

**ASSESSMENT OF IMMUNE POTENCY OF SARS-CoV-2 VLP
VACCINE IN MICE, ENHANCED THROUGH DIFFERENT
ADJUVANTS AND LIPOSOME COMPLEXATION**

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By
İrem Fatma Abraş
August 2023

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By İrem Fatma Abraş

August, 2023

We certify that we have read this dissertation and that in our opinion is fully adequate, in scope and in quality, as a thesis for degree of Master of Science.



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ABSTRACT

ASSESSMENT OF IMMUNE POTENCY OF SARS-CoV-2 VLP VACCINE IN MICE, ENHANCED THROUGH DIFFERENT ADJUVANTS AND LIPOSOME COMPLEXATION

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M.Sc. in Molecular Biology and Genetics

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August 2023

SARS-CoV-2, emerging in December 2019 in Wuhan, China, led to a swift global pandemic declaration in March 2020, prompting widespread vaccine development. Most vaccines target specific regions, mainly the spike protein. In our lab, we have employed an innovative virus-like particle (VLP) vaccine approach encompassing all four structural proteins of the virus: spike, nucleocapsid, membrane, and envelope. Our study utilizes sterically stabilized cationic liposomes (SSCL) to encapsulate VLPs expressing the Delta variant spike protein, along with various adjuvants: CpG ODN, poly(I:C), and 2'3'-cGAMP. We characterized liposomes using tunable resistive pulse sensing for size and concentration. In C57BL/6 mice, we administered primary and two booster injections on Day 0, Day 15, and Day 73, respectively, collecting blood samples at intervals (Day 14, Day 28, Day 42, Day 72, and Day 90). To assess vaccine impact on mouse humoral immunity, we conducted ELISAs for total IgG, IgG1, and IgG2c antibodies against recombinant Spike and the receptor-binding domain (RBD). IgG titers increased until Day 42, remained stable or slightly decreased on Day 72, and significantly rose on Day 90. We calculated IgG2c/IgG1 ratios, reflecting Th1 immune responses, revealing enhanced cellular immunity potential in groups with adjuvants compared to the VLP-only group. This study underscores the effectiveness of our VLP vaccine strategy in stimulating robust immune responses and opens avenues for further research and development.

Keywords: SARS-CoV-2, VLP, Liposome, CpG, poly(I:C), 2'3'-cGAMP, vaccine

ÖZET

FARKLI ADJUVANTLAR VE LİPOZOM KOMPLEKSASYONUyla GELİŞTİRİLMİŞ SARS-CoV-2 VLP AŞININ FARELERDEKİ BAĞIŞIKLIK GÜCÜNÜN DEĞERLENDİRİLMESİ

İrem Fatma Abraş

Moleküler Biyoloj ve Genetik, Yüksek Lisans

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SARS-CoV-2, Aralık 2019'da Çin'in Wuhan kentinde ortaya çıkarak milyonlarca insanı hızla enfekte etti. Mart 2020'de COVID-19 pandemisi olarak ilan edildi ve dünya çapında birçok bilim insanını virüse karşı aşı geliştirmeye yöneltti. Geliştirilen ve geliştirilmekte olan aşılardan çoğu spike proteini veya spike proteinin belirli bir bölgesini hedeflemektedir. Laboratuvarımız, virüsün dört yapısal proteinini de; spike, nucleocapsid, membrane and envelope dahil eden bir virüs benzeri parçacık (VLP) aşı stratejisi kullanmıştır. Bu çalışmada, Delta varyantı spike'ı eksprese eden VLP'leri üç adjuvanın; CpG ODN, poly(I:C) ve 2'3'-cGAMP, farklı kombinasyonları ile birlikte kapsüllemek için sterik olarak stabilize edilmiş katyonik lipozomlar (SSCL) hazırlandı. Lipozomları boyut ve konsantrasyon açısından karakterize etmek için ayarlanabilir dirençli darbe algılama yöntemi kullanıldı. Aşı formülasyonları ile in vivo deney, C57BL/6 fareleri üzerinde gerçekleştirildi. Primer ve iki rapel enjeksiyon sırasıyla 0. Gün, 15. Gün ve 73. Günde gerçekleştirildi. Hayvanlardan düzenli aralıklarla (14. Gün, 28. Gün, 42. Gün, 72. Gün ve 90. Gün) kan alındı. Rekombinant Spike ve reseptör bağlama alanına (RBD) karşı toplam IgG, IgG1 ve IgG2c ELISA'ları, aşı formülasyonlarının farelerin humoral bağışıklık tepkileri üzerindeki etkisini değerlendirmek için yapıldı. IgG titreleri 42. güne kadar arttı, 72. günde aynı kaldı veya hafif azaldı ve 90. günde önemli ölçüde arttı. IgG2c/IgG1 oranları, Th1 immün yanıtı ile korele olduğundan, hücresel immün yanıtları tahmin etmek için kullanıldı. Yalnızca VLP kapsüllü grupla karşılaştırıldığında, adjuvanlı gruplar ya Th1/Th2 dengeli ya da Th1 taraflı profil gösterdi. Bu çalışma, VLP aşı stratejimizin güçlü bağışıklık yanıtlarını uyarmadaki etkinliğinin altını çiziyor ve daha fazla araştırma ve geliştirme için yollar açıyor.

Anahtar Kelimeler: SARS-CoV-2, VLP, Lipozom, CpG, poly(I:C), 2'3'-cGAMP, aşı

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DEDICATION



To my Father...

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ABBREVIATIONS

Angiotensin-Converting Enzyme 2	ACE2
Alkaline phosphatase	ALP
Antigen presenting cell	APC
Bovine serum albumin	BSA
Cyclic guanosine monophosphate-adenosine	cGAMP
Cyclic GMP-AMP synthase	cGAS
Coronavirus disease 2019	COVID-19
C-type lectin receptor	CLR
Cytosine phosphate guanine	CpG
CpG oligodeoxynucleotide	CpG ODN
Cytotoxic T lymphocyte	CTL
Dendritic cell	DC
Double membrane vesicle	DMV
Deoxyribonucleic acid	DNA
Dulbecco's Phosphate Saline Buffer	DPBS
Envelope protein	E
Enzyme Linked Immunosorbent Assay	ELISA
Endoplasmic reticulum	ER
Endoplasmic reticulum-Golgi intermediate compartment	ERGIC
Fetal bovine serum	FBS

