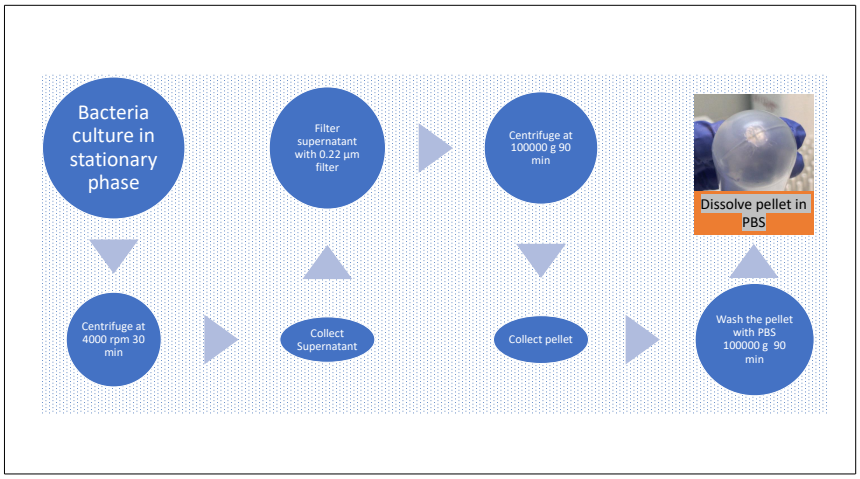


Figure 2.1. Membrane vesicle isolation protocol

To eliminate host cell-derived nucleic acids, the harvest was treated with 200 U/ml of Denarase (c-LEcta, Germany) at +4°C overnight. This solution, containing OMVs, was concentrated in PBS using an Amicon 100 kDa filter (Vivaspin® 20, Sartorius, Germany) and treated with 1% sodium deoxycholate at 37°C for 30 minutes. The solution underwent diafiltration using PBS to remove the detergent. OMVs were then purified on a Hi-Screen Capto Core 400 column (Cytiva, Sweden) using the ÄKTA-GO fast protein liquid chromatography system (Cytiva).

2.3. Protein Quantification

Protein content was quantified using a BCA protein assay kit (ThermoFischer Scientific, USA) following the manufacturer's instructions.

2.3.1. Quantification of Protein Concentration by BCA Assay

Concentration of OMVs were determined by Pierce™ BCA protein assay kit (ThermoFischer Scientific, USA). 25 µl of samples (OMVs 5X diluted in 1XPBS) and 25 µl of 2-fold serially diluted BSA standards ranging from 2000 µg/ml to 31.25 µg/ml, were transferred onto 96-well flat bottom plates. 200 µl of working reagent, which was prepared by 1 volume of Solution A and 50 volumes of Solution B, was mixed with OMVs and standards. After incubation at 37°C for 30 minutes, OD values at 562 nm were measured by microplate reader (Synergy HT). By using linear standard curve which was constructed by BSA standards, concentration of OMVs were calculated.

2.4. Outer Membrane Vesicles Characterization

2.4.1. Characterization of OMVs by Atomic force microscopy (AFM) and Scanning Electron Microscopy (SEM)

Atomic force microscopy (AFM) using XE-100E model AFM (PSIA with XEI 1.6 software incorporated) was performed to characterize the shape and size of the extracellular vesicles. Purified OMVs were diluted 1:100 in sterile PBS (Life Technologies) and adsorbed onto freshly cleaved mica sheets. The adsorbed samples dried at ambient temperature for 30 min before analysis. Micrometer-scale Imaging was obtained in non-contact dynamic mode with a 0.73– 0.79 Hz scanning rate (NanoMagnetics Instruments, USA) according to manufacturer's instructions. OMV samples for AFM studies were prepared by dropping 10µl of solution containing 1µg exosomes onto mica slides and either 5x5 µM or 1x1 µM and in some experiments 0.5x0.5 µM areas were scanned. All data analyzed using an NMI ImageAnalyzer.

Additionally, a 10 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 before imaging on an environmental scanning electron microscopy (SEM) (Zeiss Sigma500 FESEM, Germany).

2.4.2. 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 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.

2.5. Production of the W +B OMV vaccine for the first experiments

The W and B OMVs were prepared separately and adjuvanted with Alum (20 µg per mouse, 2% Alhydrogel®), or K3-CpG ODN (10 µg per mouse). The OMVs (OMV W and/or OMV B) adsorbed to the adjuvant Alum at a protein/adjuvant ratio of 1:4 and to the adjuvant CpG at a protein/adjuvant ratio of 1:10 (for low dose groups)/1:2 (for high dose groups) and two adsorbed OMVs were combined 1:1 in each mixt vaccine. Each planned vaccine dose prepared according the group label seen in Table 2.2.

Table 2.2. Overview of the immunization groups (First animal experiment)

Group title	Mouse strain	No of mice	Vaccine
Group 1	BALB/c	5	Control (PBS)
Group 2	BALB/c	5	W OMV (5 µg)
Group 3	BALB/c	5	B OMV (5 µg)
Group 4	BALB/c	5	W OMV (5 µg) + CpG (10 µg)
Group 5	BALB/c	5	B OMV (5 µg) + CpG (10 µg)
Group 6	BALB/c	5	W OMV (5 µg) + AlOH (20 µg)
Group 7	BALB/c	5	B OMV (5 µg) + AlOH (20 µg)
Group 8	BALB/c	5	W OMV (2.5 µg) + B OMV (2.5 µg)
Group 9	BALB/c	5	W OMV (2.5 µg) + B OMV (2.5 µg) + CpG (10 µg)
Group 10	BALB/c	5	W OMV (2.5 µg) + B OMV (2.5 µg) + AlOH (20 µg)
Group 11*	BALB/c	5	W OMV (OMV 1 µg + CpG 10 µg) (Low Dose)
Group 12*	BALB/c	5	W OMV (OMV 5 µg + CpG 10 µg) (High Dose)
Group 13*	BALB/c	5	B OMV (OMV 1 µg + CpG 10 µg) (Low Dose)
Group 14*	BALB/c	5	B OMV (OMV 5 µg + CpG 10 µg) (High Dose)
Group 15*	BALB/c	5	W OMV (OMV 0.5 µg + CpG 5 µg) + B OMV (OMV 0.5 µg + CpG 5 µg) (Low Dose)
Group 16*	BALB/c	5	W OMV (OMV 2.5 µg + CpG 5 µg) + B OMV (OMV 2.5 µg + CpG 5 µg) (High Dose)
Group 17	BALB/c	5	Control (Available vaccine-Bexero)
Group 18	BALB/c	5	Control (Available vaccine-Nimenrix)

* Dehydration rehydration technology was applied.

2.6. Dehydration rehydration technology for ligand loading within OMV

OMVs and ligands were mixed at 1:2 or 1:10 ratio according to the low dose groups vs high dose groups, respectively. After mixing OMVs and ligands, the solution was snap-frozen in liquid nitrogen and then lyophilized overnight using VirTis BenchTop K Freeze Dryer (SP Industries, New York, USA). At this step, OMV/ligand powder is obtained and there is no OMV loaded with ligands. Ligand encapsulation within the OMVs was achieved during controlled-rehydration step. Nuclease free ddH_2O (1:10 ratio of original solution volume) was added onto dehydrated OMV/ligand powder and vigorously vortexed for 15s every 3min for a duration of 30 min at RT. At the end of 30min, PBS (1:10 original OMV/ODN solution volume) was added and gently vortexed 15s every 3min for 10 min in order to regain the buoyancy of the solution. Loaded OMVs were diluted to desired ligand concentration by adding required volume of 1XPBS. OMV formulations were stored at 4°C until use.

2.6.1. Characterization of the W and B OMVs applied with dehydration rehydration technology.

Characterization of the W and B OMVs after dehydration rehydration technology were also performed via TRPS.

2.7. Comparator vaccines

The comparator vaccines were the ACYW conjugate vaccine Nimenrix™ (Pfizer) and Bexsero (GlaxoSmithKline), all licensed as one human dose of 0.5 mL. Nimenrix™ contains 10 ug/mL of each of the polysaccharide A, C, W and Y, conjugated to tetanus toxoid and Bexsero contains 50 ug/mL OMV, which is including PorA P1.4 recovered group B NZ98/254 and 100 ug/mL of each protein 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 two-week interval. The dose for NimenrixTM was 8 µg/0.2 ml, while for Bexsero, it was 5 µg/0.1 ml.

2.8. Animal Studies

2.8.1. Maintenance of Animals

Animal studies were conducted at Bilkent University, Animal Housing Facility of the Department of Molecular Biology and Genetics. In vivo vaccination studies were conducted using BALB/c mice. Animal housing conditions were regulated using 12 hours dark/light cycles with steady temperatures (22°C ± 2) and ad libitum food-water sources. The protocol was approved by the Ethical Committee of the Bilkent University (Protocol number: 2019/27).

2.8.2. Immunization of mice

2.8.2.1. First Animal Experiment

Groups of Balb/c mice were immunized with OMVs or comparator vaccines according to the groups listed in Table 2.2 on day 0 and day 15 and bled on day 14 and day 35. 6-8 weeks old, female mice were immunized subcutaneously. Mice immunized with PBS only (n=5) were used as controls. Same dosing and time schedule were used for the study in each vaccination group.

2.8.2.2. Second Animal Experiment

Best groups were evaluated and chosen for the second experiment. Additionally, OMV doses were increased up to 10 µg and CpG concentration was increased up to 30 µg in study groups as compared to previous experiment. MenB and MenW OMVs were combined (5 µg each per mouse) and formulated with either Alum (20 µg per mouse, 2% Alhydrogel®), or K3-CpG ODN (30 µg per mouse). Groups of Balb/c mice were immunized with OMVs or comparator vaccines according to the groups listed in Table 2.3 on day 0 and day 15 and bled on day 14, day 35 and day 75. 6-8 weeks old, female mice were immunized subcutaneously. Mice immunized with PBS only (n=14) were used as controls. Same dosing and time schedule were used for the study in each vaccination group.

Table 2.3 Overview of the immunization groups (Second animal experiment)

Group title	Mouse strain	No of mice	Vaccine
Group 1	BALB/c	14	Control (PBS)
Group 2	BALB/c	14	W OMV (5 µg) + B OMV (5 µg)
Group 3	BALB/c	14	W OMV (5 µg) + B OMV (5 µg) + AlOH (20 µg)
Group 4	BALB/c	14	W OMV (5 µg) + B OMV (5 µg) + CpG-K3 (30 µg)
Group 5	BALB/c	14	W OMV [OMV 5 µg + CpG-K3 15 µg] + B OMV [OMV 5 µg + CpG-K3 15 µg]
Group 6	BALB/c	14	W OMV (5 µg) + B OMV (5 µg) [CpG-K3 30 µg]
Group 7	BALB/c	14	Control (Bexero) (5 µg)(25 µg/0.5 mL OMW B) (100 µg/each mouse)
Group 8	BALB/c	14	Control (Nimenrix) (2 µg)(5 µg/0.5 mL W) (200 µg/each mouse)

2.8.2.3. Third Animal Experiment

According the results of the previous animal studies, a third animal experiment was performed. MenB and MenW OMVs were combined (2.5 µg each per mouse) and formulated with either Alum (20 µg per mouse, 2% Alhydrogel®), or K3-CpG ODN (30 µg per mouse), or a combination of both. Groups of Balb/c mice were immunized with OMVs according to the groups listed in Table 2.4 on day 0 and day 15 and bled on day 14, day 35 and day 75. 6-8 weeks old, female mice were immunized subcutaneously. Mice immunized with PBS only (n=16) were used as controls. Same dosing and time schedule were used for the study in each vaccination group. OMV concentration was adjusted as 5 µg in each group and a new group in which both CpG and Alum were used in the same vaccine formulation was designed in this experiment.

Table 2.4. Overview of the immunization groups (Third animal experiment)

Group title	Mouse strain	No of mice	Vaccine
Group 1	BALB/c	16	Control (PBS)
Group 2	BALB/c	16	W OMV (2.5 µg) + B OMV (2.5 µg) + AlOH (20 µg)
Group 3	BALB/c	16	W OMV (2.5 µg) + B OMV (2.5 µg) + CpG-K3 (30 µg)
Group 4	BALB/c	16	W OMV (2.5 µg) + B OMV (2.5 µg) + AlOH (20 µg) + CpG-K3 (30 µg)

2.9. IgG ELISA

Immulon 2HB ELISA plates (SPL Life Sciences, Korea) were coated with 50 µl/well of isolated OMVs (0.5 µg/ml) in PBS at +4°C overnight. The coated plates were then blocked with 200 µl/well of 5% BSA-PBS-T solution and incubated for 4 hours at room temperature. Afterward, the plates were washed three times with PBS-Tween (0.05%, PBS-T) and finally washed two times with dH2O. Mice sera diluted at 1:50 were serially diluted four-fold with 5% BSA in PBS-Tween (0.05%, PBS-T) into the wells. Next, 50 µl of the serially diluted sera was transferred into ELISA plates and incubated at 4°C overnight. After subsequent washing, 50 µl of ALP-conjugated goat anti-mouse IgG, anti-mouse IgG1, or anti-mouse IgG2a (all IgGs were from Southern Biotech, USA) antibodies were added at a 1:1000 dilution and incubated for 2 hours at room temperature. Following the last washing step, a PNPP (Thermo Scientific, USA.) substrate solution was prepared according to the manufacturer's instructions and added (50 µl/well) for the development of the plates. Consecutive optical density values at 405nm were measured using a microwell plate reader (SpectraMax Molecular Devices, USA) for 5 hours. The final log10 titers were calculated in GraphPad Prism 9 software.

2.10. Serum Bactericidal Activity (SBA) Assay

Neisseria meningitidis MenB, MenW, and MenX target strains were incubated with heat-inactivated animal sera collected 2 months after the booster injection to assess the complement-mediated killing activity of vaccine-induced antibodies. Pooled baby rabbit serum (3-4 weeks sterile, Pel-Freez) was used as the complement source, and sera from non-immunized mice served as negative controls. Bacteria were sub-cultured on blood agar plates (BDTM Columbia agar with 5% sheep blood) and incubated for 18 hours at 37°C with 5% CO_2. Colonies were then inoculated in 4 ml of Brain Heart Infusion broth (BHI) and incubated on a shaker at 37°C, 210 rpm until reaching an OD600 of 0.3. A working dilution of bacteria (1:625 for MenB and MenX; 1:1200 for MenW) was prepared in PBS containing 1% (w/v) BSA and 0.1% (w/v) glucose (Assay buffer). All sera tested in the Serum Bactericidal Assay (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 a 1/72 dilution for MenB; 1/8 dilution for MenW and MenX) to 40 µl of assay buffer previously added to the wells. This mixture was then transferred to 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 MenX), and 10 µl of active baby rabbit serum (BRS) as an external source of complement. The plate was incubated for 1 hour at 37°C, 180 rpm on a rotating shaker. Subsequently, 35 µl of the reaction mixtures were allowed to flow down a blood agar plate using the tilt method. The agar plates were incubated overnight at 37°C with 5% CO_2. 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 ≥ 90% reduction (≥ 50% reduction for the MenX strain) in CFUs after 1 hour compared to the average for controls.

2.11. SDS-PAGE analysis

Protein electrophoresis was conducted using 12% SDS-PAGE. Molecular weight markers and the OMV preparations of *N. meningitidis* serogroup B and W were dissolved in Laemmli buffer containing bromophenol blue. The samples were then heated at 95°C for 5 minutes, and the separation was carried out in a Mini–Protean II 2-D Cell (BioRad, USA) using Mini-PROTEAN TGX stain-free gel. Subsequently, the protein bands were stained with Coomassie brilliant blue and visualized using a ChemiDoc imager (Bio-Rad).

2.12. Sample Preparation for Proteomics Experiments

Fifty micrograms of protein from each sample were transferred to microtubes. The proteins were reduced with dithiothreitol (DTT) to achieve a final concentration of 5 mM at room temperature and then alkylated with iodoacetamide (IAA) to reach a final concentration of 50 mM, all while in the dark for 1 hour.

Additional DTT was added to reach a final concentration of 10 mM. Proteins were precipitated using the methanol/chloroform precipitation protocol and dissolved in a solution containing 8 M urea and 50 mM Tris buffer at pH 8.5. The urea concentration was then diluted to 1M with 50 mM Tris buffer at pH 8.5. Trypsin was dissolved in 50 mM Tris buffer at pH 8.5 and added to the protein solution at a ratio of 1:100 (w/w, trypsin/protein). The mixture was incubated overnight. Trifluoroacetic acid was added to the incubated solution to stop the enzymatic digestion, setting the final concentration of trifluoroacetic concentration to 0.5% (v/v). The samples were cleaned using Sep-Pak (Waters) according to the manufacturer's protocol. Subsequently, the samples were dried using a Speed-Vac centrifugal evaporator and then dissolved in MilliQ water (18.2 MW.cm) to achieve a protein concentration of 0.5 µg/µl.

2.12.1. Proteomics Analysis

To identify the protein entities shared between different sample groups, a Venn diagram was made using the R package “venn” [70]. The significance of the overlap was computed using a hypergeometric test. The EnrichR v01.07.2022 [71], a multi-domain gene list enrichment analysis platform (https://amp.pharm.mssm.edu/Enrichr/), with the PANTHER Pathways library was used for functional annotation within the scope of this study.

2.12.2. Mass Spectrometry

LC-MS/MS analyses were conducted using a trapped ion mobility spectrometry (TIMS) – time-of-flight mass spectrometer equipped with a CaptiveSpray nanoBooster source (Bruker Daltonics, Bremen, Germany) coupled to an Ultimate 3000 RSLCnano system (Dionex, Thermo Scientific, USA). The mass spectrometer and the LC were controlled by Hystar 4.1 (Bruker Daltonics), and data analysis was performed using DataAnalysis 5.0 (Bruker Daltonics). One microliter of the purified peptide solution was loaded onto a C18 nanotrap column (C18 PepMap 100, 100 µm x 2cm, 5 µm, 100 Å; Dionex) with a loading solvent (water, 0.05% TFA) at a flow rate of 15 µL/min for 125 minutes. The analytes were separated on a C18 analytical column (Acclaim PepMap 100, 75 µm x 50 cm, 2 µm, 100 Å; Dionex) at a

column oven temperature of 60°C. Elution was carried out at a flow rate of 0.4 µL/min with solvent A (water containing 0.1% FA, v/v) and solvent B (acetonitrile containing 0.1% FA, v/v). A linear gradient of 2–35% solvent B for 90 minutes was applied, followed by column washing and reconditioning. Ionization using CaptiveSpray nanoBooster was enhanced with acetonitrile at 0.2 bar. The source parameters were as follows: dry gas 3.0 L/min with 150°C; capillary voltage 1600 V. Mass spectra were acquired within a mass range of 150-3000 m/z and a spectra rate of 2 Hz. MS/MS parameters included a 3.0 sec. cycle time, absolute threshold 2500 cts, smart exclusion 5x, exclusion after one spectrum, and release after 0.40 min.

2.12.3. LC-MS/MS Data Analysis

Qualitative LC-MS/MS data in string format were parsed to extract protein symbols for each of the three sample groups separately. Venn diagrams were generated using the "ggVennDiagram" function in the R package ggVennDiagram v1.2.3. Ontology analyses for over-representation were conducted using the R package ReactomePA v1.28.053. A STRING v12.0 PPI network was generated with a minimal interaction score of 0.400 (and an FET p-value of 5e-02). A hypergeometric test was performed to compute the statistical significance of the overlap between two groups of proteins based on the Venn diagram.

2.13. HEK-TLR4 endotoxicity

HEK-Blue™ hTLR4 cells (Invivogen, France) 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% CO_2. Twenty µl of sample supernatants were mixed and then incubated with 180 µl of QUANTI-Blue solution (Invivogen) for 30 minutes. The optical density (OD) values were measured at 655 nm.

2.14. In vivo Toxicity

Groups of female mice (BALB/c, 6–8 weeks old, n=5) were intraperitoneally injected with varying doses of W+B OMVs (1.66, 5, 15, and 45 μg per animal, with an equal amount of 50% for both W and B OMVs). Their body weight, food consumption, and water intake were monitored over a five-day period.

2.15. Statistical Methods

Statistical differences in all treatment groups were analyzed using GraphPad Prism 9 statistical software. Groups were compared by one-way ANOVA with Dunnett's multiple comparisons 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. Purification and Characterization of *N. meningitidis* OMVs

Native outer membrane vesicles (OMVs) were extracted from the culture supernatant of *Neisseria meningitidis* serogroup W (ST-11) and serogroup B (ST-32). The purification process, depicted in Figure 2.1, involved loading Denarase-treated and clarified cell culture supernatant onto a HiScreen CaptoCore 400 multimodal size-exclusion/ion-exchange chromatography column.

The resulting pooled flow-through fraction was then subjected to ultrafiltration and diafiltration. The structural characterization of OMVs was conducted using atomic force microscopy (AFM, as shown in Figure 3.1), scanning electron microscopy (SEM, as depicted in Figure 3.2), tunable resistive pulse sensing (TRPS, as illustrated in Figure 3.3), and SDS-PAGE to visualize major membrane proteins (Figure 3.5).

Atomic force microscopy images of *N. meningitidis* serogroup W and B OMVs at 1 x 1 μm area were shown in Figure 3.1.

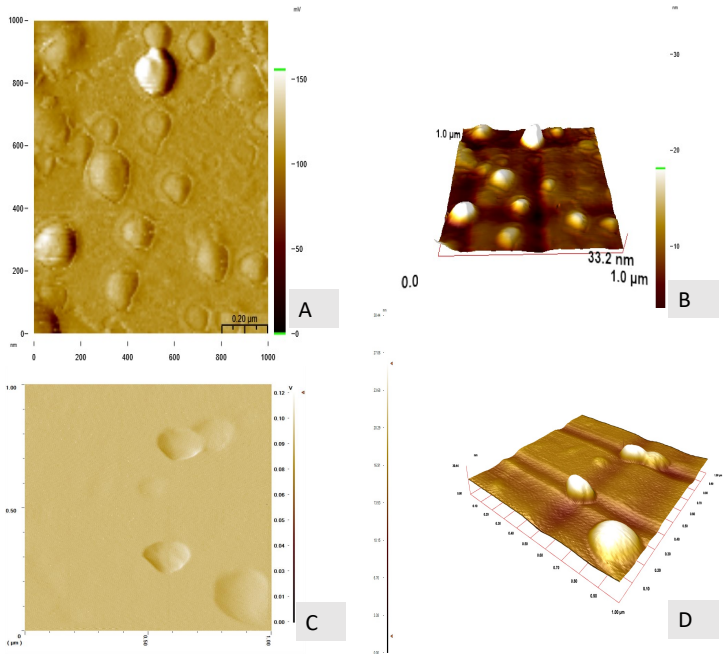


Figure 3.1. Atomic force microscopy images of *N. meningitidis* serogroup OMVs at 1 x 1 µm area (256x256). **A.** W OMVs **B**. Tomographic image of W OMVs**. C.** B OMVs. **D.** Tomographic image of B OMVs.

Scanning electron microscopy images of individual MenW and MenB OMVs were shown in Figure 3.2.

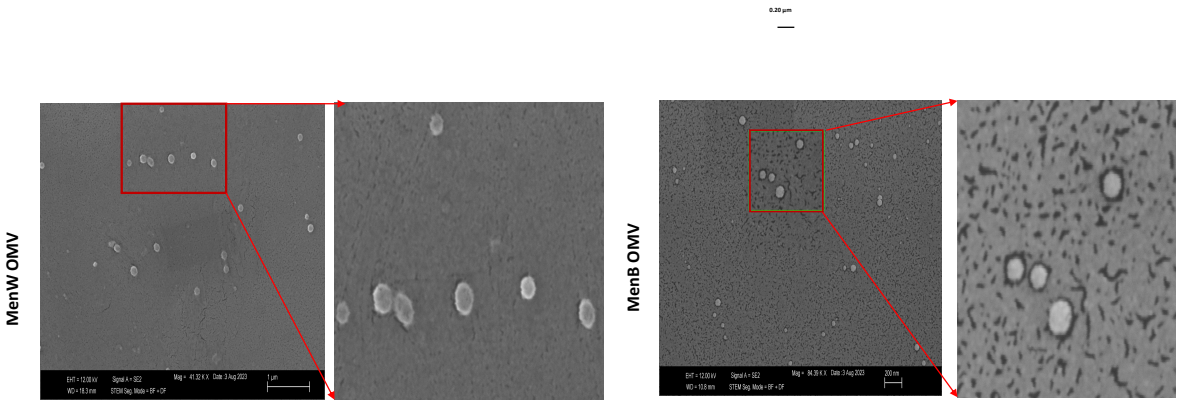


Figure 3.2. Scanning electron microscopy images of individual MenW and MenB strain OMVs. Representative images from three independent experiments are shown.

The size distribution measurement (nm) and particle number of OMVs were shown via TRPS in Figure 3.3. Characterization of the W and B OMVs after the dehydration rehydration technology was also performed via TRPS and shown in Figure 3.4.

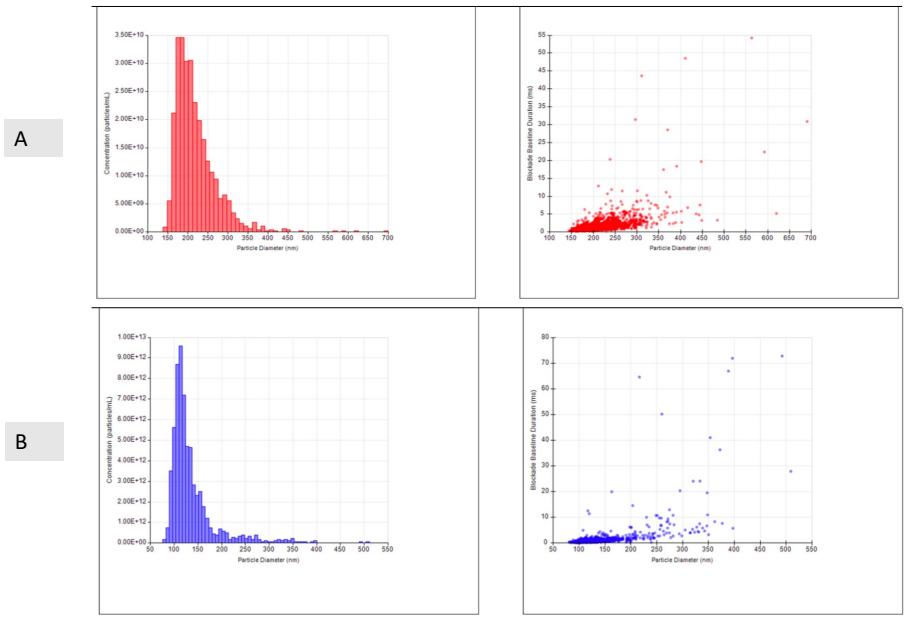
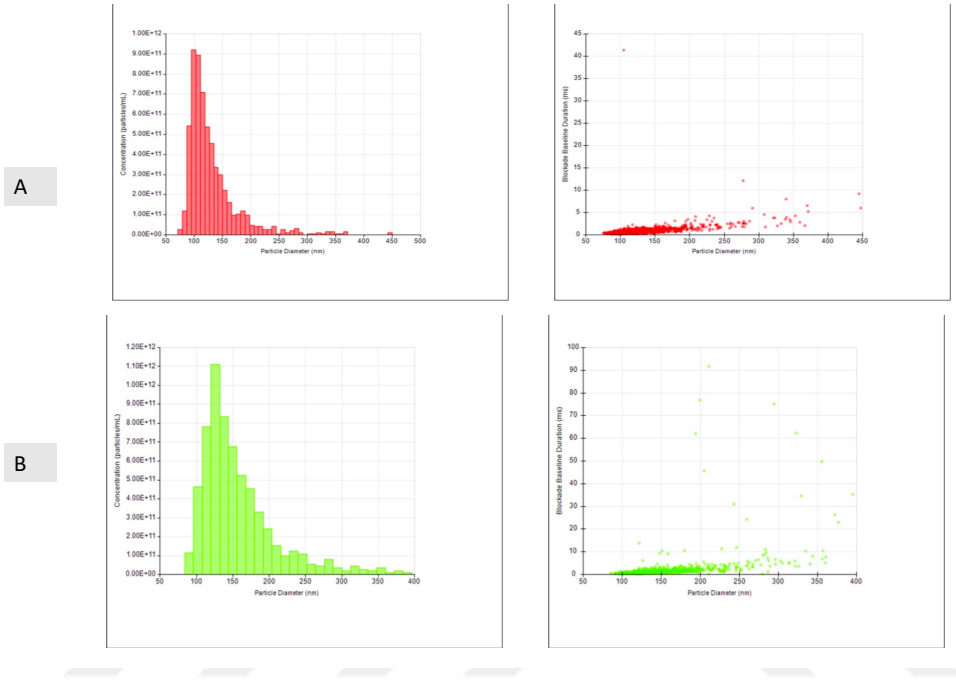


Figure 3.3. A. TRPS results of MenW OMVs. Particle diameter of OMVs were mean 219 (144-690) nm. Measured concentration of MenW OMVs was 2.83e+11 particles/ml. **B.** TRPS results of the MenB OMVs. Particle diameter of OMVs were mean 136 ± 50.5 (81-509) nm. Measured concentration of MenB OMVs was 6.32e+11 particles/ml.

Figure 3.4. OMVs and ligands were mixed at 1:2 or 1:10 ratio according to the low dose groups vs high dose groups, respectively in the first animal experiment. **A.** TRPS results of the MenW OMVs applied with dehydration rehydration technology. Particle diameter of OMVs were mean 131 ± 46.9 (75-447) nm. Measured concentration of MenW OMVs was 6.08e+11 particles/ml. **B.** TRPS results of the MenB OMVs applied with dehydration rehydration technology. Particle diameter of OMVs were mean 157 ± 51.7 (85-395) nm. Measured concentration of MenB OMVs was 3.20e+11 particles/ml.

AFM, SEM, and TRPS analyses collectively confirmed the spherical, vesicular nature of the OMVs, measuring 92±33 nm and 97±38 nm in diameter for MenW and MenB OMVs, respectively. Coomassie brilliant blue-stained SDS-PAGE analysis (Figure 3.5) of the OMVs revealed the presence of five

predominant membrane proteins: NadA, PorA, PorB, RmpM, and OpC. The result of protein electrophoresis was shown in Figure 3.5.



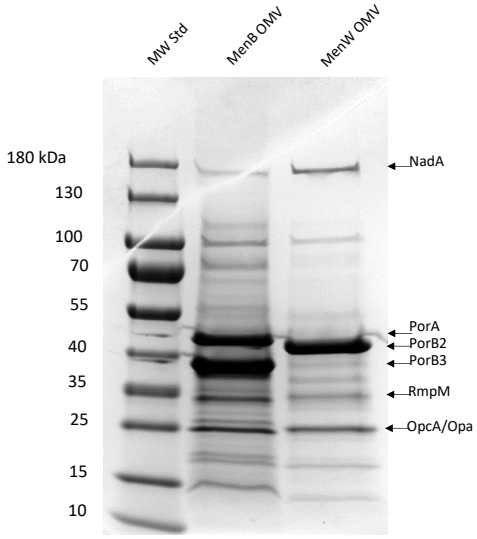


Figure 3.5. Coomassie brilliant blue-stained SDS-PAGE analysis of the OMVs of *Neisseria meningitidis* serogroup W and B.

3.2. The results of HEK-TLR4 endotoxicity experiments

To assess the LPS content within OMVs obtained from strains isolated with or without the presence of sodium deoxycholate, TLR4 activation was tested using HEK-Blue reporter cells expressing human TLR4. Following stimulation with varying concentrations of OMVs (Figure 3.6), comparable TLR4 activation profiles were observed between LPS and native OMVs. However, OMVs purified after detergent treatment exhibited significantly diminished h-TLR4 response, necessitating 10 to 100-fold higher concentrations of OMVs to achieve similar levels of TLR4 activation as seen with LPS. Collectively, these findings support that extracellular OMV nanovesicles derived from two strains of Neisseria meningitidis display convincing outer membrane vesicle features with reduced LPS activity.

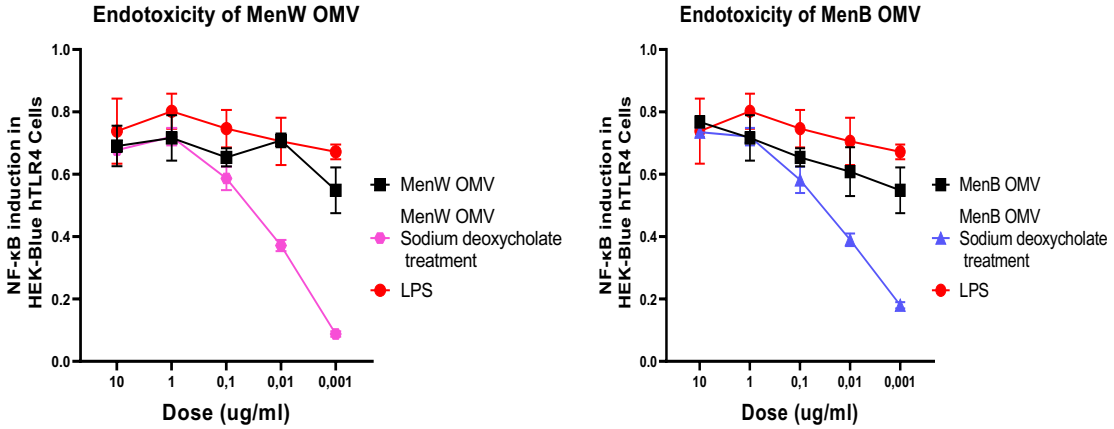


Figure 3.6. Comparison of endotoxic responses from LPS, native OMVs, and SDOC-treated OMVs.

3.3. The results of toxicity experiments in mice

To determine the optimal dosage of OMVs for use as an antigen in vaccine formulation, we examined the potential systemic toxicity of both high and low doses in mice. The mice were intraperitoneally injected with varying doses of OMVs (1.66, 5, 15, and 45 µg per animal), and their mortality rate, body weight,

food consumption, and water intake were monitored for a five-day period. Throughout the dosing and recovery phases, no OMV-related mortality was observed. While no significant impact on body weight was noted in mice that received low OMV doses (1.66 and 5 µg per animal), those exposed to high doses (15-45 µg per animal) exhibited noteworthy weight loss compared to the control group (Figure 3.7.A). Additionally, no significant changes in food and water consumption were detected in groups administered with low doses, whereas high OMV doses had a significant effect on these parameters (Figure 3.7. B-C). Consequently, based on these findings, an optimal dose of 5 µg of OMVs was identified as having no systemic toxic effects for vaccine formulation.

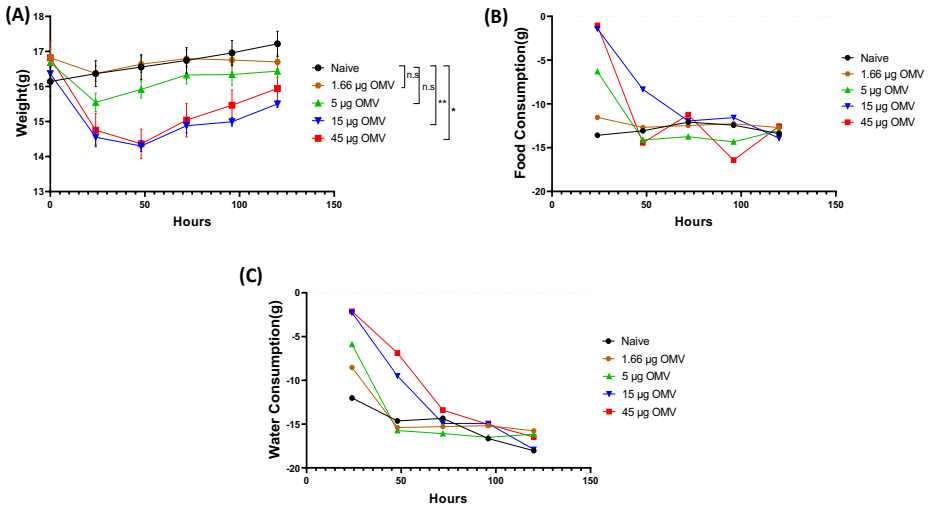


Figure 3.7. (A) Time course of body weight after OMV injection. (B) food and (C) water consumption were evaluated daily for five days after injection. Values are the average ± SEM of animals in each group (n=6). Mann Whitney test. *P < 0.05, **P < 0.01.

3.4. Proteomics Analysis of 2 OMV-derived Study Vaccines for N. *meningitidis*

Anabolic pathways are highly enriched in the shared proteins of MenW and MenB OMV vaccine groups. The final product of histidine biosynthesis by catalytic activities of the metabolite interconversion

enzymes makes up a substantial part of the cargo encapsulated in the OMV-B and OMV-W of N. *meningitidis.* Stress response and FA anabolism are linked to the shared set of proteins between these two different OMV vaccine groups. Two group comparisons are shown in Figure 3.8. A Venn diagram was used to identify, with high statistical significance (*p-value* < 6.2e-24), the degree of overlap between the protein entities shared between different sample groups mentioned above, reporting 252 proteins at the intersection (Fig 3.8.a). A pathway-level enrichment analysis identified histidine biosynthesis as significantly enriched in these shared proteins (Fig 3.8.b).

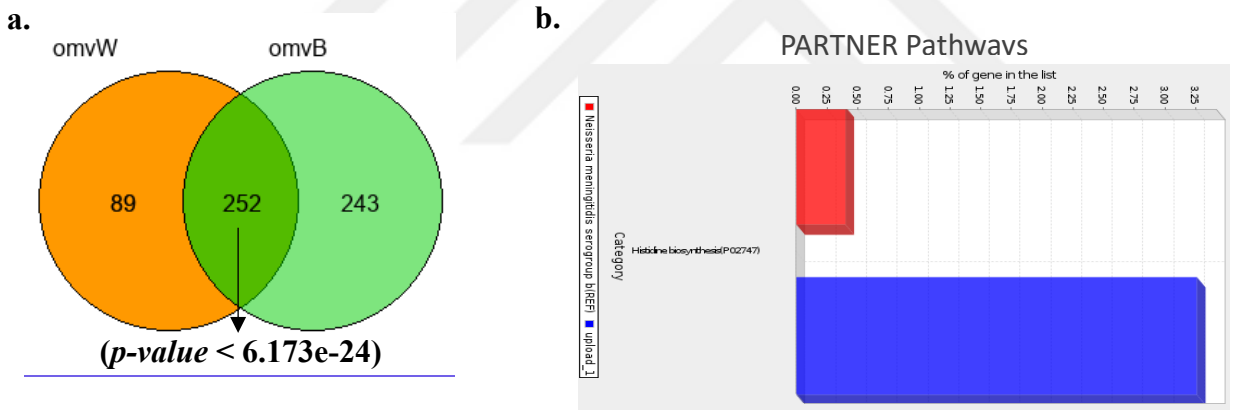


Figure 3.8. a) The comparison of the cargo encapsulated in the OMV-B and OMV-W of N. *meningitidis* following proteomics analyses. The significance of the overlap between protein entities in the compared groups calculated by a hypergeometric test is given in parenthesis. b) Compared to the reference levels (red), the functional enrichment results of this intersection reported histidine biosynthesis as the only significant pathway (blue) enriched in both groups.

After proteomics profiling of these OMVs, we found that OMV B and OMV W are quantitatively richer in content than the licensed vaccine (Figure 3.9.A). When the degree of overlap of the protein content among these three different sample groups was analyzed, the ontology term "cell outer membrane" appeared

enriched within the shared proteins by all groups, as expected (Figure 3.9.B). Of note, a more comprehensive functional enrichment analysis managed to identify, as expected, energy metabolism that is enriched in the same subgroup of proteins (Figure 3.9.C) for all vaccines investigated. As a result, we concluded that the proteomics results of the OMV cargo are both biologically relevant and highly comparable to the protein content of the licensed vaccine.

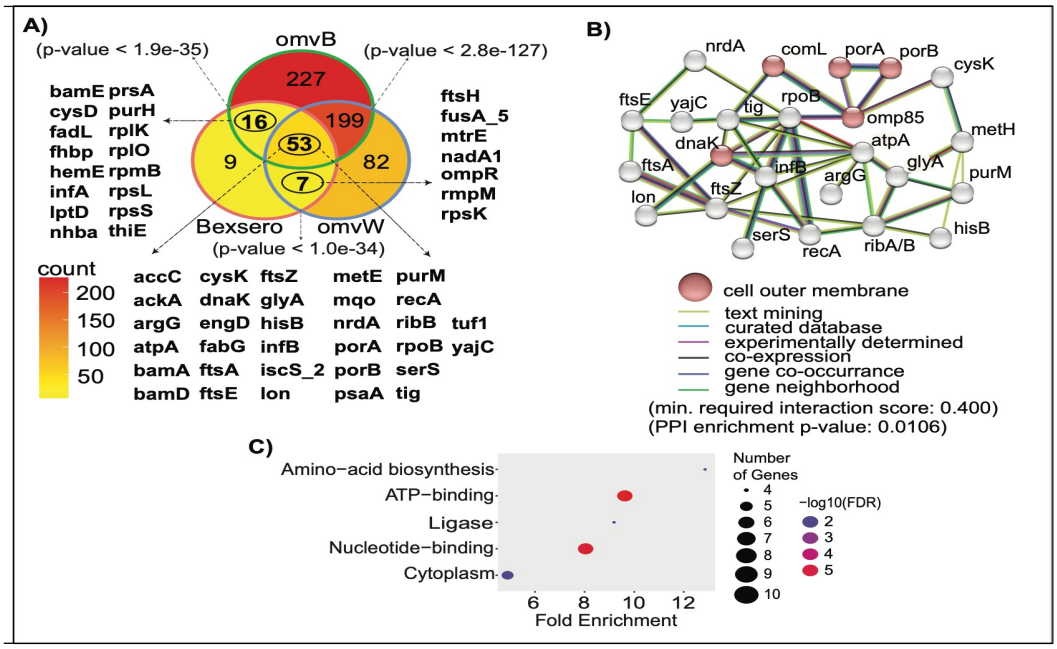


Figure 3.9. Proteomics Profiling of OMV-Based Meningococcal Vaccines

(A) A Venn diagram showing the degree of overlap of the protein content among three different vaccine groups. The color code reflects the number of unique or shared proteins in each sector. The p-values in parentheses, derived from a hypergeometric test, reflect the statistical significance of the overlap between the corresponding pairs of sample groups. The protein contents in three key sectors (circled in the diagram) were disclosed after filtering out redundant ribosomal proteins. (B) Taking the shared proteins among all groups (32; the number of proteins left after filtering out ribosomal proteins) as input, a STRING PPI analysis identified enrichment of the cell outer membrane in a subnetwork of these proteins, as expected. Nodes represent genes, and edges represent interactions. The color-coded edge thickness indicates interaction strength by the type of interaction between any pairs of proteins in the network. PPI enrichment p-value < 0.0106; minimum required interaction score, 0.400. (C) Ontology level enrichment by GO identifiers of energy metabolism terms within the same query list as in B.