Fusion peptide	FP
Heptapeptide Repeat	HR
Interferon	IFN
Immunoglobulin	Ig
Interleukin	IL
Lipopolysaccharide	LPS
Membrane protein	M
Middle East respiratory syndrome related coronavirus	MERS-CoV
Major histocompatibility complex	MHC
Messenger ribonucleic acid	mRNA
Myeloid differentiation primary response gene 88	MyD88
Nucleocapsid protein	N
Nuclear factor- κ B	NF- κ B
Natural killer	NK
NOD-like receptor	NLR
Non-structural proteins	NSP
N Terminal domain	NTD
Optical density	OD
Open reading frame	ORF
Pathogen-associated molecular patterns	PAMP
Polyinosinic:polycytidylic acid	Poly(I:C)
Poly(ethyleneglycol)	PEG
Pattern recognition receptors	PRR
p-Nitrophenyl Phosphate	PNPP
Receptor-binding domain	RBD
RIG-1 like receptor	RLR

Ribonucleic acid	RNA
Replication transcription complex	RTC
Spike protein	S
Severe acute respiratory syndrome coronavirus	SARS-CoV
Severe Acute Respiratory Syndrome Coronavirus 2	SARS-CoV-2
Serum separating tubes	SST
Stimulator of interferon genes	STING
TANK-binding kinase	TBK
T cell receptors	TCR
Follicular T cells	Tfh
T helper cells	Th
T helper cells class 1	Th1
T helper cells class 2	Th2
T helper cells class 17	Th17
Toll-like receptors	TLR
Transmembrane serine protease 2	TMPRSS2
Tumour Necrosis Factor	TNF
Regulatory T cells	Treg
Virus-like particle	VLP
Wild-type	WT
World Health Organization	WHO

1.1 SARS-CoV-2

The pneumonia outbreak caused by a novel coronavirus in December 2019 in Wuhan city of China, was reported to be a global pandemic on 11 March 2020 as it spread rapidly worldwide. The disease caused by the virus is called Coronavirus Disease-2019 (COVID-19), and the virus responsible for the condition named Severe Respiratory Disease Syndrome-Coronavirus-2 (SARS-CoV-2), which is a single-stranded RNA virus (Bhardwaj et al., 2021). To date (07.12.2022), there have been more than 641 million confirmed cases and 6.6 million deaths worldwide (WHO Coronavirus (COVID-19 Dashboard, 2022). The virus belongs to the *Betacoronavirus* genus of the *Coronaviridae* family (Bhardwaj et al., 2021). Before SARS-CoV-2, SARS-CoV and MERS-CoV were the two viruses from the coronavirus family that emerged and caused outbreaks in 2002 and 2012, respectively, but they did not manage to cause a pandemic (Pustake et al., 2022). SARS-CoV-2 shares a 79.5% sequence with SARS-CoV and 40% with MERS-CoV, and the mortality rates of the diseases caused by these viruses are 4.2%, 11%, and 34%, respectively (Hu et al., 2020).

1.1.1 SARS-CoV-2 Structure

Coronaviruses get their name because of the crown-like shape formed by the spike proteins protruding from the outer surfaces of the viruses (Yang et al. 2021). They are positive-sense single-stranded RNA viruses, of which SARS-CoV-2 has a genome size of approximately 30kb (Malone et al., 2022). The RNA molecule consists of a 5' cap essential for replication and transcription of the genome and a 3' poly(A) tail for genome stability (Park et al., 2022, Brant et al., 2021). The SARS-CoV-2 genome encodes 16 non-structural proteins (NSPs), four structural proteins, and six accessory proteins. The partially overlapping open reading frames (ORF) ORF1a and ORF1b, which occupy 70% of the viral genome, encode two polyproteins, pp1a and pp1ab, that are cleaved into NSPs by viral proteases. NSPs assemble into replication and transcription machinery to initiate viral RNA replication and transcription, and they also have a role in immune evasion. The four structural proteins are Spike (S), Envelope (E), Nucleocapsid (N), and Membrane (M), which make up the virion. The functions of accessory proteins are still unknown (Figure 1.1.A, 1.1.B) (Brant et al., 2021).

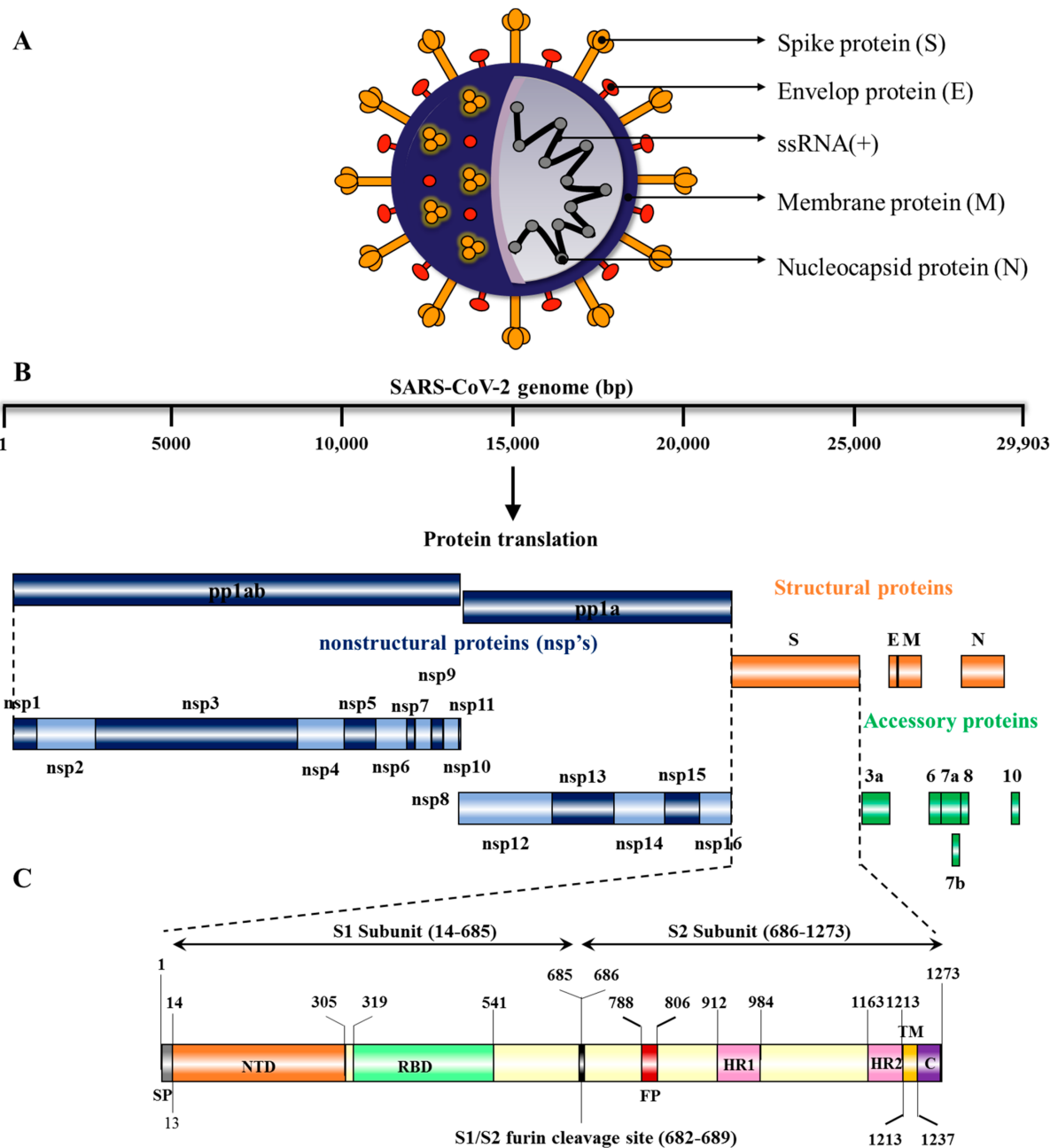


Figure 1.1 Structure and genomic organization of SARS-CoV-2: **(A)** *Structure of SARS-CoV-2*: The figure depicts the single-stranded RNA molecule and all the structural proteins, spike (S), envelope (E), nucleocapsid (N), membrane (M) that make up the virion. **(B)** *Genome and proteins of SARS-CoV-2*: 29,903 nucleotides make up the SARS-CoV-2 genome, which encodes non-structural, structural and accessory proteins. **(C)** *Structure of the Spike protein*: The spike protein comprises signal peptide (SP), S1 subunit, and S2 subunit. The S1 subunit consists of N terminal domain (NTD) and receptor binding domain (RBD). The S2 subunit consists of fusion peptide (FP), heptapeptide repeat sequence 1 (HR1), HR2, TM domain, and cytoplasm domain (C). (Adapted from; Tulumilli et al., 2022)

The trimeric S protein is a transmembrane glycoprotein found on the surface of the viral envelope. It is responsible for recognizing and binding to the host cells. It consists of 1273 amino acids and has two functional subunits, S1, and S2, responsible for host cell attachment mediation and membrane fusion, respectively (Yang et al., 2021 & Yadav et al., 2021) (Figure 1.1.C). The S1 subunit contains a receptor binding domain (RBD) with a high affinity for the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell membrane. The N terminal domain (NTD) is the other domain of S1, which is also thought to be responsible for mediating the conformational changes of the S protein during host cell infection (Yang et al., 2021). The S2 subunit has the domains of fusion peptide (FP), heptapeptide repeat sequence 1 (HR1), HR2, TM domain, and cytoplasm domain (C), which are responsible for membrane fusion (Huang et al., 2020). The S protein has two conformational states; prefusion state and postfusion state. RBD is inaccessible by ACE2 in the prefusion state, whereas it can be accessed in the postfusion state (Peng et al., 2021).

The transmembrane E protein is the smallest structural protein of the virus, having a role in the pathogenesis of the disease, assembly, and release of the virus (Yadav et al., 2021). It has N terminal ectodomain, transmembrane domain and C terminal domain and in total, it is made up of 75 amino acids (Yan et al., 2022). The E protein carries out its role in viral assembly by localizing to the endoplasmic reticulum (ER) and Golgi body membranes. It also enhances host antiviral response by activating host inflammasome through its ion channel role, regulating Ca^{2+} pumping out of the ER (Yang et al., 2021).

The M protein is the major constituent of the viral envelope which also has roles in the assembly and release of viral particles and the processing and packaging of viral proteins. A short N-terminus, a triple transmembrane domain, and a long C-terminal cytoplasmic domain make up the 222-amino acid M protein. The M protein contributes to interferon signalling, interfering with the host immune response (Yan et al., 2022).

The N protein packages the RNA of the virus into a ribonucleoprotein complex to protect the genome (Yang et al., 2021). It is 419 amino acids long and has an N terminal domain, a linker region, and C terminal domain, all participating in RNA binding (Bai et al., 2021 & Yadav et al., 2021). In addition to RNA packaging, N protein also evades the host's immune response, and it accomplishes this by inhibiting interferon production and antagonizing RNAi, the cell-intrinsic antiviral defence mechanism (Bai et al., 2021)

1.1.2 SARS-CoV-2 Lifecycle

The binding of the S with the ACE2 receptor starts the lifecycle of SARS-CoV-2. RBD of the S1 is responsible for binding, and the binding triggers proteolytic cleavages by the proteases found on the host cell membrane needed for the fusion of the host membrane with the viral membrane. Furin cleaves at S1/S2 site to separate the subunits, and the transmembrane serine protease 2 (TMPRSS2) cleaves the S2' site located within the S2 subunit to release the fusion peptide required for membrane fusion (Figure 1.2). At the same time, HR1 and HR2 form a six-helix bundle fusion core to bring the viral and host membranes closer together. After the membrane fusion occurs, viral RNA is released into the cytoplasm. Viral RNA hijacks the host's translation machinery to produce the polyproteins (pp1a and pp1ab) encoded by the ORFs. The polyproteins are cleaved into NSPs to form the viral replication transcription complex (RTC) (Zhang et al., 2021). Nsp3 and Nsp4 induce double membrane vesicles (DMVs) generation from ER, essential for keeping together the proteins and other factors needed for viral replication (Roingard et al., 2022).