3.5. ELISA Results

3.5.1 ELISA Results of the First Experiment

Mice were immunized with two doses of the various vaccine fromulations (Table 2.2). Antigen-specific Ig G responses were measured in the sera of the vaccinated mice. Total IgG, IgG1, and IgG2a against OMV W and OMV B (coating 10 μg/ml) were performed at 3 weeks after booster injection. W-OMV + B-OMV + Al (1:1 combination of the Men W and Men B OMV; total 5 μg protein) and W-OMV + B-OMV + CpG (loaded within OMVs) (1:1 combination of the Men W and Men B OMV; total 5 μg protein) formulations induced high levels of anti-W-Ig G and anti-B-Ig G (Figure 3.10). The immune response against Men W and Men B OMV was higher with 5 μg doses than with 1 μg doses. The monovalent vaccine (5 μg of B) induced a significantly higher response against Men B OMV than 2.5 + 2.5 μg of the corresponding bivalent vaccine; however, there was no significant difference in the IgG response between the monovalent and the bivalent OMV vaccine formulations. Best groups are selected for the second experiment.

A

B

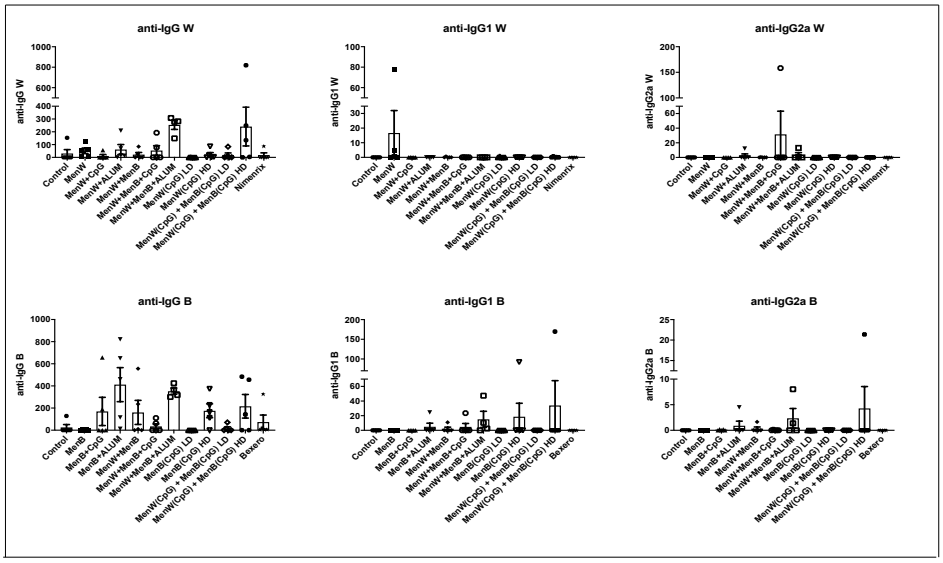


Figure 3.10. A summary of total ELISA results of the first experiment. Total IgG, IgG1, and IgG2a against OMV W and OMV B (coating 10 µg/ml) (3 weeks after booster injection). **A.** anti-Ig G W, anti-Ig G1 W and anti-Ig G2a W results of vaccinated animals and comparison of available vaccine Nimenrix. **B.** anti-Ig G B, anti-Ig G1 B, and anti-Ig G2a B results of vaccinated animals and comparison of available vaccine Bexero.

3.5.2. ELISA Results of the Second Experiment

According to the results of the first animal experiment, mice were immunized with two doses of the new vaccine fromulations. In this experiments, OMV and adjuvant doses were increased (Table 2.3). Total IgG, IgG1, and IgG2a against OMV W and OMV B (coating 10 µg/ml) were performed at 2 months after booster injection. All bivalent WB OMV vaccines adjuvanted with either Alum or CpG induced significantly higher IgG specific for both W-OMV and B-OMV than controls and mice immunized with Nimenrix. A higher anti-Ig G response against B OMV was shown in mice immunized with Bexero. Interestingly, high anti-Ig G against W were also shown in those Bexero immunized mice. The bivalent vaccine (5 µg W + 5 µg B) adjuvanted with CpG (30 µg) induced a significantly higher Ig G and Ig G2a response against Men W OMV than 5 + 5 µg CpG loaded (30 µg) vaccine and available Bexero vaccine. While bivalent vaccine (5 µg W + 5 µg B) adjuvanted with CpG (30 µg) induced high and similar Ig G response against Men B OMV, Ig G2a response was significantly higher than the Bexero vaccine (Figure 3.11 and Figure 3.12).

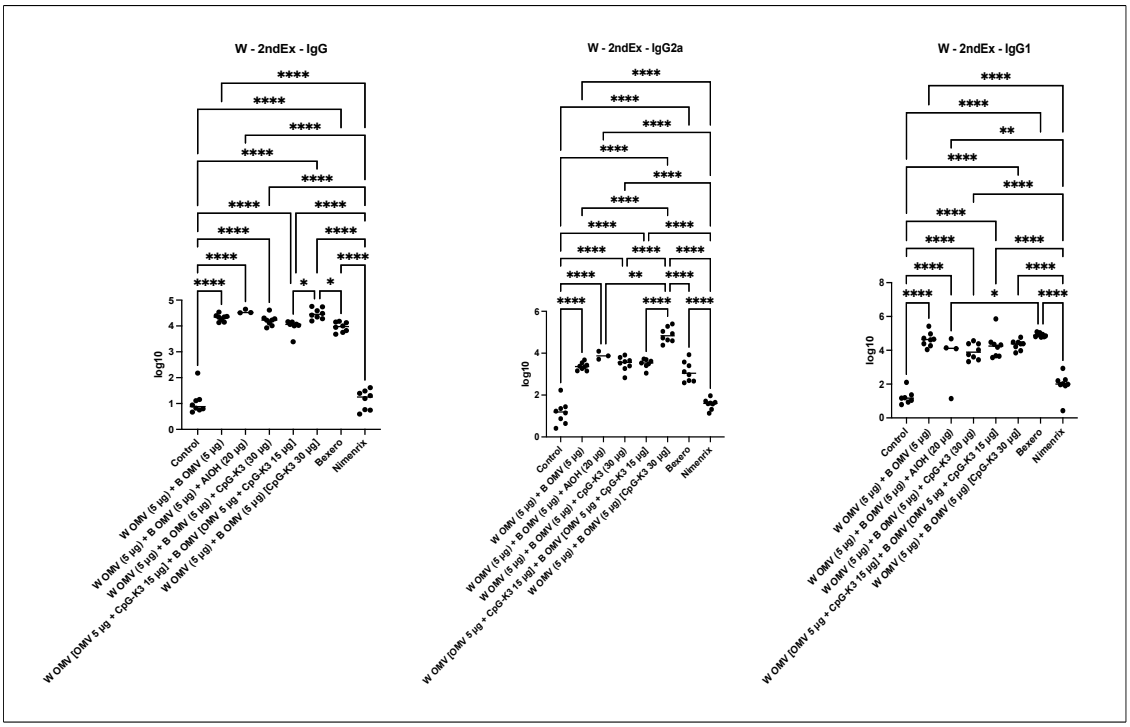


Figure 3.11. A summary of total ELISA results of the second experiment. Total IgG, IgG1, and IgG2a against OMV W (coating 10 µg/ml) (2 months after booster injection) and comparison of available vaccine Nimenrix and Bexero.

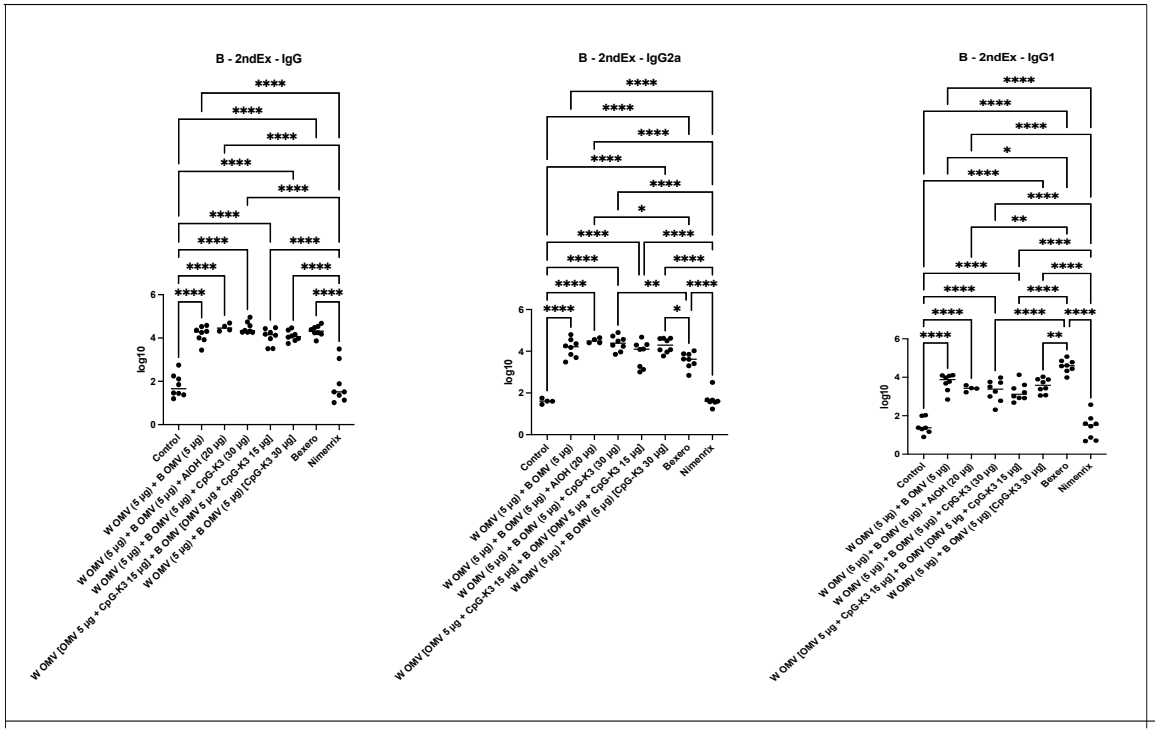


Figure 3.12. A summary of total ELISA results of the second experiment. Total IgG, IgG1, and IgG2a against OMV B (coating 10 µg/ml) (2 months after booster injection) and comparison of available vaccine Nimenrix and Bexero.

3.5.3. ELISA Results of the Third Experiment

The Ig G response was also compared with Ig G results of the best groups in the previous (first) animal experiment. Since there was no any significant difference between the groups, we planned the third experiment with low OMV and adjuvant doses (Table 2.4). In this experiment, there was a new formulation including two adjuvants simultaneously. Total IgG, IgG1, and IgG2a against OMV W and OMV B (coating 10 µg/ml) were performed at 2 months after booster injection and compared available vaccines (from the second experiment). All bivalent W+B OMV formulations adjuvanted with Al and/or CpG induced anti-W IgG, which is significantly higher than in controls and sera from Nimenrix immunized mice. Again, mice immunized with Bexero induced higher Ig G levels against W as compared

to Nimenrix group. All bivalent W+B OMV vaccine formulations induced IgG against B at least as well as Bexero and there was no difference between the groups (Figure 3.13).

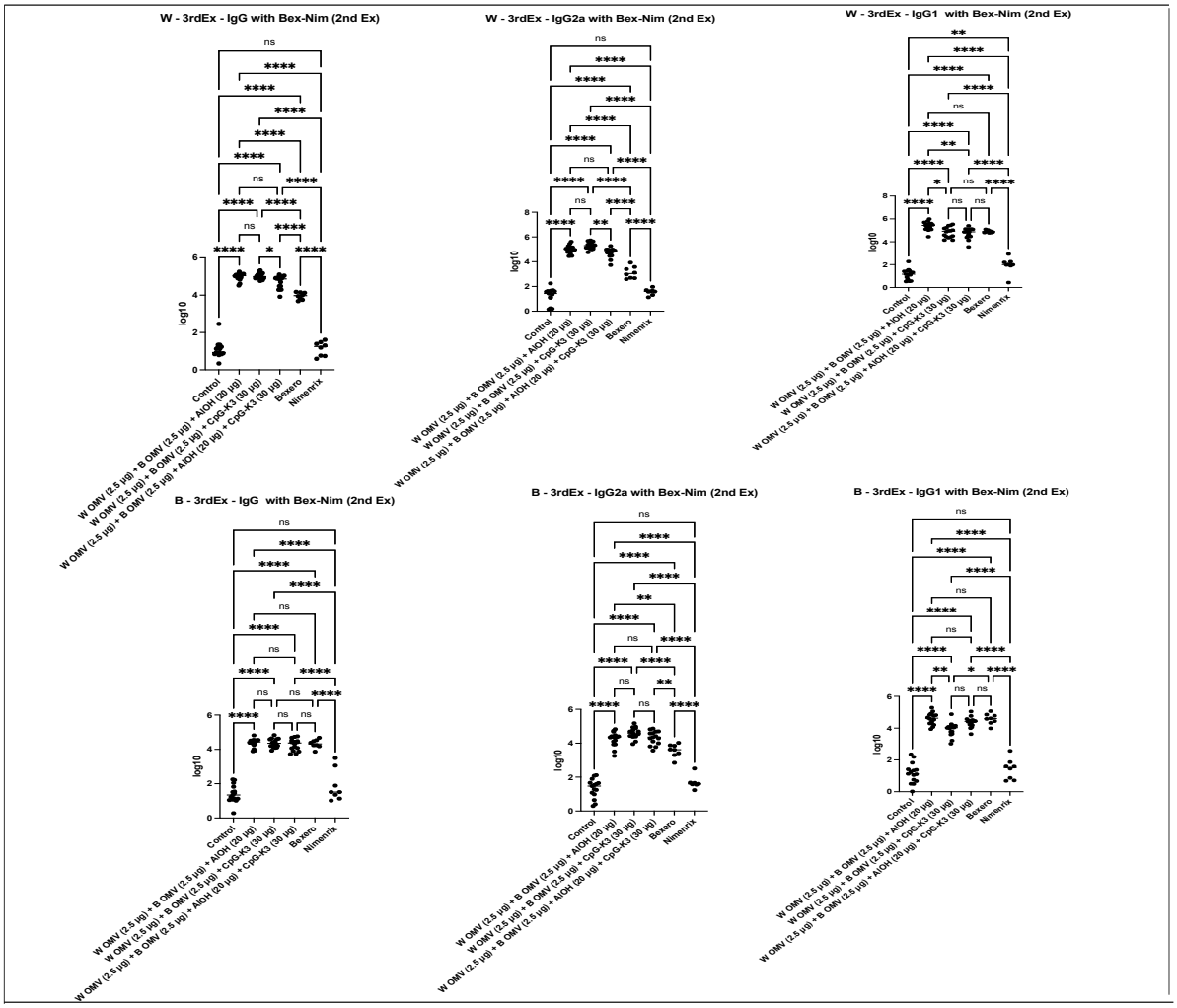


Figure 3.13. A summary of total ELISA results of the third experiment. Total IgG, IgG1, and IgG2a against OMV W and B (coating 10 µg/ml) (2 months after booster injection) and comparison of available vaccine Nimenrix and Bexero.

3.5.4. Final Immunogenicity Experiments of W+B OMV Vaccine

To evaluate if a combination of OMVs from serogroups B and W, the most prevalent serogroups in the Middle East and Europe, can be used as a vaccine, potentially offering protection against a broader spectrum of meningococci, we developed a bivalent vaccine and conducted a comparative analysis with

commercially available vaccines. To assess the immunogenicity of the B+W 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 B+W OMVs or in combination with K3 CpG ODN (30 µg/mouse) or Alum (20 µg/mouse). A conjugate vaccine ACYW (NimenrixTM, Pfizer) and serogroup B vaccine 4CMenB (Bexsero, GSK) 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). Mice immunized with W+B OMV or adjuvanted counterparts had higher anti-MenW and MenB-specific IgG, IgG1, and IgG2a levels after booster injections (Figure 3.14). As expected, the W+B 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 4CMenB vaccine showed the direct protection afforded by 4CMenB against MenW:cc11 disease [72]. Consisting 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 (Figure 3.14.A). W+B OMV+Alum elicited 14-fold more anti-MenW OMV IgG when compared to its formulated W+B OMV counterpart (Fig. 3.14.A), indicating that the Alum-adjuvanted OMV vaccine is more immunogenic than only W+B OMV. Mice vaccinated with W+B OMV and its adjuvanted counterparts exhibited similar antibody responses to Bexsero (Figure 3.14.B).

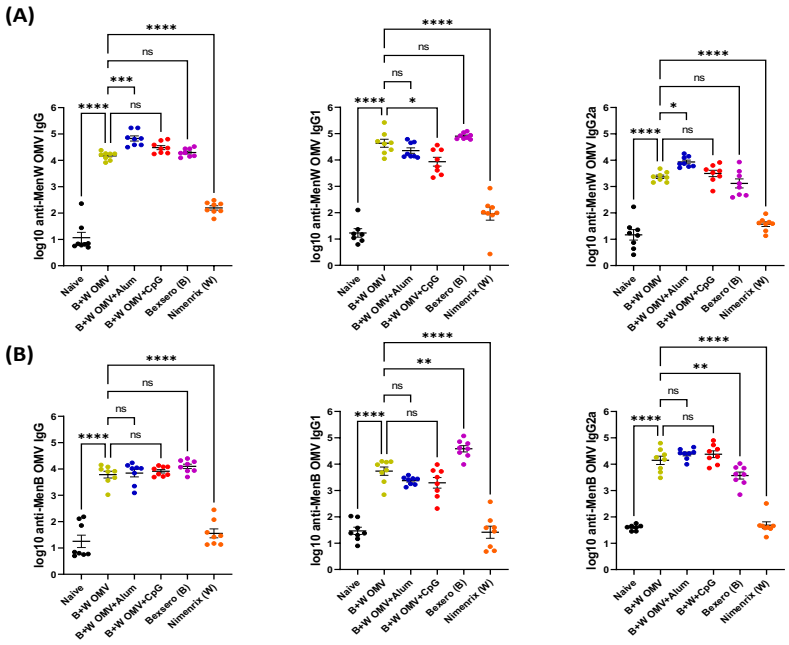


Figure 3.14. W+B OMV Vaccine Elicits Robust Antibody Responses in Mice

BALB/c mice (n=8/group) were immunized on days 0 and 14 with 5µg MenW OMVs plus 5µg MenB OMVs without or with Alum (20µg/mouse), without or with K3 CpG ODN (30µg/mouse) or with Alum + CpG ODN. Comparator BALB/c mice (n=8/group) were injected on days 0 and 14 with 100µl of 4CMenB vaccine (Bexsero®, GSK, includes 25µg MenB OMV in 0.5ml for the human dose) or 200µl of MenACYW conjugate vaccine (NimenrixTM, Pfizer). Sera were collected 2 weeks post-boost and assessed for (A) MenW-OMV and (B) MenB-OMV-specific IgG, IgG1, and IgG2a by ELISA. Vaccinated groups were compared by one-way ANOVA with Dunnett's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data are presented as Mean ± SEM.

3.6. Comparison of the W+B OMV Vaccine and Commercially Available Vaccines in SBA

Mice immunized with the W+B OMV vaccine and its adjuvanted counterparts developed bactericidal antibodies against the MenW vaccine strain clonal complex ST-11 and MenB clonal complex ST-32 two months after the booster injection. Notably, all groups that received the W+B OMV vaccine exhibited higher efficacy in eliciting SBA titers against the MenW strain, in comparison to the Nimenrix vaccine (Figure 3.15.A and 3.16.A). All vaccine groups except the W+B OMV+Alum induced similar SBA titers

against the MenB strain, while the W+B OMV+Alum group had significantly higher SBA titers compared to Bexsero (Figure 3.15.B and 3.16.B).

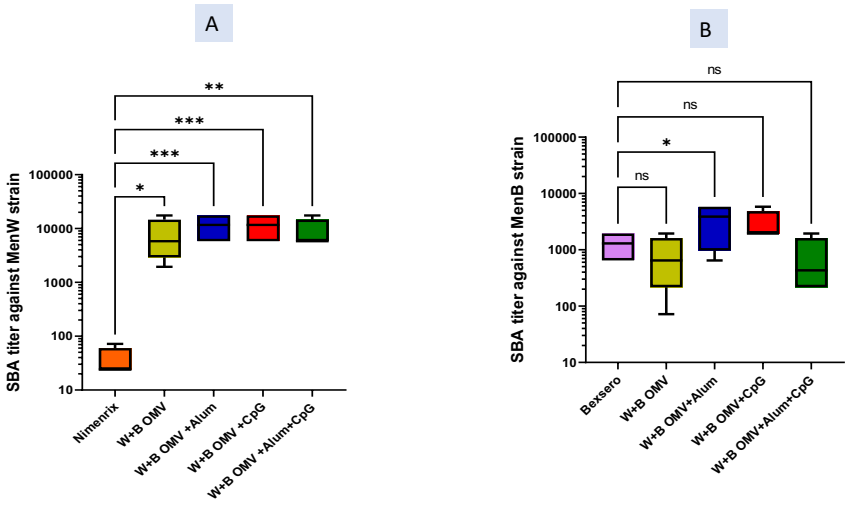


Figure 3.15. Serum Bactericidal Activity (SBA) Titers in Sera

SBA responses against (A) MenW and (B) MenB strains after 2 doses of the W+B OMV vaccine, NimenrixTM, and Bexsero®. Data in (A) and (B) are shown as box plots; whiskers show min to max data points. Vaccinated groups were compared by one-way ANOVA with Dunnett's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data are presented as Mean ± SEM.