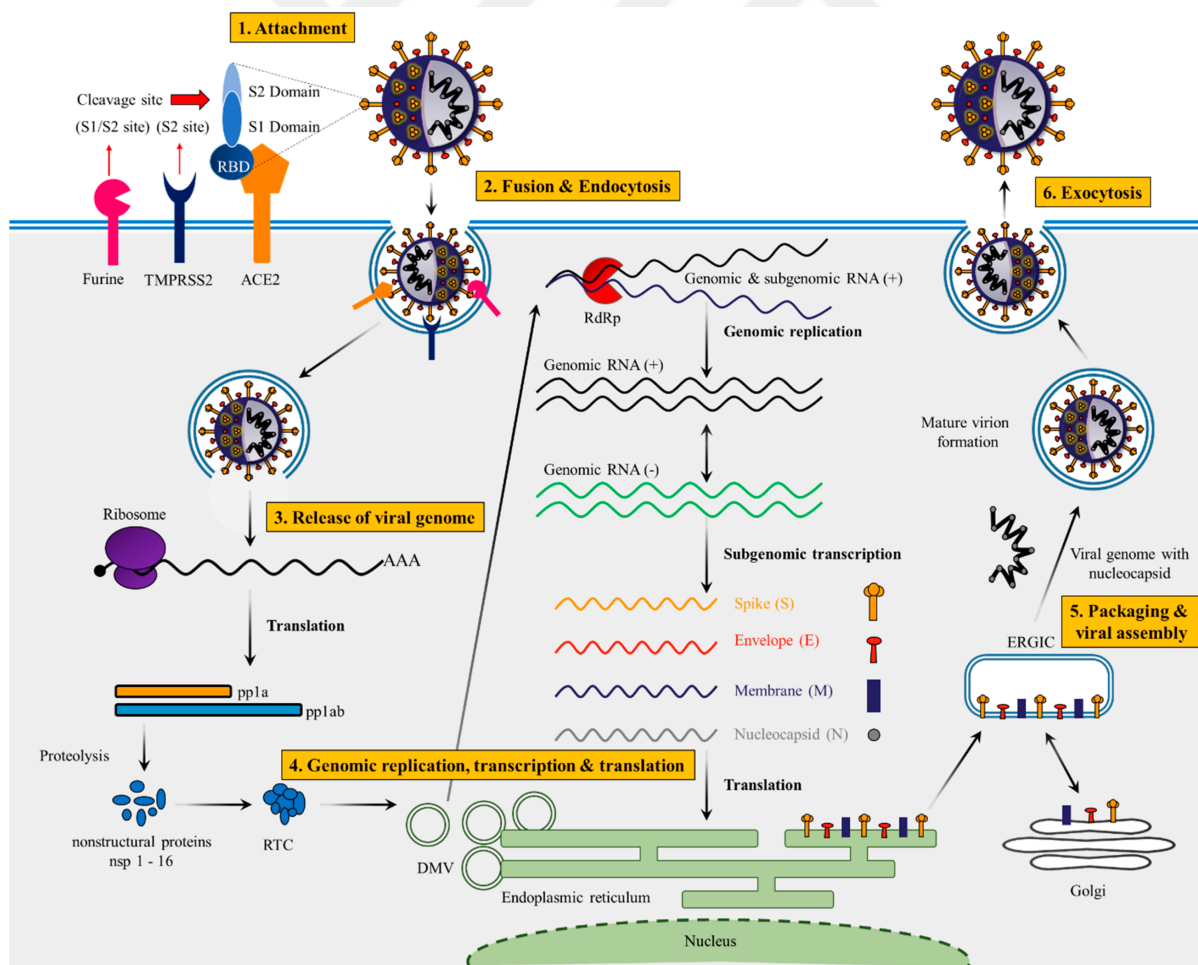


Figure 1.2. Lifecycle of SARS-CoV-2. (Adapted from; Tulimilli et al., 2022)

RNA-dependent RNA polymerase (RdRp) is required for the replication of genomic RNA and the subgenomic RNA synthesis, which is needed for the production of structural proteins (Tulimilli et al., 2022). N protein forms a nucleoprotein complex by combining with the genomic RNA, and it assembles with S, E, and M in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) to create a mature virion. Finally, the exocytosis of the virion completes the lifecycle (Bhardwaj et al., 2022).

1.2 Immune System

The immune system has developed to fight and eliminate the harmful pathogenic microbes and toxins the host encounters. The immune system must distinguish pathogens from host cells to destroy pathogens without harming the host. The innate and adaptive immune systems are the two arms of the immune system that work in synergy to generate an effective immune response against a pathogen (Medina, 2016). Innate immune system is the first line of defence when there is an infection, and it is nonspecific in terms of pathogen recognition to generate a rapid response. On the other hand, the adaptive immune system needs time to generate antigen-specific responses, and it develops a memory for the specific pathogen to destroy it when the host encounters the same pathogen. Macrophages and dendritic cells are the antigen presenting cells (APCs) of the innate immune system that process and express the antigens to the T cells of the adaptive immune system, making a bridge between two arms of the immune system (Marshall et al., 2018). B cells are the members of the adaptive immune system that act as APCs and produce antibodies needed for eliminating pathogens (Medina, 2016).

1.2.1 Innate Immunity

The innate immune system harbours four barriers that utilize different mechanisms to protect the host from invading pathogens. The anatomical barriers like skin and mucous membranes prevent the entry of microbes. Physiological barriers like low pH and temperature inhibit growth and kill pathogens. The complement system belongs to this category as its components opsonize the pathogens and make them suitable for phagocytes. Macrophages and neutrophils are the components of the phagocytic barrier; their function is to clear away the pathogens by engulfing and directly killing the pathogens and infected cells. Lastly, inflammatory barriers ensure immune cells are recruited to the infected area. Neutrophils, eosinophils, mast cells, basophils and natural killer cells are also the cellular components of the innate immune system (Marshall et al., 2018).

The innate immune system uses its germline-encoded pattern recognition receptors (PRRs) to detect invading microorganisms through their particular structures called pathogen associated molecular patterns (PAMPs). There are four families of PRRs identified; Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs). TLR family has the highest number of characterized members among other families. Each TLR recognizes different PAMPs. Some TLRs are found in the cell wall to recognize certain PAMPs like Lipopolysaccharide (LPS) and flagellin, while others are found in endosomal membranes to detect nucleic acids originating from microorganisms. Generally, PRR signalling triggers the expression of proinflammatory cytokines, type I interferons (IFNs), chemokines, and other proteins. Some TLRs induce type I IFN and proinflammatory cytokine expression through the adaptor proteins MyD88 and TRIF. Type I IFNs are essential in antiviral defences (Takeuchi et al., 2010)

1.2.1.1 Innate Immune Response against SARS-CoV-2

When SARS-CoV-2 enters the respiratory airways of the host, tissue-resident immune cells recognize the virus to generate a local immune response and facilitate the arrival of other innate immune cells to the area (Ricci et al., 2021). Although the roles of each PRR against SARS-CoV-2 have not been fully verified, there are some experimental data and predictions regarding this issue. As depicted in Figure 1.3, different PAMPs of the SARS-CoV-2 are recognized by different PRRs. Signalling pathways induced by the PRRs leads to the production of inflammatory cytokines and chemokines, which cause disease pathogenesis (Karki and Kanenganti, 2022). In asymptomatic and mild cases, infection is controlled by the transcription of antiviral IFN, proinflammatory cytokines, and chemokines (Ricci et al., 2021). In severe cases, the combination of TNF and IFN γ activates the inflammatory cell death, PANoptosis, which causes the release of more cytokines, leading to cytokine storm. Cytokine storm is the cause of severe disease pathogenesis and death (Karki and Kanenganti, 2022).

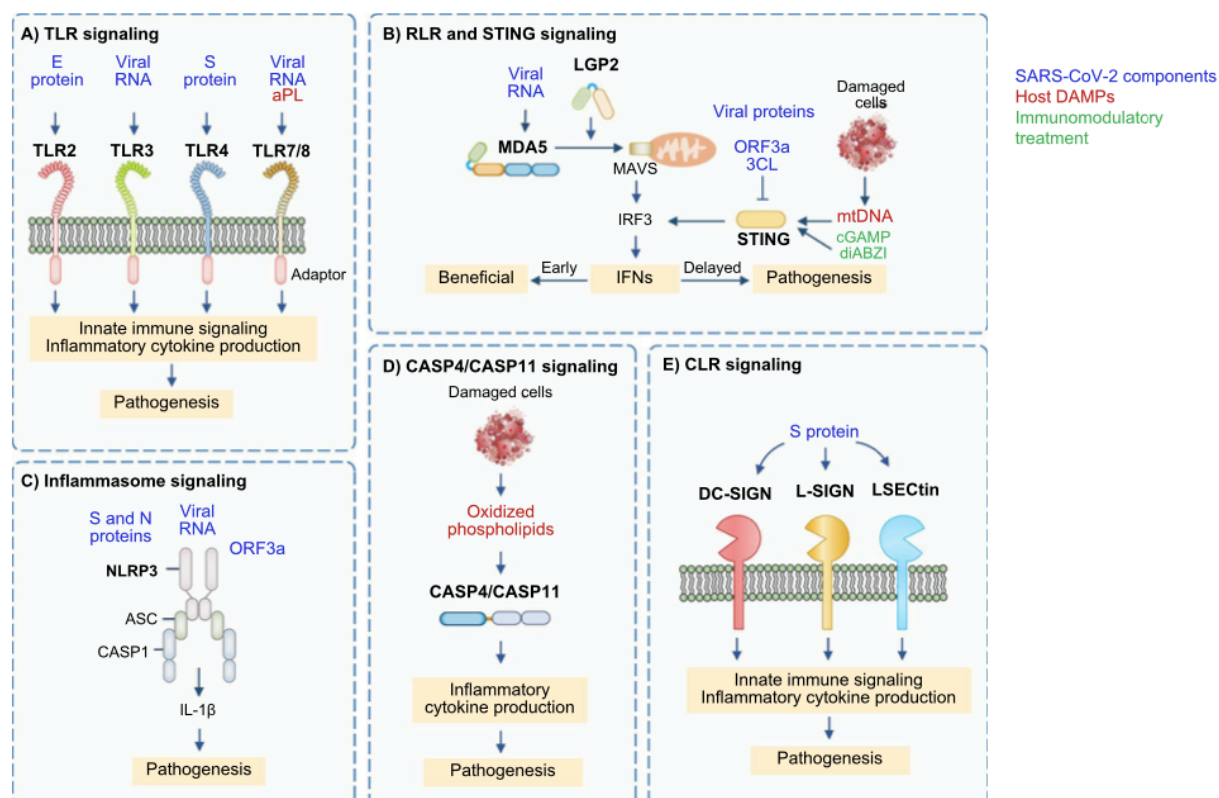


Figure 1.3. Possible PRR components taking part in the pathogenesis of SARS-CoV-2 infection (Adapted from Karki and Kanenganti, 2022).

1.2.2 Adaptive Immunity

The adaptive immune system is responsible for detecting non-self antigens, creating a pathogen-specific immune response, and developing immunologic memory against that specific pathogen. Adaptive immunity has two branches; cell-mediated immunity and humoral immunity, where T cells and B cells are the cellular components, respectively. Antigen binding receptors of the T and B cells have unique antigen specificity thanks to the random rearrangement of receptor genes during cell maturation. T cell receptors (TCRs) recognize antigens on the other cells only when bound to major histocompatibility complex (MHC) molecules. CD4 helper T (Th) cells and CD8 cytotoxic T cells are two classes that become activated upon antigen presentation. MHC class I molecules are found in all nucleated cells and antigen presentation by MHC class I activates cytotoxic T cells, whose function is to kill the infected cells. MHC class II molecules are found only on APCs, and MHC class II bound antigens activate the Th cells. There are different Th cell responses, as shown in Figure 1.4. Their primary role is to regulate the immune response through secreting cytokines that direct other immune cells (Marshall et al., 2018 & Tomar and De, 2014)