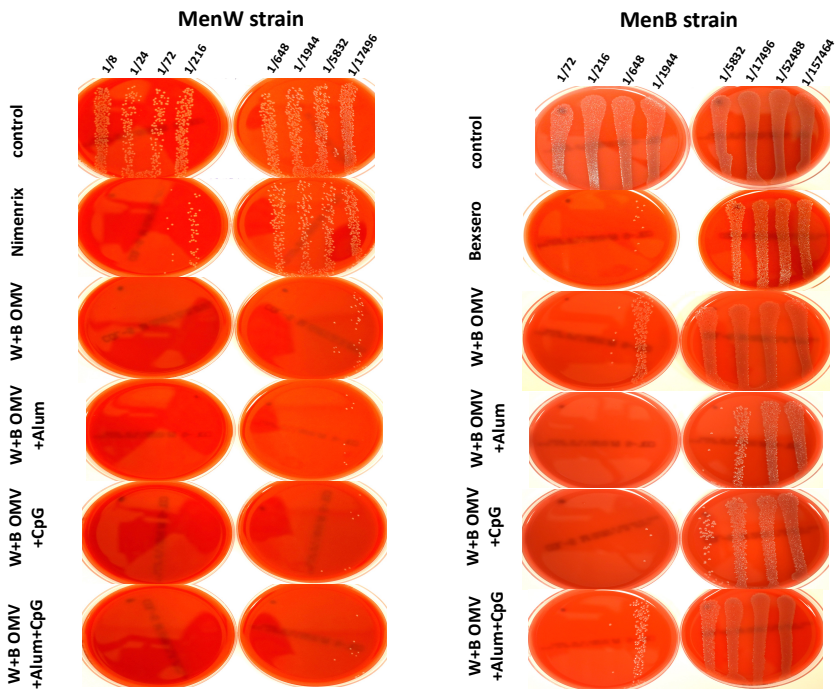


Figure 3.16. SBA responses against MenW (A) and MenB (B) strains after 2 doses of the W+B OMV vaccine, NimenrixTM, and Bexsero®. Representative pictures of blood agar plates from each vaccinated group showing CFUs. The bactericidal antibody titer was expressed as the reciprocal of the final serum dilution yielding ≥ 90% killing after 1 hour as compared with the average for controls.

Furthermore, W+B OMV+Alum vaccine and Bexsero were tested against the MenX strain, and W+B OMV+Alum was found to be strongly bactericidal against this strain as well compared to Bexsero (Figure 3.17).

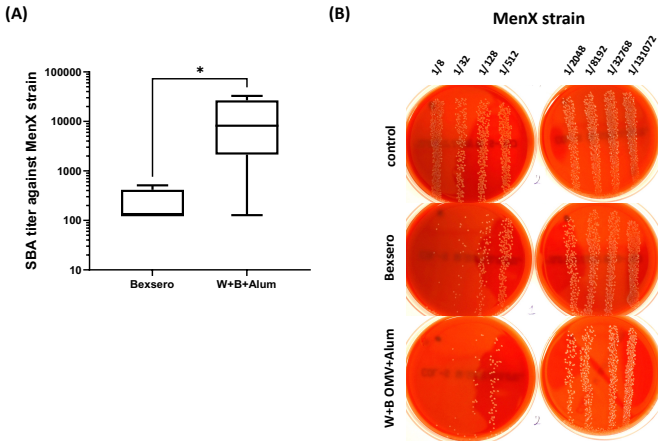


Figure 3.17. A Cross-reactivity by W+B OMV+Alum Vaccine Sera

(A) Serum bactericidal activity (SBA) responses against MenX strain (non-B meningococci) after 2 doses of the W+B OMV+Alum vaccine and Bexsero®. Data in (A) is shown as a box plot; whiskers show min to max data points. (B) Representative pictures of blood agar plates from each vaccinated group showing CFUs. The bactericidal antibody titer was expressed as the reciprocal of the final serum dilution yielding ≥ 50% killing after 1 hour as compared with the average for controls. Vaccinated groups were compared by Mann Whitney test. *P < 0.05.

4. CHAPTER 4

DISCUSSION

We have here studied safety, immunogenicity and the SBA-based protective capacity of bivalent meningococcal WB vaccines that are not found together in any currently available vaccines licensed for human in mice and high levels of IgG antibodies against both B and W OMV were detected in mice receiving the B + W OMV vaccine adjuvanted with CpG and/or Al $(OH)_3$. Since serum bactericidal antibodies (SBA) are considered as the "Gold Standard" for correlate for protection against meningococcal disease, we determined SBA titers, and OMVs from MenW and MenB strains were shown to induce bactericidal antibodies in immunized mice. Furthermore, the B OMV vaccine was shown to induce IgG antibodies against MenW of the same magnitude as the titers induced by the W OMV vaccine. Furthermore, the B + W OMV vaccine was shown to induce higher Ig G antibody levels against MenW as compared to antibody levels induced by the ACWY-conjugate vaccine, Nimenrix and induce as high as Ig G antibody levels against MenB as compared to available vaccine, Bexero. Additionally, combining two OMVs in one vaccine did not seem to interfere with the immune response to the individual OMV types.

In this study, we have developed an immunogenic and potentially protective bivalent MenW+MenB OMV vaccine that could also protect against the most common serotypes not only in Turkey but also in Europe. After serogroup W and B, serogroup A is the most common serogroup in Turkey and serogroups X and Y are also seen in cases [23-25]. In Europe, in recent years, B is the most common type and W has risen to the second place with a very striking increase. This makes the coverage of our vaccine very important within Europe, as well. In addition, serogroups C and Y, which are common in Europe, encourage us to make our vaccine a molecule that covers more serogroups [29]. That is why, we tried to understand the possible role of possible overlapping OMV proteins between the meningococcal serogroups. Therefore,

the substantial SBA titers of the MenW+MenB OMV+Alum combination against the MenX serotype, which is not included in any commercial vaccine available, gave us hope in regarding with the breadth of protection of our bivalent OMV vaccine.

The selection of the suitable adjuvant to be included in the vaccine is as important as the selection of the target antigen. To enhance the immunogenicity of inactivated vaccines, adjuvants, such as aluminum compounds and salts, have been used for many years [55]. Aluminum hydroxide has also been an adjuvant option for MenB OMV vaccines in Norway, New Zealand, and Cuba [56, 57,73]. However, aluminum-containing adjuvants have limitations, such as local reactions, increased IgE-mediated responses, ineffectiveness with certain antigens, and an inability to enhance cell-mediated immune responses, particularly cytotoxic T cell responses [55]. Since aluminum hydroxide primarily stimulates a humoral B cell response, new adjuvants have been explored in our novel vaccine platforms. One of these adjuvants is CpG-based adjuvants, which have never been used in vaccine studies developed against meningococci. The effectiveness of this adjuvant has not been evaluated until now.

It was firstly reported by Yamamoto et. al. that synthetic ODNs induce interferon-gamma (IFN-gamma) secretion via activating natural killer (NK) cells. Palindromic sequences found in ODNs might be a stimulator for this induction [74]. The “danger signal” caused by unmethylated CpG DNA released by bacteria during an infection stimulate the innate immune system of the host. CpG motifs have various role in the functions of the immune cells such as B and T lymphocytes, NK cells, and dendritic cells (DCs). These cells create T helper 1 (Th1)-biased immune response via cytokines and chemokines [59].

The Toll-like receptor (TLR) 9 which are predominantly expressed by B cells and plasmacytoid dendritic cells (pDCs) recognizes CpG motifs by immune cells. In contrast to other family members of TLRs, TLR9 is found within the cell; therefore, CpG DNA should be internalized by immune cells to interact with TLR9. CpG-mediated signaling is triggered by the interaction of TLR9 with CpG ODN. TLR9 interacts with myeloid differentiation marker 88 (MyD88) that gathers IL-1 receptor-associated kinase 4 (IRAK4) to stimulate tumor necrosis factor receptor-associated factor (TRAF) 6. TRAF6 culminate in the existence

of some transcription factors such as nuclear factor (NF)-kB and thus, gene expression of cytokines/chemokines is upregulated by those elements [75-77]. TLR9 molecules and TLR9 secreted cell types might be different in various species. Three different CpG ODN classes including “K”, “D”, and “C” type ODNs have been defined. K-type was discovered firstly and thanks to phosphorothioate backbone, the susceptibility to DNAse digestion is low and in vivo half-life is longer than phosphodiester ODNs. D-type ODNs result in the maturation of APCs and secretion of IFN-alpha. C-type ODNs directly stimulate IL-6 secretion and IFN-alpha production, thus combining the features of K- and D-type ODNs [59].

CpG DNA induce the cytokine and chemokine production via APCs to develop adaptive (antigen specific) immunity and CpG ODNs are used in many labs to boost immunogenicity. Klinman et al. reported in their early experiments that CpG ODN added to ovalbumin (OVA) to induce antibody production increased about threefold antibody production. Moreover, co-localizing CpG ODN with OVA might increase anti-OVA IgG up to 10-fold. Adjuvant effect of CpG was demonstrated according to the findings of this report [78]. The findings of other laboratories and new studies also supported the adjuvant-like characteristics of CpG ODNs, particularly in case linking to antigen directly or encapsulating in liposome vesicles [63, 79]. A conjugate including antigen and ODN cause high CTL response, which is mediated by DNA-binding receptors on APCs. This knowledge demonstrates one more time that both antigen and adjuvant mixtures cause the optimal stimulation of the immune system [80]. CpG ODN may improve immune response elicited by conventional vaccines. These effects are the result of the functional maturation of APCs promoted by CpG ODNs [81, 82]. Because mucosae including respiratory, gastrointestinal, vaginal or rectal are the main entrance of the pathogens, it was also investigated whether the effect of CpG ODNs on mucosal immunity. Intranasal administration of CpG ODN adjuvanted inactive influenza virus vaccine induced high saliva and genital tract levels of flu-specific antibodies in addition the serum levels [82]. More than 600 preclinical studies have been published examining CpG-adjuvanted vaccine immunity. Studies have been carried out with vaccines against a wide range of pathogens such as anthrax, leishmania, influenza and measles viruses, hepatitis B, and it has been shown that humoral immune

response is stimulated [58]. ODN and antigen-containing conjugates are preferentially uptake by professional antigen presenting cells (APC), resulting in a significantly higher CTL response. In addition, the duration of the immune response can be improved with CpG-based adjuvants. For example, CpG ODNs dramatically extended the protection induced by the licensed human anthrax vaccine, Anthrax Vaccine Adsorbed (AVA). Moreover, protective antibody titers persisted for more than one year compared to AVA alone [64]. CpG-based adjuvants also affect the cellular immune response. One study reported that the number and lifespan of CD8+ T cells were significantly increased by administration of CpG ODN two days before peptide inoculation [65].

CpG ODN was applied repeatedly with Engerix-B (available hepatitis B vaccine) to healthy and non-immune volunteers in a study [83]. It was reported that quicker antibody response (anti-hepatitis B IgG) was shown in cases received the mixture of vaccine and CpG ODN than that in cases received vaccine alone. Moreover, the about 15-45-fold higher geometric mean titer of the serum anti-hepatitis B IgGs were detected among cases treated with CpG ODN plus vaccine than that in only vaccine recipients. In another study, Fluarix (available influenza vaccine) co-administered with CpG ODN significantly induced high anti-hemagglutinin titers in cases with pre-existing anti-flu antibodies [84, 85]. Thus, CpG ODNs function as adjuvants when combined with protein-based vaccines via increasing the response of antigen-specific antibody and cell-mediated immunity. Several studies also reported that these CpG-combined vaccines boost antigen-specific Ig A titers at mucosal sites in addition to systemic Ig G levels [82,86]. Furthermore, CpG ODN is of ability to magnify vaccine-specific immunity. Additionally, CpG ODNs might be used in a couple of groups with decreased immune function, including newborns, the older people, and immunodeficient individuals [59]. Heplisav-B (HepB-CpG), a CpG adjuvanted vaccine against hepatitis B, was approved by the ACIP in February 2018 [66]. For the vaccine formulation we designed in our study, "CpG-based adjuvants" were preferred as adjuvant and in fact and because of the effective vaccine response, to reduce the amount of antigen in the formulation was possible in our study.

The Bexsero® vaccine was shown to induce high IgG antibodies and SBA titers against MenW. Amino acids play essential roles in the survival of organisms, and many of them are involved in immune functions and host-pathogen crosstalk. Histidine is one of the essential amino acids, which is responsible in the regulation of various cellular processes and catalytic activities. Histidine biosynthesis pathways of bacteria have been broadly characterized and despite of the knowledge gap, some products of histidine metabolism has been revealed to induce the anti-inflammatory responses during an infection scenario. This finding also might be a sign of immune modulatory effects of histidine catabolism [87, 88]. In the present study, histidine biosynthesis products found in both MenW and MenB OMVs might contribute to the anti-MenW IgG response in mice injected with MenB OMV-based vaccines. Consistently, it was reported that two available commercial MenB vaccines may protect against MenB and non-MenB IMD because of the shared common proteins such as fHbp [31]. Furthermore, it was proved that OMV including 4CMenB vaccine has an increased immunogenic benefit, which might be a result of cooperativity between antibodies induced by OMV or adjuvant property of OMVs [39]. In light of these data, OMV-based meningococcal vaccines seem to be promising candidates for the prevention of IMD with broader serotype coverage, but this is an idea that definitely needs to be proven with new experiments including new serotypes.

There are some limitations of the current study. First, meningococcal OMVs have been largely studied as candidate vaccines. The isolation of OMVs involves treatment with detergents to reduce the possible adverse effects associated with lipopolysaccharide (LPS), which is a very potent PAMP. However, LPS is considered as a strong immune adjuvant and has been reported to skew T-cell responses toward a Th1-type, which might be crucial for expanding the coverage and protection against meningococcal disease [89]. Additionally, detergent treatment has a risk of removing protective antigens from outer membrane, such as fHbp, and might lead to reduction of OMV stability, which affects the keeping process in long-term [90, 91]. Although there are some recommended genetic modifications to improve the yield of OMVs, we decreased the antigen dosage, instead of detergent treatment, and did not experience any systemic side effects in animals. Second, we could not improve our proteomics data analysis any further to

perform in-dept analysis of the interaction network or to generate a ranked list of each shared genes by pathogenicity/immunogenicity.

Conclusion

The results presented here are the first 'proof of principle' that a bivalent W+B OMV vaccine may have the potential to give broad protection against meningococcal disease. Moreover, such a vaccine could also protect against other serotypes that express identical subcapsular antigens, as shown here for a serogroup X strain. Vaccines covering W and B serotypes available in our country are not in the national immunization schedule of Turkey, and the cost of these vaccines to the family of a child under the age of one is around 400 dollars (12.206 TL), and the child must be exposed to separate injections. With this study, we believe that by bringing these needed molecules together with this method, we will contribute to reducing both the cost and the number of doses, where the minimum wage in 2024 is 17.002 TL in Türkiye. In addition, the method seems promising for countries with more limited resources, such as Africa, as it is easier to implement compared to other methods and allows the use and combination of local strains. The B + W OMV vaccine, as a result, induced high levels of antibodies to both MenB and MenW strains, levels that were shown to be higher or equal to the levels induced by licensed meningococcal vaccines. OMV vaccines are relatively simple and inexpensive to manufacture, and a bivalent W+B OMV vaccine could serve as an alternative or a supplement to the existing polysaccharide/conjugate vaccines against epidemics of meningococcal disease in the Europe and Middle East.

5. CHAPTER

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91. Ferrari G, Garaguso I, Adu-Bobie J, et al. Outer membrane vesicles from group B *Neisseria meningitidis* delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. Proteomics 2006;6:1856-1866

CURRICULUM VITAE

Name	Yasemin
Surname	Ozsurekci
Place Of Birth	Antalya/Turkey
Date Of Birth	18.12.1979
Site	Hacettepe University Faculty Of Medicine Department Of Pediatrics
Language	English
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Date	**Education**
1997-2003	Ankara University Faculty of Medicine
2004-2009	Pediatric Residency (Hacettepe University Faculty of Medicine)
2010-2013	Fellowship Residency (Hacettepe University Faculty of Medicine, Department of Pediatric Infectious Diseases)
2016-	Ph. D., Molecular Biology and Genetics (Bilkent University)

Good Clinical Practice Trainings	
03.06.2011	Vienna School of Clinical Research Introduction to GCP

Academical Titles	
2004	Resident
2007-2009	Observer
2010-2013	Fellowship Resident
2013-2018	Pediatric Infectious Diseases Specialist
2018-2018	Assistant Professor
2018-	Associate Professor

Work Experiences	
2004-2009	Hacettepe University Faculty of Medicine Department of Pediatrics
2007	Harvard University Faculty of Medicine, Department of Immunology/Rheumatology
2009	Harvard University Faculty of Medicine, Newborn Medicine
2009-2010	Kahramanmaras Pazarcik State Hospital (Mandatory Service)

2010-2013	Hacettepe University Faculty of Medicine, Department of Pediatric Infection Diseases
2013-2018	Hacettepe University Faculty of Medicine, Department of Pediatric Infection Diseases
2018-	Hacettepe University Faculty of Medicine, Department of Pediatric Infection Diseases

Projects

1. Preoperatif barsak temizliği ve/veya perioperatif probiyotik uygulamasının barsak anastomozu üzerine etkileri. HÜBAB (Destek Projesi), Project no: TDS-2015-6415. Sub-investigator.

2. Biomarker Identification via Invivo Microbial Antigen Discovery. Discovery of Secreted Circulating and In Vivo Amplified Bacterial Antigens. Grant award number: HDTRA1-16-1-0055. Sub-investigator.

3. Phylogeography of Francisella Tularensis Subspecies Holarctica from the Country of Turkey. Department of Homeland Security Science and Technology Directorate award number: NBCH2070001 and Cowden Endowment in Microbiology at Northern Arizona University. Project managers [Yasemin Ozsurekci (Turkey) and David M. Wagner (USA)]. Study period: 2011-2013.

4. Genotyping of Turkish Brucella Isolates Using Multiple-Locus Variable-Number Tandem Repeat Analysis and Whole Genome Sequencing. Department of Homeland Security Science and Technology Directorate award number: NBCH2070001 and Cowden Endowment in Microbiology at Northern Arizona University. Project managers [Yasemin Ozsurekci (Turkey) and David M. Wagner (USA)]. Study period: 2010-2015.

5. A Phase III, observer blind, randomized, non-influenza vaccine comparator-controlled, multi-country and multi-centre study of the efficacy of GSK Biologicals' quadrivalent, inactivated, split virion, seasonal influenza vaccine candidate, GSK2282512A (FLU Q-QIV), administered intramuscularly in healthy children 3 to 8 years of age. Sub-investigator. GSK Biological's vaccine clinical study. Study period: 2010-2011.

6. A Phase III, randomised, open, controlled, multicentre, primary vaccination study to evaluate the immunogenicity and persistence of 1 and 2 doses of GlaxoSmithKline

Biologicals' meningococcal conjugate vaccine MenACWY-TT in toddlers (after 1 month and up to 5 years) and to demonstrate noninferiority of co-administration of MenACWY-TT and Pfizer's 13-valent pneumococcal conjugate vaccine Prevenar 13^{TM} versus separate administration of the 2 vaccines. Sub-investigator. GSK Biological's vaccine clinical study. Study period: 2011-2016.

7. A Phase III Clinical Trial to Study the Tolerability and Immunogenicity of V503, a Multivalent Human Papillomavirus (HPV) L1 Virüs-Like Particle (VLP) Vaccine, in 16- to 26- year-old Men and 16- to 26- year-old Women (Protocol no: V503-003). Sub-investigator. MSD vaccine clinical study. Study period: 2014-2015.

8. A Phase III Clinical Trial to Study the Tolerability and Immunogenicity of a 2-dose regimen of V503, a Multivalent Human Papillomavirus (HPV) L1 Virüs-Like Particle (VLP) Vaccine, administered in Preadolescents and Adolescents (9- to 14-year-olds) with a comparison to Young Women (16- to 26-year-olds). (Protocol no: V503-010). Sub-investigator. MSD vaccine clinical study. Study period: 2014-2018.

9. Preoperatif barsak temizliği ve/veya perioperatif probiyotik uygulaması sonrası barsak anastomoz hattındaki yara iyileşme sürecinin kollajen yapımı ve anjiogenezis açısından immünohistokimyasal yöntemler kullanılarak değerlendirilmesi. Project manager. HÜBAB (Hızlı Destek Projesi), Project no: THD-2017-11585. Bugget: 25080,04 TL. Study period: July 2017-November 2018.

10. Çocuk Hastalarda Pnömonide Endotelyal Aktivasyon Belirteçleri ve Oksidatif Stresin Yeri. Project manager. TÜBİTAK (3001-Başlangıç Ar-Ge Projeleri Destekleme Programı), Project no: 216S316. Bugget: 56.899,30 TL. Study period: March 2018-March 2019.

11. Çocuklarda akut ve subakut ensefalitlerin etiyolojisinde viral ve otoimmün etkenlerin dağılımı: Prospektif bir çalışma. Sub-investigator. HÜBAB (Hızlı Destek Projesi), Project no: THD-2017-15724. Bütçe: 29430 TL. Study period: October 2017-April 2019.

12. Kırım-Kongo Kanamalı Ateşi'ne (KKKA) Karşı Viral Enfeksiyonu Önleyici ve/veya Viral Genom Hedefli Terapötik Stratejilerin Geliştirilmesi. (Araştırmacı/Uzman). TÜBİTAK (1001). Proje no: 119S440. Bugget: 581500 TL. Study period: 01.12.2019-01.08.2022.