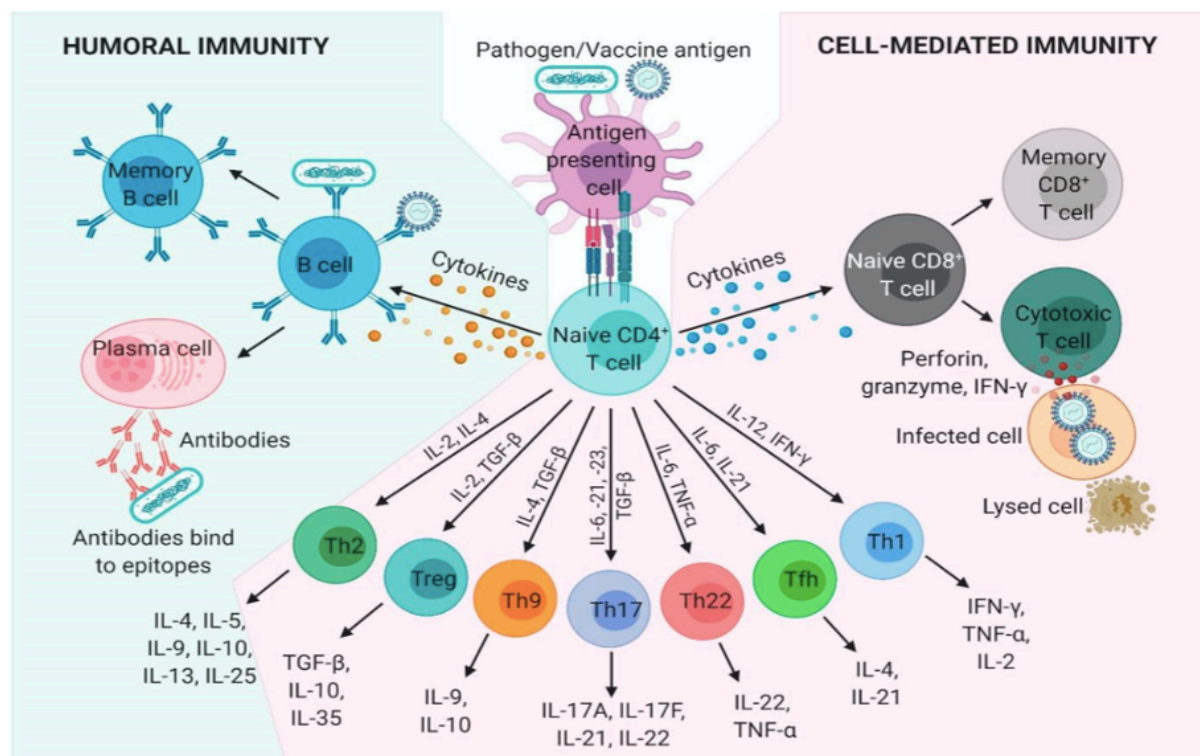


Figure 1.4. Cellular components of the adaptive immune response (Adapted from Schijns et al., 2021)

B cells become activated after recognizing antigens through their membrane-bound immunoglobulin receptors. Once activated, they either differentiate into antibody-secreting plasma cells or memory cells. Antibodies are divided into five major classes, IgM, IgG, IgA, IgD and IgE, which differ in their function such as complement fixation and antigen opsonization. Th cells instruct the plasma cells which type of antibody to produce (Marshall et al., 2018).

1.2.2.1 Adaptive Immune Response against SARS-CoV-2

In cases of infections with intracellular pathogens, like viral infections, antibodies alone cannot fight and eliminate the infection; cell-mediated immune responses are indispensable (Marshall et al., 2018). CD4⁺ T cells were observed to be significant in generating responses to control the SARS-CoV-2 infection. They differentiate into Th1 cells to elicit antiviral activities by releasing IFN γ and other cytokines. Other notable functions of CD4⁺ cells include helping CD8⁺ cells to express the strong cytotoxic activity and participating in tissue repair. Tfh differentiation is essential to assist B cells in developing effective humoral immunity. T cell responses against SARS-CoV-2 are reported to be rapid as one day after the onset of symptoms (Sette & Crotty, 2021). B cell responses can be detected one week after symptom onset through

IgA, IgM and IgG production, of which IgG is the more stable. Differentiated B cells reach the mucosal tissues, where the infection is, to prevent disease transmission and provide resistance against reinfection. Antibodies against SARS-CoV-2 primarily recognize the virus's S and N proteins, but the neutralising antibodies' main target is the RBD. Severe COVID-19 cases showed higher titers of antigen-specific antibodies and neutralising antibodies than mild cases, possibly due to increased viral load (Qi et al., 2022).

1.3 Vaccine Development

Vaccines are essential contributors to immunity against infections. The emergence of the first vaccine dates back to the 18th century, when Edward Jenner's inoculation brought immunity to a little boy against smallpox. Thanks to advances in vaccine development, smallpox has been completely eradicated, and most of humanity has become immune to polio, Hepatitis A and many other diseases (Saleh et al., 2021). There are different sorts of vaccines in terms of the type of antigenic component, including live attenuated, inactivated, subunit, toxoid, conjugate, recombinant, adenovirus vector and mRNA vaccines (Clem, 2011 & Saleh et al., 2021). Effective vaccines should stimulate the immune system to generate long-term protection against pathogens through memory cell production by the adaptive immune system (Clem, 2011). Adjuvants are used in vaccine formulations to improve the efficacy of vaccines (Facciola et al., 2022).

1.3.1 Antigen Delivery Systems

Live attenuated, inactivated or killed, and subunit vaccines are traditional types of vaccines used by humans for years against various infectious diseases. Although they can elicit strong immune responses against the antigens, several concerns exist regarding the safety, stability and efficacy of these vaccines. To address these concerns, new strategies in vaccine development had to be implemented, and evaluation of new vaccine delivery systems begun (Trovato & De Berardinis, 2015). Nanoparticle delivery systems are one of the platforms that present numerous advantages in vaccine development. Nanoparticle delivery systems include virus-like particles, liposomes, exosomes, lipid nanoparticles, inorganic nanoparticles, immunostimulatory complexes, emulsions and polymeric nanoparticles. In general, nanoparticles augment the immunogenicity of the vaccines by increasing the stability of antigens, by promoting the processing of antigens by APC, by enabling delivery of antigens

and adjuvants at the same time and most importantly, by acting as immune-stimulating adjuvants themselves. Physiochemical properties of nanoparticles including size, shape and charge are significant characteristics to consider and they can be modified to enhance the cellular uptake, hence the immunogenicity of the vaccine (Bezbaruah et al., 2022).

1.3.1.1 Liposomes

Liposomes are tiny vesicles composed of two layers of phospholipids. Gregoriadis and his colleagues are the first to report that liposomes combined with antigens can induce immune responses (Schwendener, 2014). They are one of the vaccine delivery systems used due to their versatility and plasticity. Creating liposomes with the desired lipid composition, size, lamellarity, and charge is possible for specific applications (Daraee et al., 2016). More than one antigen and adjuvant can be encapsulated within the liposomes or linked to the surfaces of the liposomes, as represented in Figure 1.5. Poly(ethyleneglycol) (PEG) is a polymer used to improve the stability of liposomes so that antigens remain in circulation longer, and the liposomes with PEG inclusion are referred to as sterically stabilized liposomes (Schwendener, 2014 & Woodle, 1995). Liposomes as vaccine delivery systems ensure that antigens and adjuvants are simultaneously delivered and protect the antigens from premature degradation (Tretiakova and Vodovozova, 2021).