13. Sağlıklı Bebeklerde Güvenlilik, Tolerabilite ve İmmünojenisite Açısından V114'ün ve Prevenar 13'ün Birbirinin Yerine Kullanılabilirliğini Değerlendiren Faz 3, Çok Merkezli, Randomize, Çift Kör Çalışma (PNEU-DIRECTION)/ V114-027 (MSD). (Sub-investigator).

14. COVID-19 Pozitif Çocuklarda Erişkinlere Kıyasla İyi Klinik Seyir Sitokin ve Kemokin Cevaplarıyla Açıklanabilir mi? Project manager. HÜBAB (Hızlı Destek Projesi), Project no: THD-2020-18774. Bütçe: 35958,14 TL. Study period: May 2020-December 2020.

15. Çocukluk Çağı Toplum Kökenli Pnömonilerinde TLR2 ve TLR4 Tanıda ve Prognozda Kullanışlı Biyobelirteçler Olabilir mi. Project manager. HÜBAB (Hızlı Destek Projesi), Project no: THD-2021-19459. Bütçe: 28996,92 TL. Study period: November 2021-July 2022.
16. CPVID-19 İlişkili MISC Sendromu Patofizyolojisi Sitokin ve Kemokin Cevaplarıyla Açıklanabilir mi? Project manager. HÜBAB (Hızlı Destek Projesi), Project no: THD-2021-19591. Bugget: 33990,0 TL. Study period: September 2021-March 2022.

International Talks

1. Ozsurekci Y. 7th Asia Pacific STD and Infectious Diseases Congress. Osaka, Japan, 23-25 October 2017. Title of talk: Retrospective investigation of Serratia species infection in Tertiary Care Children's Hospital.
2. Ozsurekci Y. 4th International Conference on Vaccines Research δ Development. Baltimore, United States of America, 13-15 November 2018. Speech title: Distribution of seasonal influenza virus and types of influenza: 4 years data from Hacettepe, Turkey.

Graduate Students Advised

Özlem Necipoğlu, MD, Pediatrician, 2022
Dilan Demir, MD, Pediatrician, 2022

University Service

2018-present, The head of hospital infectious diseases control committee in Children’s Hospital, Hacettepe University.

Awards

1. Ozsurekci Y, Karadag Oncel E, Atilla P, Müftüoglu S, Kara A. Investigation of the Effects of Systemic Voriconazole on the Developing Retina in Pigmented Rats-Animal Study. 56th Turkish National Pediatrics Congress, 2012, Girne, Cyprus (**Project Support Award**).
2. Ceyhan M, Ozsurekci Y, Gürler N, Ozkan S, Sensoy G, Belet N, Hacimustafaoglu M, Celebi S, Keser M, Listener EC, Alhan E, Baki A, Oner AF, Uzun H, Kurugol Z, Aycan AE, Gurbuz V, Karadag Oncel E, Celik M, Ozkaya Parlakay A. Serotype distribution of Streptococcus pneumoniae Causing Parapneumonic Empyema in Turkey. 8th National

Pediatric Infectious Diseases Congress, Congress Book, Oral presentation-14, May 2013, Antalya, Turkey (**Scientific Research Competition, First Prize).**

3. Ceyhan M, Ozsurekci Y, Aydin MM, Akcali KC, Talim B, Karadag Oncel E, Gurbuz V, Aycan AE, Onbasilar I, Buzgan T. Determination of Diphtheria Toxin in Myocardial Tissue in Rabbits and a Female Case by Immunofluorescence Antibody Method. 5th National Vaccine Symposium, 25-29 September, 2013, Ankara. (**18th Pfizer Scientific Research Competition, Honorable Mention**).

4. Ceyhan M, Ozsurekci Y, Gürler N, Öksüz L, Aydemir S, Ozkan S, Yuksekkaya S, Keser Emiroglu M, Gultekin M, Yaman A, Kiremitçi A, Yanık K, Karli A, Ozcinar H, Aydin F, Bayramoglu G, Zer Y, Gulay Z, Gayyurhan ED, Gul M, Özakin C, Güdücüoğlu H, Perçin D, Akpolat N, Ozturk C, Camcioglu Y, Karadag Oncel E, Celik M, Virtual L, Uslu H. Invasive Disease in Children in Turkey Serotype Distribution of Causing Streptococcus Pneumoniae: 2008-2014. 9th National Pediatric Infectious Diseases Congress, Congress Book, Oral presentation-11, April 2015, Antalya, Turkey (**Scientific Research Competition, Second Prize**).

5. Ceyhan M, Ozsurekci Y, Gürler N, Camcioglu Y, Salman N, Celik M, Keser Emiroglu M, Akin F, Tezer H, Ozkaya Parlakay A, Tuygun N, Ozkan S, Tamburaci D, Listener EC, Karbuz A, Uluca Ü, İskender Caca A, Alhan E, Çay Ü, Kurugol Z, Karadag Oncel E, Hatipoglu N, Şiraneci R, İnce T, Gülnar Ş, Belet N, Gayyurhan ED, Coskun E, Yilmaz F, Hacimustafaoglu M, Celebi S, Celik Ü, Ozen M, Akaslan A, Devrim İ, Kuyucu N. Bacterial agents causing meningitis and seroprevalence of different serogroups of Neisseria meningitidis, Haemophilus inluenzae type B and Streptococcus pneumoniae during 2013-2014 in Turkey: A multicenter prospective surveillance study. 6th National Vaccine Symposium, 21-25 October, 2015, Ankara (**19th Pfizer Scientific Research, Competition First Prize).**

6. Ozsurekci Y. 7. Asia Pacific Infectious Diseases and Sexually Transmitted Diseases Congress. HÜBAB (**Scientific Achievement Support**), Project no: TBB-2017-13874.

7. Ozsurekci Y, Lucidarme J, Borrow R, Ceyhan M. Characterization of invasive Neisseria meningitidis strains from Turkey, during a period of increased serogroup B disease, 2013-2017. 8th National Vaccine Symposium, 26-29 September, 2019, Ankara (**21st Pfizer Scientific Research Competition, First Prize**).

Peer-Reviewed Publications

1. Kose M, Pekcan S, Dogru D, Akyuz C, Ozcelik U, **Ozsurekci Y**, Gulhan B, Demircin M, Kiper N. Gorham-Stout Syndrome With Chylothorax: Successful Remission by Interferon Alpha-2b. Pediatric Pulmonology 2009; 44: 613-615.
2. **Ozsurekci Y**, Yavuz ST, Alanay Y, Utine GE, Kalayci O. Cowden syndrome with bronchial asthma. Turk J Pediatr 2010; 52: 330-332.
3. Cataltepe O, Arikan MC, Ghelfi E, Karaaslan C, **Ozsurekci Y**, Dresser K , Li Y, Smith TW, Cataltepe S. Fatty Acid Binding Protein 4 is Expressed in Distinct Endothelial and Non-endothelial Cell Populations in Glioblastoma. Neuropathol Appl Neurobiol. 2012;38:400-410.
4. **Ozsurekci Y**, Ceyhan M, Celik M, Kara A, Cengiz AB, Ozgen-Mocan B. Suppurative cervical adenopathy and pharyngeal mass due to tularemia unresponsive to medical treatment. Turk J Pediatr 2011; 53: 554-7.
5. Ceyhan M, Karadag Oncel E, Badur S, Ciblak MA, Alhan E, Celik US, Kurugol Z, Saz EU, **Ozsurekci Y**, Celik M, Parlakay AO. Effectiveness of a new bioequivalent formulation of oseltamivir (Enfluvir(®)) on 2010-2011 seasonal influenza viruses: an open phase IV study. Int J Infect Dis. 2012;16:273-278.
6. **Ozsurekci Y**, Kara A, Cengiz AB, Celik M, Ozkaya-Parlakay A, Karadag Oncel E, Ceyhan M. Brain abscess in childhood: a 28-year experince. Turk J Pediatr 2012;54:144-149.
7. Karadag-Oncel E, **Ozsurekci Y**, Yurdakok M, Kara A. Is liposomal amphotericin B really safety in neonates? Early Hum Dev 2013;89:35-36.
8. Yildiz C, **Ozsurekci Y**, Gucer S, Cengiz AB, Topaloglu R. Acute kidney injury due to acyclovir. CEN Case Rep 2013;2:38-40.
9. Karadag-Oncel E, **Ozsurekci** Y, Kara A, Karahan S, Cengiz AB, Ceyhan M. The value of mean platelet volume in the determination of community acquired pneumonia in children. Ital J Pediatr. 2013 Mar 8;39:16
10. **Özsürekçi Y**, Parlakay AÖ, Cengiz AB, Çelik M, Karadağ Öncel E, Kara A, Kiper N, Cinel G, Oğuz B. Atypical presentation in hydatid disease: hemoptysis. Turkiye Parazitol Derg. 2013;37(1):64-8.

11. Ceyhan M, **Ozsurekci** Y, Gürler N, Ozkan S, Sensoy G, Belet N, Hacimustafaoglu M, Celebi S, Keser M, Dinleyici EC, Alhan E, Baki A, Oner AF, Uzun H, Kurugol Z, Aycan AE, Gurbuz V, Karadag Oncel E, Celik M, Ozkaya Parlakay A. Serotype distribution of Streptococcus pneumoniae causing parapneumonic empyema in Turkey. Clin Vaccine Immunol. 2013;20:972-976.

12. Karadağ-Öncel E, **Ozsürekçi Y**, Ozkaya-Parlakay A, Celik M, Cengiz AB, Haliloğlu M, Ceyhan M, Kara A. Fasciola hepatica infection: clinical and radiological findings in pediatric patients. Turk J Pediatr. 2012 Jul-Aug; 54(4):362-7.

13. Karadağ-Öncel E, **Ozsürekci Y**, Aytaç S, Kara A, Cengiz AB, Ceyhan M. Implantable Vascular Access Port-Associated Bloodstream Infection Caused by Rhizobium radiobacter: A Case Report. Turk J Pediatr. 2013 Jan-Feb;55(1):112-5.

14. **Ozsurekci Y**, Arasli M, Oncel EK, Caglayik DY, Kaya A, Icagasioglu FD, Engin A, Korukluoglu G, Elaldi N, Ceyhan M. Can the mild clinical course of crimean-congo hemorrhagic fever in children be explained by cytokine responses? J Med Virol. 2013; 85:1955-9.

15. Ozkaya-Parlakay A, Ceyhan M, Celik M, **Ozsurekci Y**, Karadag-Oncel E, Sensoy G, Belet N, Keser Emiroglu M, Aycan AE, Caglar M. Evaluation of Pneumococcal PCR and Urinary Antigen Detection in Children. Clin Lab 2013;59(11-12):1409-11.

16. Karadag-Oncel E, Ciblak MA, **Ozsurekci Y**, Badur S, Ceyhan M. Viral etiology of influenza-like illnesses during the influenza season between December 2011 and April 2012. J Med Virol. 2014 May;86(5):865-71

17. Unal S, Alanay Y, Cetin M, Boduroglu K, Utine E, Cormier-Daire V, Huber C, **Ozsurekci Y**, Kilic E, Simsek Kiper OP, Gumruk F. Striking hematological abnormalities in patients with microcephalic osteodysplastic primordial dwarfism type II (MOPD II): A potential role of pericentrin in hematopoiesis. Pediatr Blood Cancer. 2014;61:302-5.

18. Karadağ Öncel E, Ozkaya Parlakay A, **Ozsürekçi Y**, Ceyhan M, Cengiz AB, Kara A. A case of glandular tularemia presenting with prolonged fever and mesenteric lymphadenopathy. Turk J Pediatr. 2013 Jul-Aug;55(4):430-432.

19. Karadag-Oncel E, Erel O, **Ozsurekci Y**, Caglayik DY, Kaya A, Gozel MG, Icagasioglu FD, Engin A, Korukluoglu G, Uyar Y, Elaldi N, Ceyhan M. Plasma oxidative stress and total thiol levels in Crimean-Congo hemorrhagic fever. Jpn J Infect Dis. 2014;67(1):22-6.

20. **Ozsurekci Y**, Karadag Oncel E, Bayhan C, Celik M, Ozkaya-Parlakay A, Arvas M, Ceyhan M. Knowledge and attitudes about human papillomaviruses and immunization among Turkish pediatricians. Asian Pac J Cancer Prev. 2013;14(12):7325-9.
21. Kara A, **Ozsurekci Y**, Tekcicek M, Karadag Oncel E, Cengiz AB, Karahan S, Ceyhan M, Celik MO, Ozkaya-Parlakay A. Length of hospital stay and management of facial cellulitis of odontogenic origin in children. Pediatr Dent. 2014 Jan-Feb;36(1):18E-22E.
22. Ceyhan M, **Ozsurekci Y**, Gurler N, Karadag Oncel E, Celik M, Aycan AE, Gurbuz V, Akyol U, Altintas O, Karabent A, Camcioglu Y, Ada M, Alhan E, Ozgur O. Bacterial Etiology and Pneumococcal Serotypes in Turkish Children with Acute Otitis Media. J Vaccines Vaccin. 2013;5:1
23. Ozkaya Parlakay A, Cengiz AB, Ceyhan M, Hascelik G, Kara A, Celik M, **Ozsurekci Y**, Karadag Oncel E. Evaluation of Multiplex Real Time Polymerase Chain Reaction and Procalcitonin in the Diagnosis of Sepsis. Clin Lab 2014;60:1075-1081.
24. Ozkaya Parlakay A, Kara A, Celik M, **Ozsurekci Y**, Karadag Oncel E, Ceyhan M, Cengiz AB. Early lactic acidosis associated with linezolid therapy in paediatric patients. Int J Antimicrob Agents 2014;44:334-6.
25. Ceyhan M, Gürler N, **Ozsurekci Y**, Keser M, Aycan AE, Gurbuz V, Salman N, Camcioglu Y, Dinleyici EC, Ozkan S, Sensoy G, Belet N, Alhan E, Hacimustafaoglu M, Celebi S, Uzun H, Oner AF, Kurugol Z, Tas MA, Aygun D, Karadag Oncel E, Celik M, Yasa O, Akin F, Coşkun Y. Meningitis caused by Neisseria meningitidis, Haemophilus Influenzae Type B and Streptococcus pneumoniae during 2005-2012 in Turkey: A Multicenter Prospective Surveillance Study. Hum Vaccin Immunother. 2014;10:2706-12.
26. **Ozsurekci Y**, Karadag Oncel E, Oguz B, Ayvaci FN, Kara A. Presternal abscess associated with deep neck infection: an important complication. APSP J Case Rep 2014;5:35
27. **Ozsurekci Y**, Komurluoglu A, Aytac S, Oguz KK, Utine E, Ceyhan M. Partial Distal Aphalangia, Duplication of Metatarsal IV, Microcephaly and Borderline Intelligence: A 4th patient with Parental Consanguinity and Additional Feature of Massive Cerebral Thrombosis. Clinical Dysmorphology 2015;24:29-33.
28. **Özsürekci Y**, Birdsell DN, Çelik M, Karadağ-Öncel E, Johansson A, Forsman M, Vogler AJ, Keim P, Ceyhan M, Wagner DM. Diverse Francisella tularensis strains cause oropharyngeal tularemia in Turkey. Emerg Infect Dis. 2015;21:173-5.

29. **Ozsurekci Y,** Kara A, Bayhan C, Karadag Oncel E, Takci S, Yolbakan S, Korkmaz A, Korukluoglu G. Co-treatment of congenital measles with vitamin A and intravenous immunoglobulin. Case Rep Infect Dis. 2014;2014:234545.
30. Karadag-Oncel E, Kara A, **Ozsurekci Y**, Arıkan-Akdaglı S, Cengiz AB, Ceyhan M, Gur D, Celik M, Ozkaya-Parlakay A. Candidemia in a Pediatric Center and Importance of Central Venous Catheter Removal. Mycoses. 2015;58:140-8.
31. Arasli M, **Ozsurekci Y**, Elaldi N, McAuley AJ, Karadag Oncel E, Tekin IO, Gozel MG, Kaya A, Icagasioglu FD, Yagci Caglayik D, Korukluoglu G, Kokturk F, Bakir M, Bente DA, Ceyhan M. Elevated chemokine levels during adult but not pediatric Crimean-Congo hemorrhagic fever. J Clin Virol 2015;66:76-82.
32. Ceyhan M, **Ozsurekci Y**, Aydin MM, Akcali KC, Talim B, Celik M, Karadag Oncel E, Gurbuz V, Aycan AE, Onbasilar I, Buzgan T. Determination of the presence of Diphtheria toxin in the myocardial tissue of rabbits and a female subject by using an immunofluorescent antibody method. J Clin Med Res. 2015; 7: 472-478.
33. Karadag-Oncel E, Gonc N, Altay O, Cengiz AB, Ozon A, Pinar A, Ceyhan M, Alikasifoglu A, Akyon Y, Kandemir N, **Ozsurekci Y**, Kara A. Prevalence of nasal carriage of methicillin-resistant Staphylococcus aureus in children with diabetes mellitus: Trends between 2005 and 2013. Am J Infect Control. 2015 Jun 3. doi: 10.1016/j.ajic.2015.04.206. [Epub ahead of print]
34. **Ozsurekci Y**, Unal S, Cetin M, Gumruk F. Comparison of ferrous sulfate, polymaltose complex and iron-zinc in iron deficiency anemia. Minerva Pediatr 2015 July 22 [Epub ahead of print].
35. Ceyhan M, **Ozsurekci Y**, Gürler N, Öksüz L, Aydemir S, Ozkan S, Yuksekkaya S, Emiroglu MK, Gültekin M, Yaman A, Kiremitci A, Yanık K, Karli A, Ozcinar H, Aydin F, Bayramoglu G, Zer Y, Gulay Z, Gayyurhan ED, Gül M, Özakın C, Güdücüoğlu H, Perçin D, Akpolat N, Ozturk C, Camcıoğlu Y, Öncel EK, Çelik M, Şanal L, Uslu H. Serotype distribution of Streptococcus pneumoniae in children with invasive diseases in Turkey: 2008-2014. Hum Vaccin Immunother. 2015 Sep 1:0. [Epub ahead of print]
36. **Ozsurekci Y**, Cengiz AB, Duzova A, Sag E, Kadayifcilar S, Dogru Ersoz D, Akcoren Z, Yuce A, Tavil B, Ayvaz D, Akyuz C, Kara Eroglu F. Pediatric-onset adult type sarcoidosis: A case report. Arch Argent Pediatr. 2015 Dec 1;113(6):e336-40.

37. Ozer M, **Ozsurekci Y**, Cengiz AB, Ozcelik U, Yalcin E, Gokoz O. Primary nasal tuberculosis in a 10-year-old girl. Canadian Journal of Infectious Diseases & Medical Microbiology. 2016;2016:9128548.

38. Ozsurekci Y, Aykac K, Cengiz AB, Bayhan C, Sancak B, Karadag Oncel E, Kara A, Ceyhan M. Is colistin effective in treatment of infections caused by multidrug-resistant (MDR) or extremely drug-resistant (XDR) gram-negative microorganisms in children? Diagn Microbiol Infect Dis 2016;85:233-8.

39. Ozsurekci Y, Arikan KO, Bayhan C, Karadag Oncel E, Aycan AE, Gurbuz V, Hascelik G, Ceyhan M. Can procalcitonin be a diagnostic marker for catheter-related bloodstream infection in children? J Pediatr 2016;92:414-20.

40. Emiralioğlu N, Ersöz DD, Oguz B, Saribas Z, Yalcin E, Ozcelik U, **Ozsurekci Y**, Cengiz AB, Kiper N. Pulmonary Mycobacterium abscessus infection in a patient with Triple A syndrome. Journal of Tropical Pediatrics 2016;0:1-4.

41. Ozsurekci Y, Aykac K. Oxidative stress related diseases in newborns. Oxid Med Cell Longev 2016;2016:2763365

42. Aykac K, **Ozsurekci Y**, Tuncer O, Sancak B, Kara A, Ceyhan M. Six cases during 2012-2015 and literature review of Chryseobacterium indologenes infections in pediatric patients. Can J Microbiol 2016 May 17:1-8 [Epub ahead of print]

43. Ceyhan M, **Ozsurekci Y**, Gurler N, Karadag Oncel E, Camcioglu Y, Salman N, et al. Bacterial agents causing meningitis during 2013-2014 in Turkey: A multi-center hospital-based prospective surveillance study. Hum Vaccin Immunother 2016;12:2940-2945.

44. Buyukcam A, Karadag Oncel E, **Ozsurekci Y**, Cengiz AB, Kuskonmaz B, Sancak B. Vancomycin-resistan Enterococcus bacteremia in a child with acute myeloid leukemia: successful treatment with daptomycin. Archivos Argentinos de Pediatria (**Accepted).**

45. Ozer M, **Ozsurekci Y**, Cengiz AB, Emiralioglu N, Dogru D, Oguz KK, Akca O, Ozkayar O. Case of Tuberculous Meningitis With Paradoxical Response In A 14-Year-Old Boy. Case Reports in Infectious Diseases (**Accepted**).

46. Ozcan HN, Gundogdu A, **Ozsurekci Y**, Karakaya J, Oguz B, Unal S, Cetin M, Ceyhan M, Haliloglu M. MRI Findings of Pulmonary Infectious in Pediatric Immunocompromised Patients: Comparison With Multidetector Computed Tomography (MDCT). Pediatric Radiology (**Accepted**).