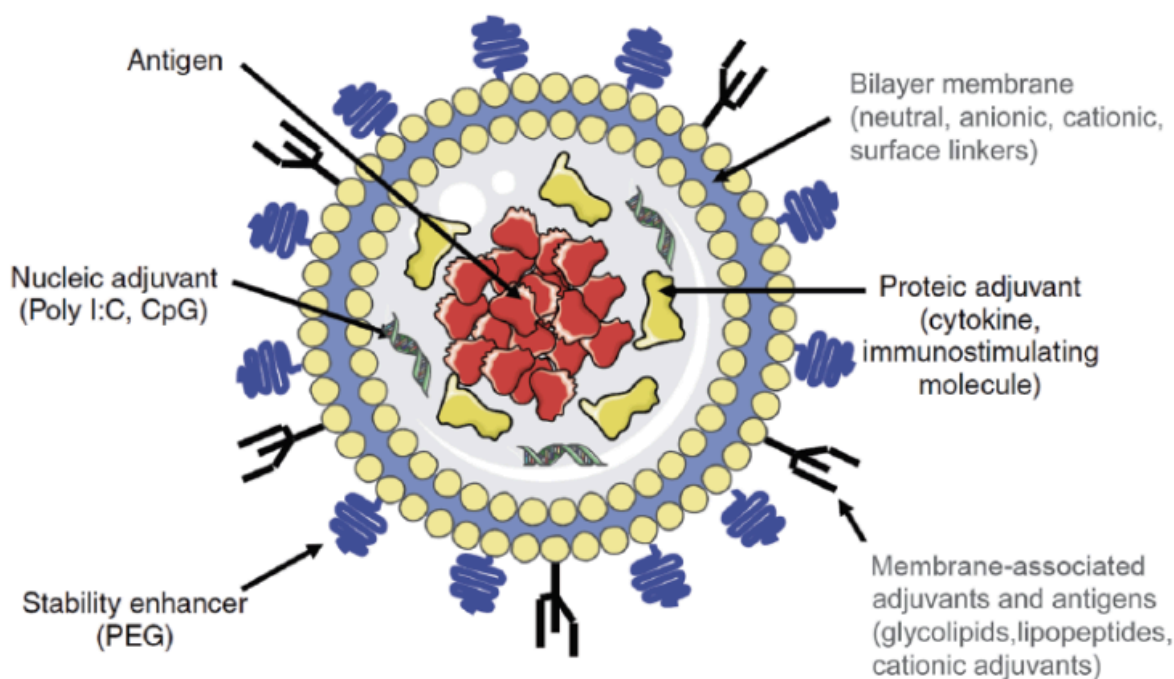


Figure 1.5. Schematic depiction of a small unilamellar vesicular liposome (Adapted from Schwendener, 2014)

The surface charge of liposomes is an important property to consider as their stability, antigen loading, and interactions with other cells and proteins depend on it, thereby affecting their immunostimulatory functions. Neutral and anionic liposomes were less effective than cationic liposomes, as many studies have demonstrated that negatively charged APCs took up the cationic liposomes more effectively (Henriksen-Lacey et al., 2010 & Wang et al., 2019).

Liposomes intrinsically activate the innate immune system, as phospholipids contribute to their phagocytosis (Tretiakova and Vodovozova, 2021). In addition, liposomes may interact with the immune system through various receptors, among which liposomes carrying TLR ligands are of great importance in vaccine development (Zahednezhad et al., 2019). Numerous studies have shown that liposomes encapsulating one or more TLR agonists with the antigen enhance immune responses to that antigen (Chatzikleanthous et al., 2021). In the context of cell mediated adaptive immune response, negatively charged dendritic cells, the main inducers of T cell mediated immune responses, mainly interact with the cationic liposomes. It is worth mentioning that encapsulating both the antigen and adjuvant is necessary to stimulate Th1 immune response (Zahednezhad et al., 2019). With liposomal vaccines, robust humoral immune responses can be achieved thanks to the liposomes' depot effect (Henriksen-Lacey et al., 2010).

1.3.1.2 Virus-Like Particles

Virus-like particles (VLPs) are structures composed of viral proteins inherited from their parental viruses without viral genetic material. The repetitive surface epitopes of VLPs have self-assembly capability, and their diameter range between 10nm and 200nm. VLPs can induce immune response due to their features inherited from parental viruses, making them excellent candidates for vaccine development (Zhang et al., 2022). VLPs have several benefits when compared to other vaccine platforms like inactivated or live-attenuated vaccines. They are safe to use since they lack the viral genome, meaning there is no risk of viral replication, hence infection. VLPs are more stable than soluble antigens, their ordered and repetitive surface epitopes are PAMPs that can induce an innate immune response, and their multivalent nature and size ensure that APCs can take them up efficiently (Crisci et al., 2013).