47. Tanır Basaranoglu S, Karadag Oncel E, Aykac K, **Ozsurekci Y**, Cengiz AB, Kara A, Ceyhan M. Invasive pneumococcal disease: from a tertiary care hospital in the post-vaccine era. Hum Vaccin Immunother 2017; 13(4): 962-964.

48. Ozsurekci Y, Aykac K, Cengiz AB, Tanır-Basaranoglu S, Sancak B, Karahan S, Kara A, Ceyhan M. Bloodstream infections in children caused by carbapenem-resistant versus carbapenem-susceptible gram-negative microorgansims: risk factors and outcome. Diagn Microbiol Infect Dis 2017;87:359-364.

49. Arikan K, **Ozsurekci Y**, Ceyhan M. Bullous pyoderma gangrenosum as the presenting sign of Acute Myeloid Leukemia in a child. J Pediatr Hematol Oncol 2017;39(4):311-313.

50. Ozcan HN, Gormez A, **Ozsurekci Y,** Karakaya J, Oguz B, Unal S, Cetin M, Ceyhan M, Haliloğlu M. Rapid lung magnetic resonance imaging in children with pulmonary infection: reply to Sadhi et al. Pediatr Radiol 2017;47(2):766.

51. Tanır Basaranoglu S, Aykac K, **Ozsurekci Y**, Bajin I, Tavil B, Gumruk F, Ceyhan M. Human Bocavirus: Can It Trigger Hemophagocytic Lymphohistiocytosis? J Pediatr Hematol Oncol 2017;39:e504-e507.

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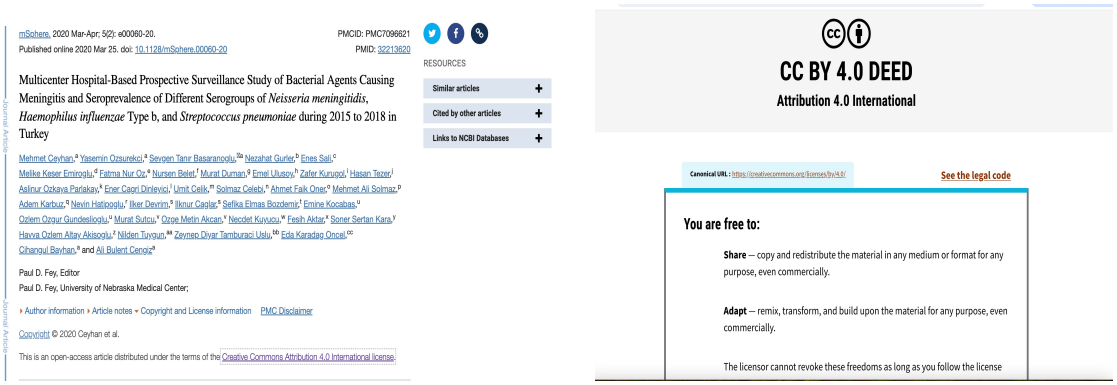

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Multicenter Hospital-Based Prospective Surveillance Study of Bacterial Agents Causing Meningitis and Seroprevalence of Different Serogroups of *Neisseria meningitidis*, *Haemophilus influenzae* Type b, and *Streptococcus pneumoniae* during 2015 to 2018 in Turkey

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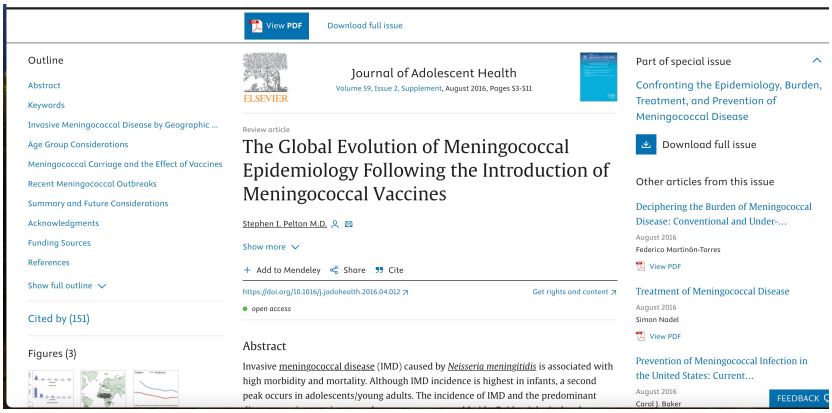

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Immunogenicity and Protective Capacity of CpG ODN Adjuvanted Alum Adsorbed Bivalent Meningococcal Outer Membrane Vesicle Vaccine

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Running Title: Effective Bivalent OMV Based Meningitidis Vaccine

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Abstract

Invasive meningococcal disease (IMD) is caused by *Neisseria meningitidis*, with the main serogroups responsible for the disease being A, B, C, W, and Y. To date, several vaccines targeting N.*meningitidis* have been developed albeit with a short-lived protection. Given that MenW and MenB are the most common causes of IMD in Europe, Turkey, and Middle East, we aimed to develop an outer membrane vesicle (OMV) based bivalent vaccine as the heterologous antigen source. Herein, we compared the immunogenicity, and breadth of SBA based protective coverage of OMV vaccine to X serotype with existing commercial meningococcal conjugate and polysaccharide (PS) vaccines in a murine model. BALB/c mice were immunized with preclinical batches of the W+B OMV vaccine, either adjuvanted with Alum, CpG ODN or their combinations and compared with a MenACYW conjugate vaccine (NimenrixTM, Pfizer) and a MenB OMV-based vaccine (Bexsero®, GSK), The immune responses were assessed through

ELISA and serum bactericidal assays (SBA). Antibody responses and SBA titers were significantly higher in the W+B OMV vaccine when adjuvanted with Alum or CpG ODN, as compared to the control groups. Moreover, the SBA titers were not only significantly higher than those achieved with available conjugated ACYW vaccines but also on par with the 4CMenB vaccines. In conclusion, the W+B OMV vaccine demonstrated the capacity to elicit robust antibody responses, surpassing or matching the levels induced by licensed meningococcal vaccines. Consequently, the W+B OMV vaccine could potentially serve as a viable alternative or supplement to existing meningococcal vaccines.

Key words: Vaccination; adjuvant; meningitis; outer membrane vesicle; *N. meningitidis; Men B; Men W serotypes*

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1 INTRODUCTION

Neisseria meningitidis is an obligate human pathogen and is the major etiological agent of bacterial meningitis and sepsis worldwide. The bacterium inhabits the upper respiratory tract asymptomatically, with a prevalence of 10–35% among healthy young adults in the global population. Invasive meningococcal disease (IMD), which has the highest incidence in infants and adolescents, can rapidly prove fatal, even with treatment (1-3). In survivors, significant sequelae such as neurological disorders, limb loss, and hearing disorders may occur in up to 20% of cases (1,4). Although N. meningitidis currently has 13 defined serogroups, the majority of invasive diseases are associated with six meningococcal serogroups: A, B, C, W, X, and Y. The distribution of serogroups that cause IMD varies by region and changes over time in many parts of the world (1,5,6). Meningococcal serogroup A (MenA) is the dominant serogroup in Africa's sub-Saharan meningitis belt and remains an important causative agent in Asia, including India. On the other hand, meningococcal serogroup C (MenC) poses a threat in North America and Europe. Recently, serogroup B (MenB) accounted for the highest percentage of global IMD cases, while serogroup W (MenW) has been responsible for major epidemics in the Middle East and Africa (6-9).

Vaccination remains the most effective approach to reduce the disease burden and limit long-term sequelae. Following its first report in 1960, the modern polysaccharide vaccine was used in an epidemic within the USA military (10). The Meningococcal ACWY polysaccharide-conjugated vaccine was licensed in the USA in 2005 (11). Strikingly, MenB has become the prominent cause of IMD in Europe and the USA. However, the structural resemblance of the serogroup B capsule to human sialic acid residues has raised concerns about its poor immunogenicity and the possibility of autoimmunity (12). Vaccine development efforts have focused on proteins, including factor H binding protein, neisserial adhesin A, and neisserial heparin binding antigen. These proteins have been formulated for MenB vaccines, of which MenB-FHbp (Trumenba, Pfizer) (13) and 4CMenB in combination with outer membrane vesicles (OMVs, hereafter, Bexero, GSK) (14) are currently approved for use in several countries worldwide (15).

Following the demonstration of serum antibodies against non-capsular antigens, outer membrane proteins (OMPs, hereafter) are considered alternative vaccine antigens to capsular polysaccharides (16). OMVs are complex structures harboring periplasmic protein components, membrane-bound proteins, enzymes, toxins, and peptidoglycan, along with other potent molecular patterns such as LPS, DNA, RNA. They initiate the proinflammatory immune response by stimulating the production of cytokines, chemokines, and antimicrobial peptides upon in vivo administration (17). These vesicles function as transport for toxins and virulence factors, facilitate biofilm formation, modulate immune and stress responses, regulate quorum sensing, and facilitate intraspecies cell-cell communication (18). Moreover, due to the resemblance of OMVs to non-living pathogens that lack the ability to cause disease, they are tested as vaccine candidates and have demonstrated protection against MenB epidemics by stimulating protective immunity. This success has been observed in New Zealand and France, where most cases are caused by the homologous strain (19-22).

Many OMV-based vaccine studies are ongoing. In one study, OMVs extracted from Men A and Men W, when formulated with an Alum adjuvant, were highly immunogenic in mice and induced bactericidal antibodies against both serogroups (16,23). Preliminary results from a Phase 1 study in Cuba have reported that the A+W OMV vaccine is safe and highly immunogenic (20). All these data suggest the possibility of successfully combining MenB OMVs with OMVs derived from other meningococcal serotypes.

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To date, vaccines targeting both ACWY and B serogroups have not been included in the same formulation due to the poor immunogenicity of MenB. We have reported that the two most dominant serotypes in Turkey are MenW and MenB, although they have changed over the years (24,25). This necessitates children receiving repeated doses of vaccines to be covered by these five serotypes, making the childhood vaccination schedule very costly. Furthermore, the recent increase in the prevalence of serotype W infections in Europe has prompted us to develop an OMV-based vaccine from Turkish MenB and MenW strains. In this study, we adjuvanted OMVs with K3 CpG ODN and/or 2% Alhydrogel® (Alum hereafter) and compared the immunogenicity of this candidate vaccine with the currently marketed two vaccine products in mice.

2 MATERIALS AND METHODS

2.1 Vaccine strains

The *Neisseria meningitidis* isolates used for preparation of OMVs were chosen as representative patient isolates from Turkey: the serogroup W (ST-11) and serogroup B (ST-32) (Table 1).

2.2 OMV isolation

Neisseria meningitidis strains were inoculated into 5 ml of Mueller Hinton broth and cultured in a rotary shaker at 37°C with 5% CO_2 overnight. Subsequently, the culture medium was diluted 1:50 with fresh broth, and the bacteria were cultured for an additional 36 hours. Culture supernatants were then harvested by centrifugation at 10,000 Xg for 30 minutes and filtered through a 0.22 μm filter membrane (Corning, USA). This solution constituted secreted OMVs. To eliminate host cell-derived nucleic acids, the harvest was treated with 200 U/ml of Denarase (c-LEcta, Germany) at +4°C overnight. This solution, containing OMVs, was concentrated in PBS using an Amicon 100 kDa filter (Vivaspin® 20, Sartorius, Germany) and treated with 1% sodium deoxycholate at 37°C for 30 minutes. The solution underwent diafiltration using PBS to remove the detergent. OMVs were then purified on a Hi-Screen Capto Core 400 column (Cytiva, Sweden) using the ÄKTA-GO fast protein liquid chromatography system (Cytiva). Protein content was quantified using a BCA protein assay kit (Pierce, USA) following the manufacturer's instructions, and the OMVs were stored at -80°C for further use.

2.3 Characterization of OMVs by AFM and SEM

Purified OMVs were diluted 1:500 in PBS and then adsorbed onto mica sheets. The adsorbed samples were air-dried, and micrometer-scale AFM imaging was conducted in non-contact

dynamic mode (NanoMagnetics Instruments, Turkey) following the manufacturer's instructions. The scans were analyzed using the NMI Image Analyzer software (Turkey). Additionally, a 10 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 before imaging on an environmental SEM (Zeiss Sigma500 FESEM, Germany).

2.4 Nanoparticle analysis of OMVs using 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 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.

2.5 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 polysaccharides A, C, W, and Y, which are 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 two-week interval. The dose for NimenrixTM was 8 µg/0.2 ml, while for Bexsero, it was 5 µg/0.1 ml.

2.6 Vaccine formulation and immunization studies

MenB and MenW OMVs were combined (2.5 µg each per mouse) and formulated with either Alum (20 µg per mouse, 2% Alhydrogel®), or K3-CpG ODN (30 µg per mouse), or a combination of both. Groups of female mice (BALB/c, 6–8 weeks old) were intraperitoneally (i.p.) injected with 5 µg of the OMV vaccine formulation or comparator vaccines, with a 2-week interval (on day 0 and day 14). Two weeks after the booster injection, mice were bled, and their sera were stored at -20°C until further use. Additional details are provided in the corresponding figure legends.

2.7 IgG ELISA

Immulon 2HB ELISA plates (SPL Life Sciences, Korea) were coated with 50 µl/well of isolated OMVs (0.5 µg/ml) in PBS at +4°C overnight. The coated plates were then blocked with 200 µl/well of 5% BSA-PBS-T solution and incubated for 4 hours at room temperature. Afterward, the plates were washed three times with PBS-Tween (0.05%, PBS-T) and finally washed two times with dH2O. Mice sera diluted at 1:50 were serially diluted four-fold with 5% BSA in PBS-Tween (0.05%, PBS-T) into the wells. Next, 50 µl of the serially diluted sera was transferred into ELISA plates and incubated at 4°C overnight. After subsequent washing, 50 µl of ALP-conjugated goat anti-mouse IgG, anti-mouse IgG1, or anti-mouse IgG2a (all IgGs were from Southern Biotech, USA) antibodies were added at a 1:1000 dilution and incubated for 2 hours at room temperature. Following the last washing step, a PNPP (Thermo Scientific, USA.) substrate solution was prepared according to the manufacturer's instructions and added (50 µl/well) for the development of the plates. Consecutive optical density values at 405nm were measured using a microwell plate reader (SpectraMax Molecular Devices, USA) for 5 hours. The final log10 titers were calculated in GraphPad Prism 9 software.

2.8 Serum Bactericidal Activity (SBA) Assay

Neisseria meningitidis MenB, MenW, and MenX target strains were incubated with heat-inactivated animal sera collected 2 months after the booster injection to assess the complement-mediated killing activity of vaccine-induced antibodies. Pooled baby rabbit serum (3-4 weeks sterile, Pel-Freez) was used as the complement source, and sera from non-immunized mice served as negative controls. Bacteria were sub-cultured on blood agar plates (BDTM Columbia agar with 5% sheep blood) and incubated for 18 hours at 37°C with 5% CO_2. Colonies were then inoculated in 4 ml of Brain Heart Infusion broth (BHI) and incubated on a shaker at 37°C, 210 rpm until reaching an OD600 of 0.3. A working dilution of bacteria (1:625 for MenB and MenX; 1:1200 for MenW) was prepared in PBS containing 1% (w/v) BSA and 0.1% (w/v) glucose (Assay buffer). All sera tested in the Serum Bactericidal Assay (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 a 1/72 dilution for MenB; 1/8 dilution for MenW and MenX) to 40 µl of assay buffer previously added to the wells. This mixture was then transferred to the next well. The reaction mixtures were prepared in a 96-well flat-bottom plate in a final volume of 40

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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 MenX), and 10 µl of active baby rabbit serum (BRS) as an external source of complement. The plate was incubated for 1 hour at 37°C, 180 rpm on a rotating shaker. Subsequently, 35 µl of the reaction mixtures were allowed to flow down a blood agar plate using the tilt method. The agar plates were incubated overnight at 37°C with 5% CO_2. 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 ≥ 90% reduction (≥ 50% reduction for the MenX strain) in CFUs after 1 hour compared to the average for controls.

2.9 SDS-PAGE analysis

Protein electrophoresis was conducted using 12% SDS-PAGE. Molecular weight markers and the OMV preparations of *N. meningitidis* serogroup B and W were dissolved in Laemmli buffer containing bromophenol blue. The samples were then heated at 95°C for 5 minutes, and the separation was carried out in a Mini–Protean II 2-D Cell (BioRad, USA) using Mini-PROTEAN TGX stain-free gel. Subsequently, the protein bands were stained with Coomassie brilliant blue and visualized using a ChemiDoc imager (Bio-Rad).

2.10 Proteomics Study

2.10.1 Sample Preparation for Proteomics Studies:

Fifty micrograms of protein from each sample were transferred to microtubes. The proteins were reduced with dithiothreitol (DTT) to achieve a final concentration of 5 mM at room temperature and then alkylated with iodoacetamide (IAA) to reach a final concentration of 50 mM, all while in the dark for 1 hour. Additional DTT was added to reach a final concentration of 10 mM. Proteins were precipitated using the methanol/chloroform precipitation protocol and dissolved in a solution containing 8 M urea and 50 mM Tris buffer at pH 8.5. The urea concentration was then diluted to 1M with 50 mM Tris buffer at pH 8.5. Trypsin was dissolved in 50 mM Tris buffer at pH 8.5 and added to the protein solution at a ratio of 1:100 (w/w, trypsin/protein). The mixture was incubated overnight. Trifluoroacetic acid was added to the incubated solution to stop the enzymatic digestion, setting the final concentration of trifluoroacetic concentration to 0.5% (v/v). The samples were cleaned using Sep-Pak (Waters) according to the manufacturer's protocol. Subsequently, the samples were dried using a Speed-Vac centrifugal evaporator and then dissolved in MilliQ water (18.2 MW.cm) to achieve a protein concentration of 0.5 µg/µl.

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2.10.2 Mass Spectrometry:

LC-MS/MS analyses were conducted using a trapped ion mobility spectrometry (TIMS) – time-of-flight mass spectrometer equipped with a CaptiveSpray nanoBooster source (Bruker Daltonics, Bremen, Germany) coupled to an Ultimate 3000 RSLCnano system (Dionex, Thermo Scientific, USA). The mass spectrometer and the LC were controlled by Hystar 4.1 (Bruker Daltonics), and data analysis was performed using DataAnalysis 5.0 (Bruker Daltonics). One microliter of the purified peptide solution was loaded onto a C18 nanotrap column (C18 PepMap 100, 100 µm x 2cm, 5 µm, 100 Å; Dionex) with a loading solvent (water, 0.05% TFA) at a flow rate of 15 µL/min for 125 minutes. The analytes were separated on a C18 analytical column (Acclaim PepMap 100, 75 µm x 50 cm, 2 µm, 100 Å; Dionex) at a column oven temperature of 60°C. Elution was carried out at a flow rate of 0.4 µL/min with solvent A (water containing 0.1% FA, v/v) and solvent B (acetonitrile containing 0.1% FA, v/v). A linear gradient of 2–35% solvent B for 90 minutes was applied, followed by column washing and reconditioning. Ionization using CaptiveSpray nanoBooster was enhanced with acetonitrile at 0.2 bar. The source parameters were as follows: dry gas 3.0 L/min with 150°C; capillary voltage 1600 V. Mass spectra were acquired within a mass range of 150-3000 m/z and a spectra rate of 2 Hz. MS/MS parameters included a 3.0 sec. cycle time, absolute threshold 2500 cts, smart exclusion 5x, exclusion after one spectrum, and release after 0.40 min.

2.10.3 LC-MS/MS Data Analysis:

Qualitative LC-MS/MS data in string format were parsed to extract protein symbols for each of the three sample groups separately. Venn diagrams were generated using the "ggVennDiagram" function in the R package ggVennDiagram v1.2.3. Ontology analyses for over-representation were conducted using the R package ReactomePA v1.28.053. A STRING v12.0 PPI network was generated with a minimal interaction score of 0.400 (and an FET p-value of 5e-02). A hypergeometric test was performed to compute the statistical significance of the overlap between two groups of proteins based on the Venn diagram.

2.11 HEK-TLR4 endotoxicity

HEK-Blue™ hTLR4 cells (Invivogen, France) 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% CO_2. Twenty µl of sample supernatants were mixed and then incubated with 180 µl of QUANTI-Blue solution (Invivogen) for 30 minutes. The optical density (OD) values were measured at 655 nm.

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2.12 In vivo Toxicity

Groups of female mice (BALB/c, 6–8 weeks old, n=5) were intraperitoneally injected with varying doses of W+B OMVs (1.66, 5, 15, and 45 µg per animal, with an equal amount of 50% for both W and B OMVs). Their body weight, food consumption, and water intake were monitored over a five-day period.

2.13 Statistical Analysis

Statistical differences in all treatment groups were analyzed using GraphPad Prism 9 statistical software. Groups were compared by one-way ANOVA with Dunnett's multiple comparisons 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 RESULTS

3.1 Purification and Characterization of N. meningitidis OMVs:

Native outer membrane vesicles (OMVs) were extracted from the culture supernatant of *Neisseria meningitidis* serogroup W (ST-11) and serogroup B (ST-32). The purification process, depicted in Figure 1A, involved loading Denarase-treated and clarified cell culture supernatant onto a HiScreen CaptoCore 400 multimodal size-exclusion/ion-exchange chromatography column. The resulting pooled flow-through fraction was then subjected to ultrafiltration and diafiltration. The structural characterization of OMVs was conducted using atomic force microscopy (AFM, as shown in Figure 1B), scanning electron microscopy (SEM, as depicted in Figure 1C), tunable resistive pulse sensing (TRPS, as illustrated in Figure 1D), and SDS-PAGE to visualize major membrane proteins.

AFM, SEM, and TRPS analyses collectively confirmed the spherical, vesicular nature of the OMVs, measuring 92±33 nm and 97±38 nm in diameter for MenW and MenB OMVs, respectively. Coomassie brilliant blue-stained SDS-PAGE analysis (Figure 1E) of the OMVs revealed the presence of five predominant membrane proteins: NadA, PorA, PorB, RmpM, and OpC. To assess the LPS content within OMVs obtained from strains isolated with or without the presence of sodium deoxycholate, TLR4 activation was tested using HEK-Blue reporter cells expressing human TLR4. Following stimulation with varying concentrations of OMVs (Figure

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1F), comparable TLR4 activation profiles were observed between LPS and native OMVs. However, OMVs purified after detergent treatment exhibited significantly diminished h-TLR4 response, necessitating 10 to 100-fold higher concentrations of OMVs to achieve similar levels of TLR4 activation as seen with LPS. Collectively, these findings support that extracellular OMV nanovesicles derived from two strains of Neisseria meningitidis display convincing outer membrane vesicle features with reduced LPS activity.

To determine the optimal dosage of OMVs for use as an antigen in vaccine formulation, we examined the potential systemic toxicity of both high and low doses in mice. The mice were intraperitoneally injected with varying doses of OMVs (1.66, 5, 15, and 45 µg per animal), and their mortality rate, body weight, food consumption, and water intake were monitored for a five-day period. Throughout the dosing and recovery phases, no OMV-related mortality was observed. While no significant impact on body weight was noted in mice that received low OMV doses (1.66 and 5 µg per animal), those exposed to high doses (15-45 µg per animal) exhibited noteworthy weight loss compared to the control group (Figure S1A). Additionally, no significant changes in food and water consumption were detected in groups administered with low doses, whereas high OMV doses had a significant effect on these parameters (Figure S1B-C). Consequently, based on these findings, an optimal dose of 5 µg of OMVs was identified as having no systemic toxic effects for vaccine formulation.

After proteomics profiling of these OMVs, we found that omvB and omvW are quantitatively richer in content than the licensed vaccine (Figure 2A). When the degree of overlap of the protein content among these three different sample groups was analyzed, the ontology term "cell outer membrane" appeared enriched within the shared proteins by all groups, as expected (Figure 2B). Of note, a more comprehensive functional enrichment analysis managed to identify, as expected, energy metabolism that is enriched in the same subgroup of proteins (Figure 2C) for all vaccines investigated. As a result, we concluded that the proteomics results of the OMV cargo are both biologically relevant and highly comparable to the protein content of the licensed vaccine.

3.2 Immunogenicity of W+B OMV Vaccine:

To evaluate if a combination of OMVs from serogroups B and W, the most prevalent serogroups in the Middle East and Europe, can be used as a vaccine, potentially offering protection against a broader spectrum of meningococci, we developed a bivalent vaccine and conducted a

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comparative analysis with commercially available vaccines. To assess the immunogenicity of the B+W OMV vaccine, 6-to 8-week-old female BALB/c mice were intraperitoneally 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 B+W OMVs or in combination with K3 CpG ODN (30 µg/mouse) or Alum (20 µg/mouse). A conjugate vaccine ACYW (NimenrixTM, Pfizer) and serogroup B vaccine 4CMenB (Bexsero, GSK) 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).

Mice immunized with W+B OMV or adjuvanted counterparts had higher anti-MenW and MenB-specific IgG, IgG1, and IgG2a levels after booster injections (Figure 3 and Fig. S2). As expected, the W+B 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 4CMenB vaccine showed the direct protection afforded by 4CMenB against MenW:cc11 disease (26). Consisting 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 (Figure 3A and Fig. S2A). W+B OMV+Alum elicited 14-fold more anti-MenW OMV IgG when compared to its formulated W+B OMV counterpart (Fig. S2A), indicating that the Alum-adjuvanted OMV vaccine is more immunogenic than only W+B OMV. Mice vaccinated with W+B OMV and its adjuvanted counterparts exhibited similar antibody responses to Bexsero (Figure 3B and S2B).

3.3 Comparison of the W+B OMV Vaccine and Commercially Available Vaccines in SBA

Mice immunized with the W+B OMV vaccine and its adjuvanted counterparts developed bactericidal antibodies against the MenW vaccine strain clonal complex ST-11 and MenB clonal complex ST-32 two months after the booster injection. Notably, all groups that received the W+B OMV vaccine exhibited higher efficacy in eliciting Serum Bactericidal Assay (SBA) titers against the MenW strain, in comparison to the Nimenrix vaccine (Figure 4A and 4C). All vaccine groups except the W+B OMV+Alum induced similar SBA titers against the MenB strain, while the W+B OMV+Alum group had significantly higher SBA titers compared to Bexsero (Figure 4B and 4D). Furthermore, W+B OMV+Alum vaccine and Bexsero were tested against the MenX

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strain, and W+B OMV+Alum was found to be strongly bactericidal against this strain as well compared to Bexsero (Fig. S3).

4. DISCUSSION

Here, we describe the assessment of safety, immunogenicity and the SBA-based protective capacity of the W+B OMV vaccine, that are not found in any currently available vaccines licensed for human. Our candidate formulations induced a strong humoral response, and throughout the study, no adverse events were recorded in immunized animals. Since serum bactericidal antibodies (SBA) are considered as the "Gold Standard" for correlate for protection against meningococcal disease, we determined SBA titers, and OMVs from MenW and MenB strains were shown to induce bactericidal antibodies in immunized mice. Furthermore, the W+B OMV vaccine yielded antibody titers against MenB as high as those induced by the available vaccine, Bexsero®. Combining two OMVs in one vaccine did not seem to interfere with the immune response to the individual OMV types.

In this study, we have developed an immunogenic and potentially protective bivalent MenW+MenB OMV vaccine that could also protect against the most common serotypes not only in Turkey but also in Europe. While MenB remains the leading cause of invasive meningococcal disease (IMD) in Europe, serogroup W is the second most common cause according to recent findings (27). Furthermore, the substantial SBA titers of the MenW+MenB OMV+Alum combination against the MenX serotype, which is not included in any commercial vaccine available, allowed us to predict the breadth of protection of our bivalent OMV vaccine.

The selection of the suitable adjuvant to be included in the vaccine is as important as the selection of the target antigen. To enhance the immunogenicity of inactivated vaccines, adjuvants, such as aluminum compounds and salts, have been used for many years (28). Aluminum hydroxide has also been an adjuvant option for MenB OMV vaccines in Norway, New Zealand, and Cuba (29-31). However, aluminum-containing adjuvants have limitations, such as local reactions, increased IgE-mediated responses, ineffectiveness with certain antigens, and an inability to enhance cell-mediated immune responses, particularly cytotoxic T cell responses (28). Since aluminum hydroxide primarily stimulates a humoral B cell response, new adjuvants have been explored in our novel vaccine platforms. One of these adjuvants is CpG-based adjuvants, which have never

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been used in vaccine studies developed against meningococci. The effectiveness of this adjuvant has not been evaluated until now.

The Bexsero® vaccine was shown to induce high IgG antibodies and SBA titers against MenW. Amino acids play essential roles in the survival of organisms, and many of them are involved in immune functions and host-pathogen crosstalk. Histidine, an essential amino acid, regulates various cellular processes and catalytic activities (32,33). Histidine biosynthesis products found in both MenW and MenB OMVs might contribute to the anti-MenW IgG response in mice injected with MenB OMV-based vaccines. OMV-based meningococcal vaccines appear to be promising candidates for preventing IMD with broader serotype coverage, but further experiments, including new serotypes, are needed to confirm this idea.

There are some limitations to the current study. First, meningococcal OMVs have been studied as candidate vaccines, but the isolation of OMVs involves treatment with detergents to reduce the possible adverse effects associated with LPS. Detergent-treated and native OMVs have different proteomic profiles, and detergent treatment has the risk of removing protective antigens from the outer membrane (34,35). In this study, we chose to decrease the antigen dosage instead of using detergent treatment and did not observe any systemic side effects in animals. Second, while we couldn't perform an in-depth analysis of the interaction network, a quantitative proteomics analysis with more biological replicates could provide further insight into the specific proteins contributing to the observed immunogenicity provided by these OMVs.

CONCLUSION

The results presented here are the first ‘proof of principle’ that a bivalent W+B OMV vaccine may have the potential to give broad protection against meningococcal disease. Moreover, such a vaccine could also protect against other serotypes that express identical subcapsular antigens, as shown here for a serogroup X strain. OMV vaccines are relatively simple and inexpensive to manufacture, and a bivalent W+B OMV vaccine could serve as an alternative or a supplement to the existing polysaccharide/conjugate vaccines against epidemics of meningococcal disease in the Europe and Middle East.

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CONFLICT OF INTEREST

Yasemin Ozsurekci receives lecture fees from Pfizer. All other Authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS: IG and MG designed the study. IG, MG, TCY, and YO analyzed the data and wrote the manuscript, TCY, YO, MY, KA, IE, conducted the in vitro and in vivo experiments. UG and BS, executed proteomics assays, VY, did the bioinformatics evaluations. All authors contributed to the article writing/editing and approved the submitted version.

DATA AND MATERIALS AVAILABILITY: All data are available in the main text or the supplementary materials.

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Tables

Table 1. Characteristics of Neisseria meningitidis vaccine strains used in this study. Results are generated from multilocus sequence type (MLST)

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-11 complex
53401	54	Turkey/2016	X	7-1	1	F4-1	5799	ST-22 complex

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Figure Legends

Figure 1: Production and Characterization of the OMV-Based Meningococcal Vaccine

(A) Schematic representation of *Neisseria meningitidis* OMV production, purification, and vaccine formulation process. (B) Atomic force microscopy images and (C) scanning electron microscopy images of individual MenW and MenB strain OMVs. Representative images from three independent experiments are shown. (D) TRPS size distribution measurement (nm) and particle number of OMVs. (E) Coomassie brilliant blue-stained SDS-PAGE analysis of the OMVs of *Neisseria meningitidis* serogroup W and B. (F) Comparison of endotoxic responses from LPS, native OMVs, and SDOC-treated OMVs.

Figure 2: Proteomics Profiling of OMV-Based Meningococcal Vaccines

(A) A Venn diagram showing the degree of overlap of the protein content among three different vaccine groups. The color code reflects the number of unique or shared proteins in each sector. The p-values in parentheses, derived from a hypergeometric test, reflect the statistical significance of the overlap between the corresponding pairs of sample groups. The protein contents in three key sectors (circled in the diagram) were disclosed after filtering out redundant ribosomal proteins. (B) Taking the shared proteins among all groups (32; the number of proteins left after filtering out ribosomal proteins) as input, a STRING PPI analysis identified enrichment of the cell outer membrane in a subnetwork of these proteins, as expected. Nodes represent genes, and edges represent interactions. The color-coded edge thickness indicates interaction strength by the type of interaction between any pairs of proteins in the network. PPI enrichment p-value < 0.0106; minimum required interaction score, 0.400. (C) Ontology level enrichment by GO identifiers of energy metabolism terms within the same query list as in B.

Figure 3: W+B OMV Vaccine Elicits Robust Antibody Responses in Mice

BALB/c mice (n=16/group) were immunized on days 0 and 14 with 2.5µg MenW OMVs plus 2.5µg MenB OMVs with or without Alum (20µg/mouse), with or without K3 CpG ODN (30µg/mouse) or with 20µg Alum+30µg CpG ODN. Comparator BALB/c mice (n=8/group) were injected on days 0 and 14 with 100µl of 4CMenB vaccine (Bexsero®, GSK, includes 25 µg MenB OMV in 0.5 ml for human dose) or 200µl of MenACYW conjugate vaccine (NimenrixTM, Pfizer). Sera were collected 2 weeks post-boost and assessed for (A) MenW-

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OMV and (B) MenB-OMV-specific IgG, IgG1, and IgG2a by ELISA. Vaccinated groups were compared by one-way ANOVA with Dunnett's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data are presented as Mean ± SEM.

Figure 4: Serum Bactericidal Activity (SBA) Titers in Sera

SBA responses against (A, C) MenW and (B, D) MenB strains after 2 doses of the W+B OMV vaccine, NimenrixTM, and Bexsero®. Data in (A) and (B) are shown as box plots; whiskers show min to max data points. (C, D) Representative pictures of blood agar plates from each vaccinated group showing CFUs. The bactericidal antibody titer was expressed as the reciprocal of the final serum dilution yielding ≥ 90% killing after 1 hour as compared with the average for controls. Vaccinated groups were compared by one-way ANOVA with Dunnett's multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data are presented as Mean ± SEM.

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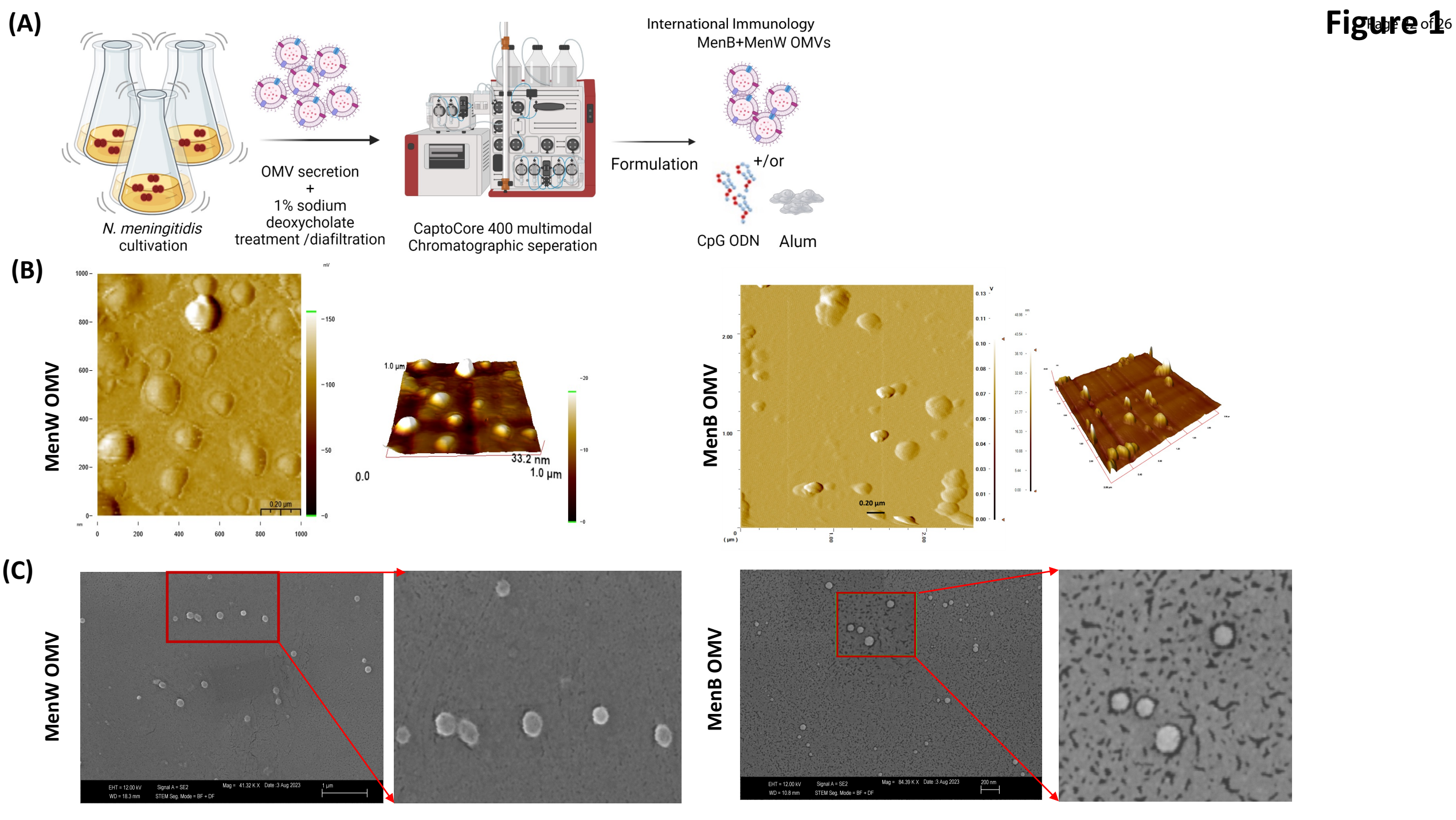
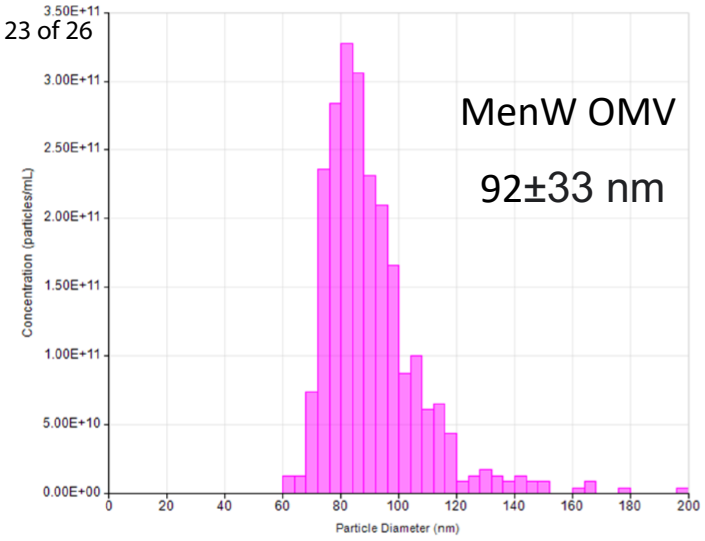
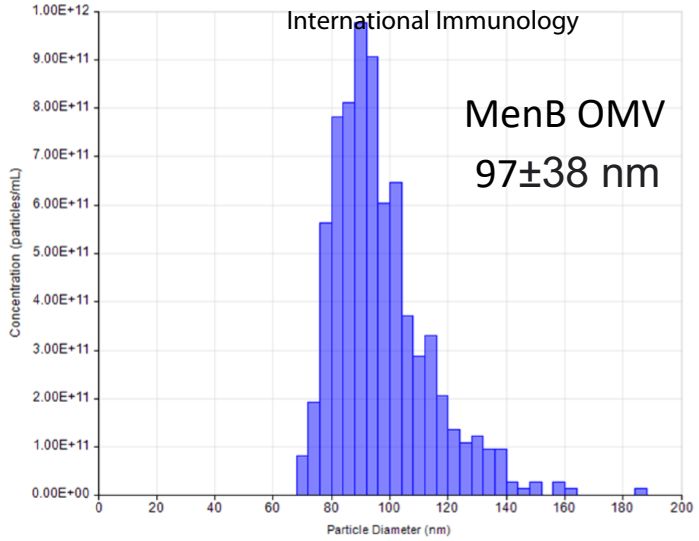
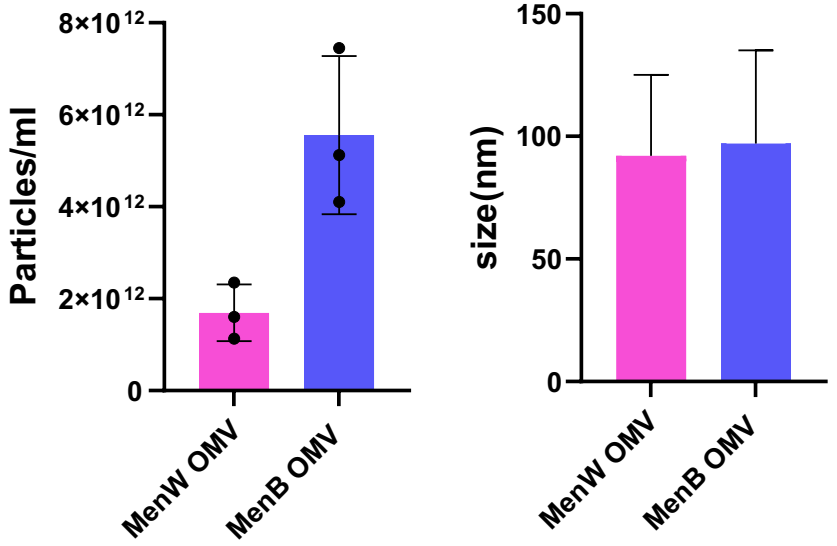
Figure 1


(A)
International Immunology
MenB+MenW OMVs
N. meningitidis
cultivation
OMV secretion
+
1% sodium
deoxycholate
treatment /diafiltration
CaptoCore 400 multimodal
Chromatographic seperation
Formulation
+/or
CpG ODN
Alum
(B)
MenW OMV
0.20 µm
1.0 µm
33.2 nm
1.0 µm
0.0
MenB OMV
0.20 µm
(C)
MenW OMV
MenB OMV

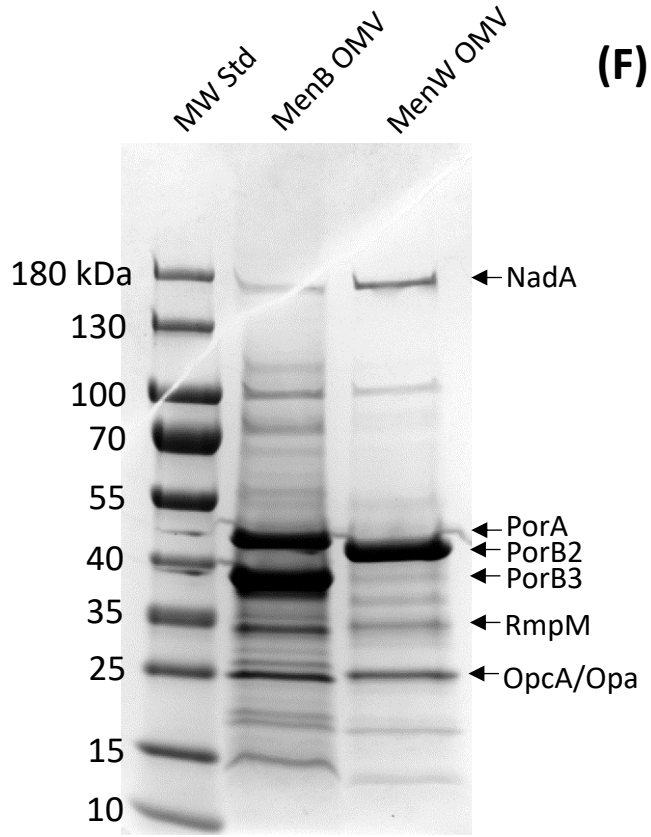
(D)


MenW OMV
92±33 nm
Concentration (particles/mL)
Particle Diameter (nm)

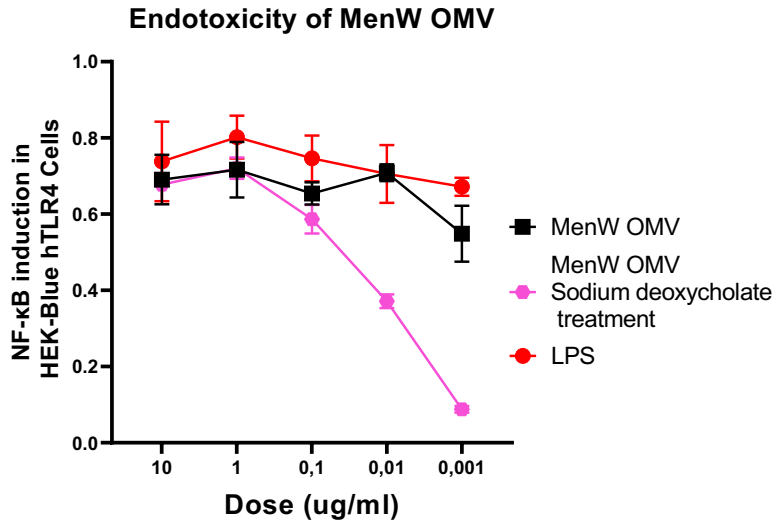

MenB OMV
97±38 nm
Concentration (particles/mL)
Particle Diameter (nm)


Particles/ml
size(nm)
MenW OMV
MenB OMV

(E)


MW Std
MenB OMV
MenW OMV
180 kDa
130
100
70
55
40
35
25
15
10
NadA
PorA
PorB2
PorB3
RmpM
OpcA/Opa

(F)


Endotoxicity of MenW OMV
NF-κB induction in HEK-Blue hTLR4 Cells
Dose (ug/ml)
MenW OMV
MenW OMV Sodium deoxycholate treatment
LPS

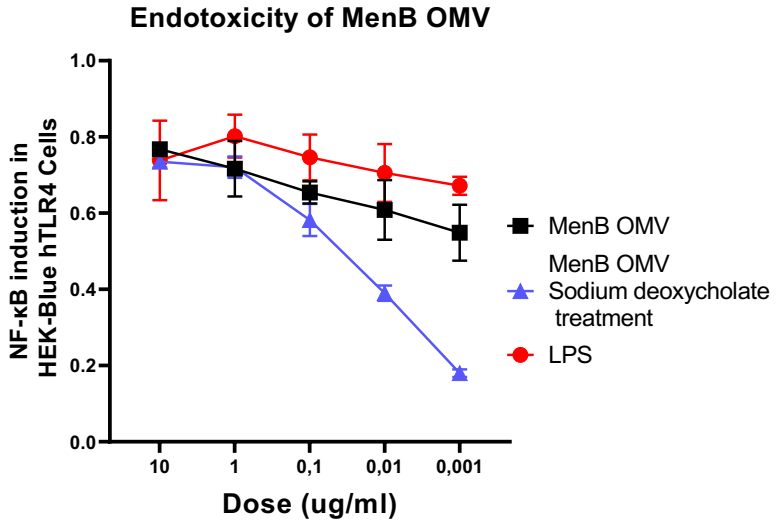

Endotoxicity of MenB OMV
NF-κB induction in HEK-Blue hTLR4 Cells
Dose (ug/ml)
MenB OMV
MenB OMV Sodium deoxycholate treatment
LPS

Figure 1

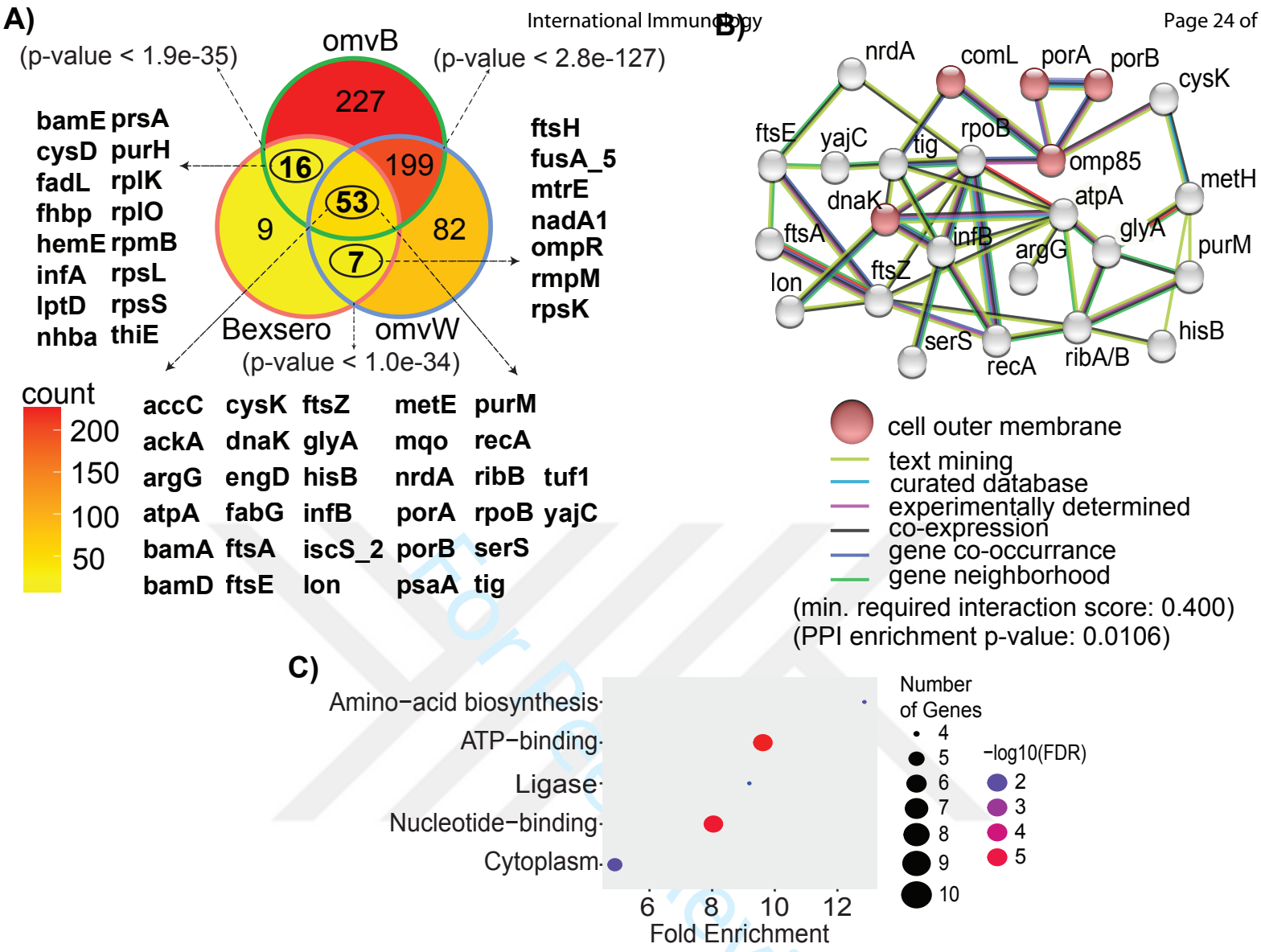


Figure 2. Proteomics profiling of OMV-based meningococcal vaccines. **A)** A Venn diagram that shows the degree of overlap of the protein content among 3 different vaccine groups. The color code reflects the number of unique or shared proteins in each sector. The p-values in parentheses, which were derived from a hypergeometric test, reflects the statistical significance of the overlap between the corresponding pairs of the sample groups. The protein contents in 3 key sectors (circled in the diagram) were disclosed after filtering out redundant ribosomal proteins. **B)** Taking the shared proteins among all groups (32; the number of proteins left after filtering out ribosomal proteins) as input, a STRING PPI analysis identified enrichment of the cell outer membrane in a subnetwork of these proteins, as expected. Nodes, genes; edges, interactions. The color-coded edge thickness explains interaction strength by the type of interaction between any pairs of proteins in the network. PPI enrichment p-value < 0.0106; minimum required interaction score, 0.400. **C)** The ontology level enrichment by GO identifiers of energy metabolism terms within the same query list as in B.

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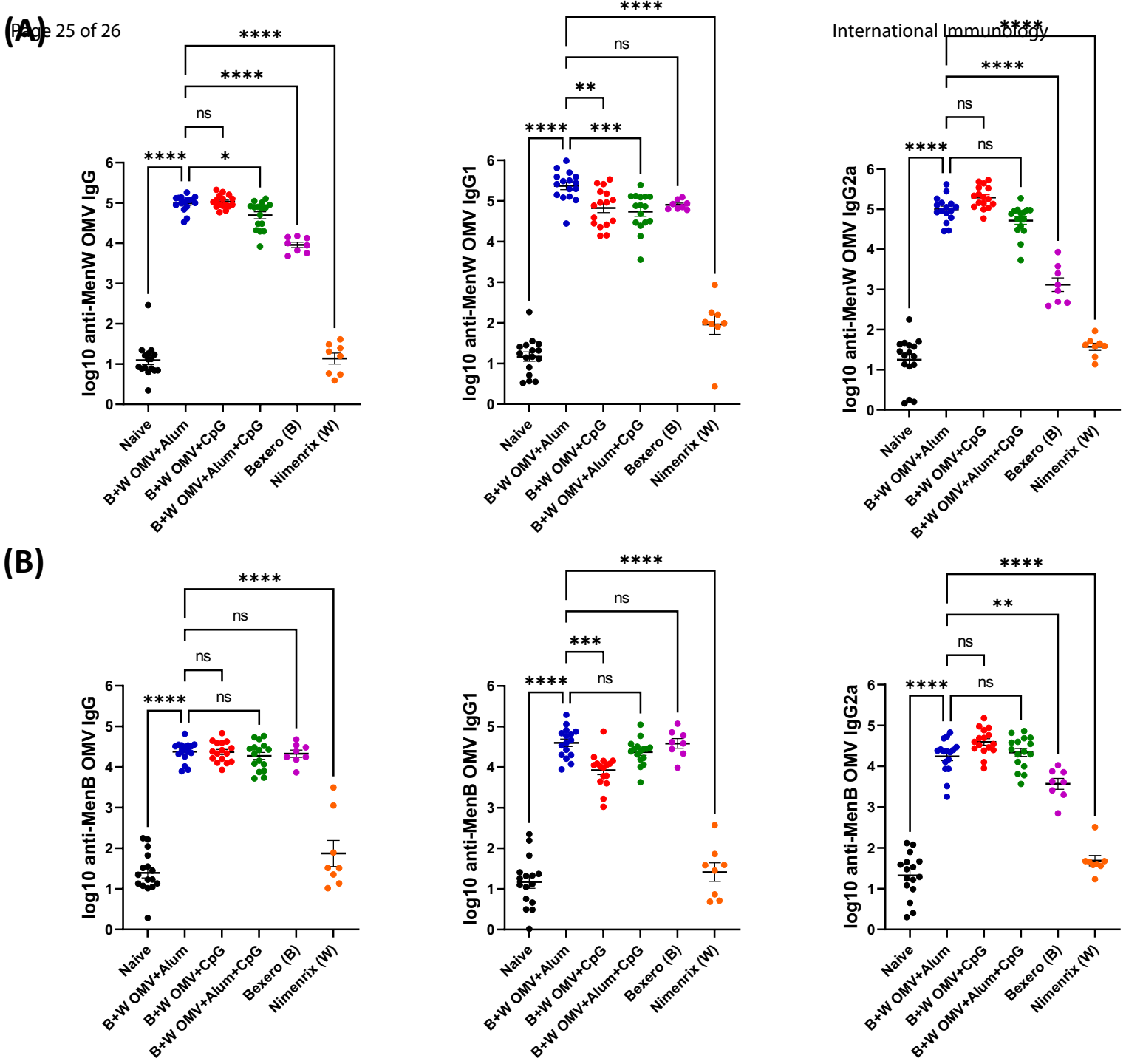
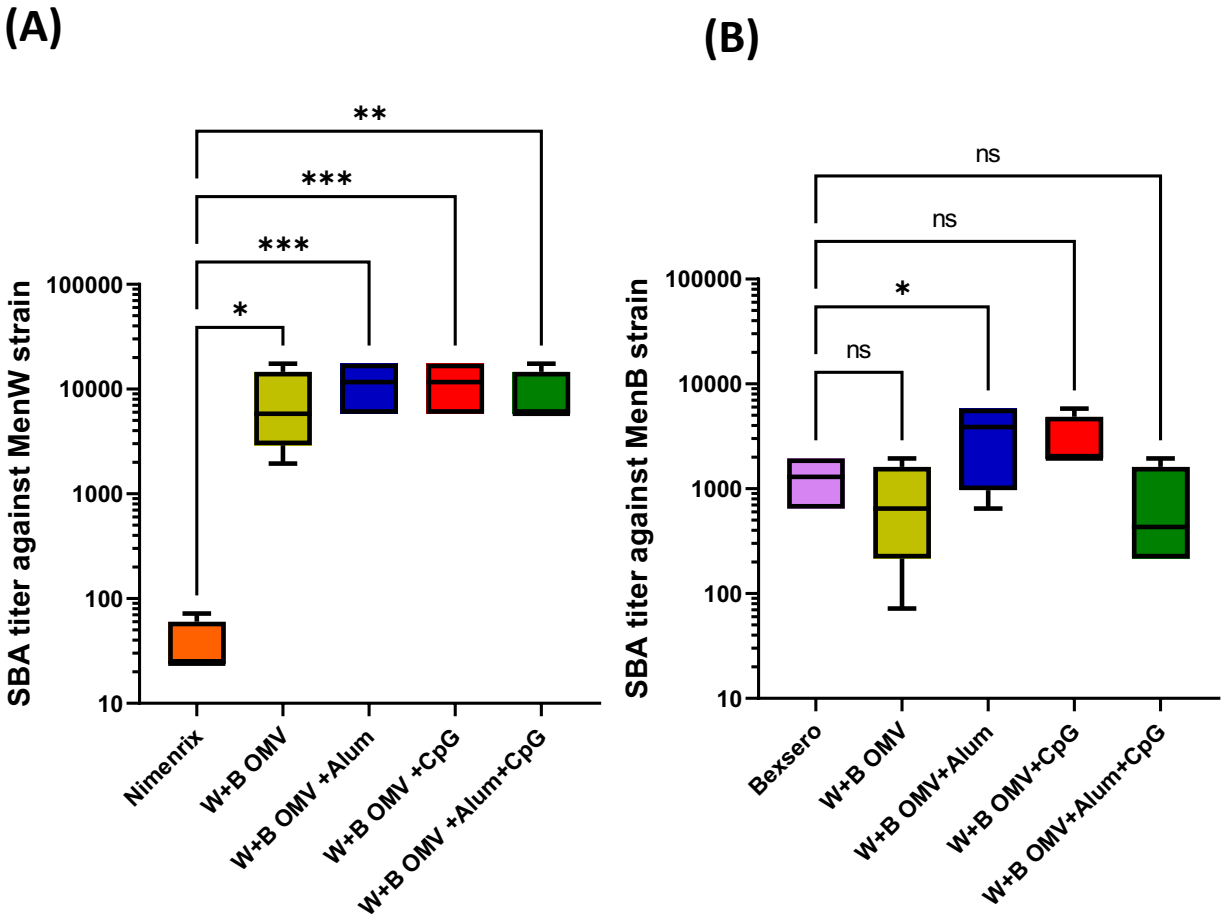
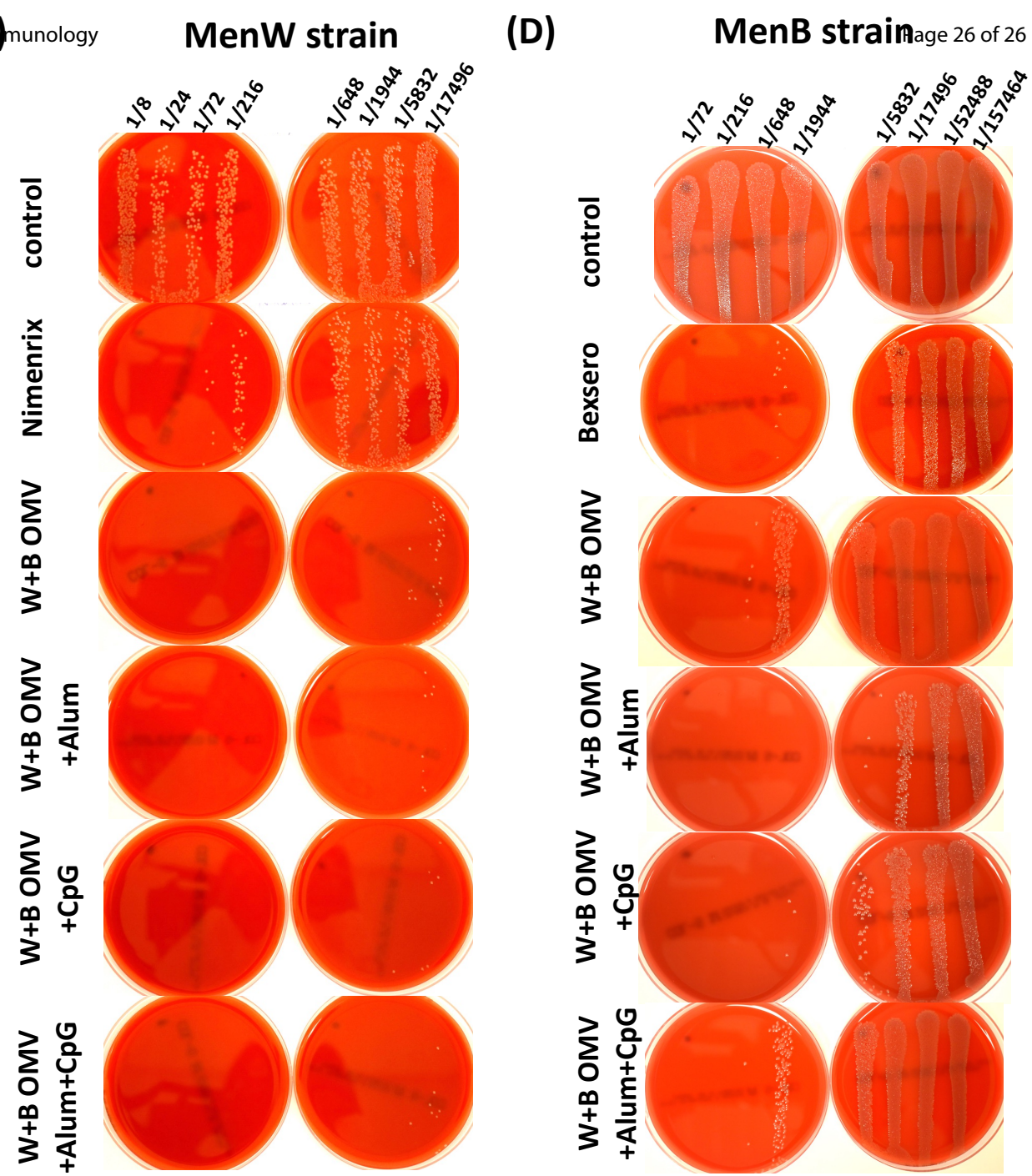
Figure 3


(A)
log10 anti-MenW OMV IgG
log10 anti-MenW OMV IgG1
log10 anti-MenW OMV IgG2a
(B)
log10 anti-MenB OMV IgG
log10 anti-MenB OMV IgG1
log10 anti-MenB OMV IgG2a
Naive
B+W OMV+Alum
B+W OMV+CpG
B+W OMV+Alum+CpG
Bexero (B)
Nimenrix (W)
****
ns
*
**
***


(A)
SBA titer against MenW strain
100000
10000
1000
100
10
**
***
***
*
Nimenrix
W+B OMV
W+B OMV +Alum
W+B OMV +CpG
W+B OMV +Alum+CpG
(B)
SBA titer against MenB strain
100000
10000
1000
100
10
ns
ns
*
ns
Bexsero
W+B OMV
W+B OMV+Alum
W+B OMV+CpG
W+B OMV+Alum+CpG


(C)
MenW strain
1/8
1/24
1/72
1/216
1/648
1/1944
1/5832
1/17496
control
Nimenrix
W+B OMV
W+B OMV +Alum
W+B OMV +CpG
W+B OMV +Alum+CpG
(D)
MenB strain
1/72
1/216
1/648
1/1944
1/5832
1/17496
1/52488
1/157464
control
Bexsero
W+B OMV
W+B OMV +Alum
W+B OMV +CpG
W+B OMV +Alum+CpG