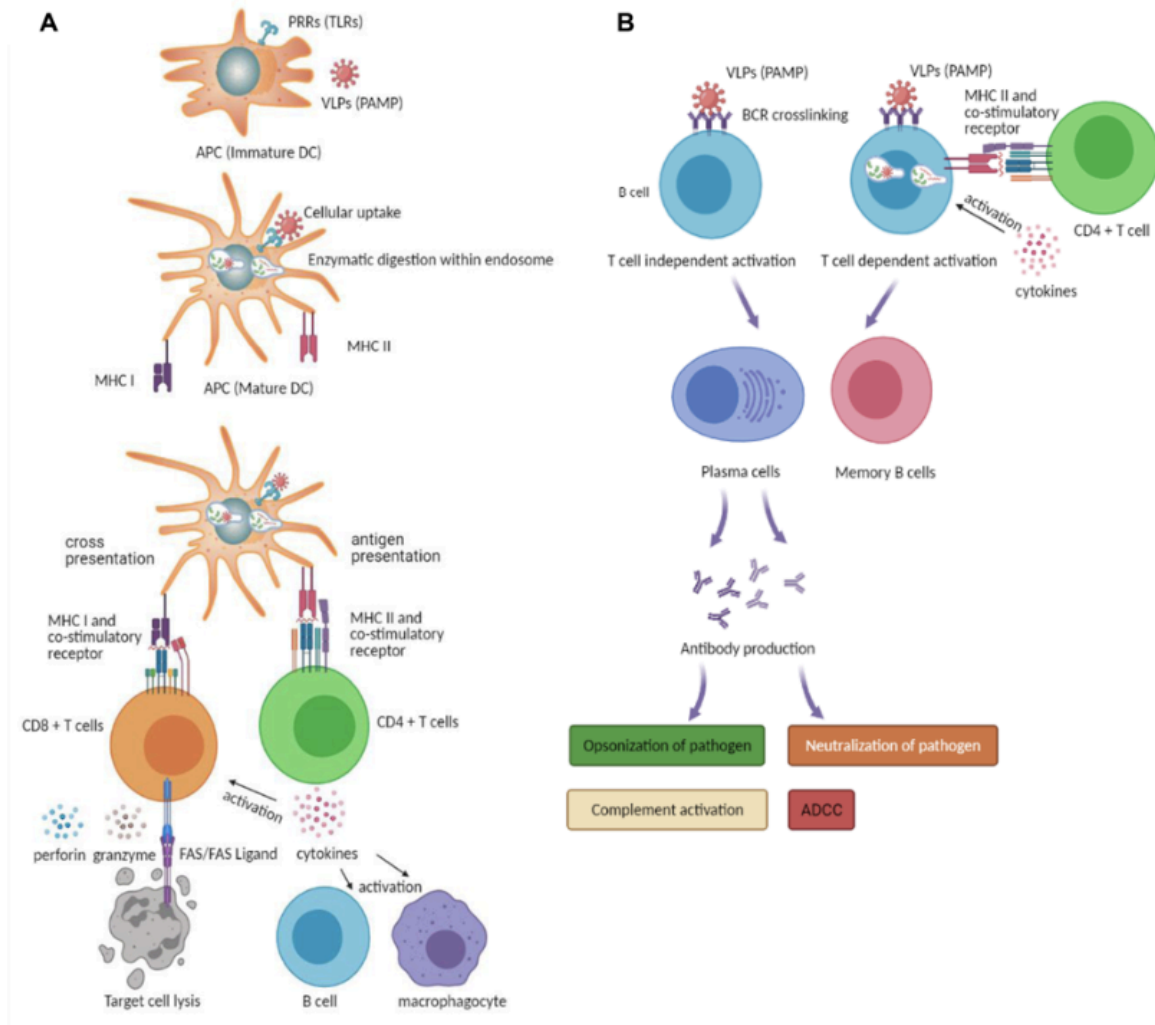


Figure 1.6. Mechanisms of (A) *cellular* and (B) *humoral* immune response stimulation by VLPs (Adapted from Zhang et al., 2023).

VLPs successfully elicit cellular and humoral immune responses, and their mechanisms are illustrated in Figure 1.6. PAMPs of VLPs bind PRRs of APCs, leading to their activation. APCs internalize the VLPs and digest them to load their antigens onto the MHC II molecules, which is required for subsequent CD4⁺ T cell activation. Antigen cross-presentation through MHC I can stimulate cytotoxic T cell immune response, which causes target cell lysis; however, VLPs do not predominantly induce a cytotoxic T cell response. Activated CD4⁺ T cells release cytokines to activate macrophages, cytotoxic T cells and B cells. The organized repetitive epitopes on the surfaces of VLPs can activate B cells in a T-cell independent manner. The resulting humoral immune response is strong, as B cells produce antibodies that opsonize pathogens, activate complement, neutralize pathogens, and participate in antibody-dependent cellular cytotoxicity (ADCC) (Zhang et al., 2023).

1.3.2 Adjuvants

Adjuvants are substances used in vaccines to generate an effective immune response against an antigen. Adjuvants are divided into two classes regarding their mechanism of action; delivery systems and immune potentiators (Facciola et al., 2022). In terms of effective immune response generation, adjuvants employ various mechanisms, including amplified antigen uptake and presentation by APCs, immune cell recruitment to the injection site, depot formation on the injection site, cytokine or chemokine stimulation, and contribution to the transport of antigen to draining lymph nodes (Awate et al., 2013).

1.3.2.1 CpG

Bacteria have unmethylated cytosine-phosphate-guanine (CpG) motifs in their genome that immune cells recognise through their TLR9 (Zhou and Deng, 2021). CpG binding stimulates the production of NF- κ B-dependent cytokines and IRF7-dependent Type I interferons through the MyD88, of which IL-12 stimulates naïve CD4⁺ T cells to develop into Th1 cells and produce IFN γ , as also depicted in Figure 1.4. Th1 cells have a significant role in developing immunity against cancer and intracellular pathogens, and they accomplish this by activating the cytotoxic T cells and boosting phagocytosis (Temizoz et al., 2015). CpG can initiate antibody production in a T-cell-independent way by directly triggering B cell proliferation and differentiation (Zhou and Deng, 2021). Three classes of synthetic CpG oligodeoxynucleotides are designed to benefit from their immunostimulatory properties; Class A CpG (also known as D-type), Class B CpG (also known as K-type) and Class C CpG. These classes of adjuvants differ in their structures and immunomodulatory activities (Adamus and Kortylewski, 2017 & Temizoz et al., 2015). Class A CpGs have a role in plasmacytoid dendritic cell (pDC) stimulation and IFN α secretion, whereas Class B CpGs are solely B-cell activators and stimulate pDC maturation, TNF α secretion. Class C CpGs have moderate effector properties of both classes (Adamus and Kortylewski, 2017).

1.3.2.2 Poly(I:C)

Polyinosinic-polycytidylic acid (Poly(I:C)) is a synthetic double-stranded(ds) RNA that mimics the viral dsRNA. It can be used as an adjuvant in vaccines against viral infections, as it is a TLR3 ligand like viral dsRNAs. Ligand binding to TLR3 results in production of Type I IFNs

and proinflammatory cytokines such as IL-12. TLR3 ligands like poly(I:C) are known to be strong promoters of Th1-skewed immune response. Yet, poly(I:C) can be toxic as they can also be detected by cytosolic RNA sensors RIG-I and MDA5, which lead to cytokine storm induction, so dosage and delivery of the poly(I:C) should be carefully considered (Hafner et al., 2013 & Temizoz et al., 2018).

1.3.2.3 2',3'-cGAMP

Cyclic guanosine monophosphate (GMP)- adenosine monophosphate (AMP) synthase stimulator of interferon genes (cGAS-STING) pathway is responsible for detecting cytosolic dsDNA molecules. It is a versatile innate immune sensing pathway detecting both self and foreign DNA, regulating antigen presentation and immune responses through Type I IFN and proinflammatory cytokine expression. cGAS gets activated after dsDNA binding and catalyses the dsDNA into 2',3'-cyclic guanosine monophosphate-adenosine monophosphate (2',3'-cGAMP). 2',3'-cGAMP binds ER resident STING, resulting in its translocation to ERGIC, where it activates TBK1. Later, TBK1 activates the transcription factors NK- κ B and IRF3, leading to the expression of Type I IFNs and interferon-stimulated genes. STING can be described as PRR as bacterial metabolites can directly activate it, which has led to investigations of STING agonists as vaccine adjuvants. Cyclic dinucleotides (CDNs) produced by mammalian and bacterial cGAS are the natural STING agonists used for this purpose (Van Herck et al., 2021 & Decout et al., 2021).

1.4 Vaccine Development Against SARS-CoV-2

After the declaration of COVID-19 as pandemic, it has become indispensable to develop an effective vaccine rapidly to control the transmission of the virus and reduce the mortality. New variants of virus emerged over time, bearing mutations that generally cause increased transmissibility. This also underlined the need for development of vaccines that can easily be adapted for variants. Conventional vaccines and next-generation vaccine platforms are implemented to produce effective vaccines against the virus (Jafari et. al., 2022). Figure 1.7 summarizes the mechanisms of six vaccine platforms used against SARS-CoV-2.

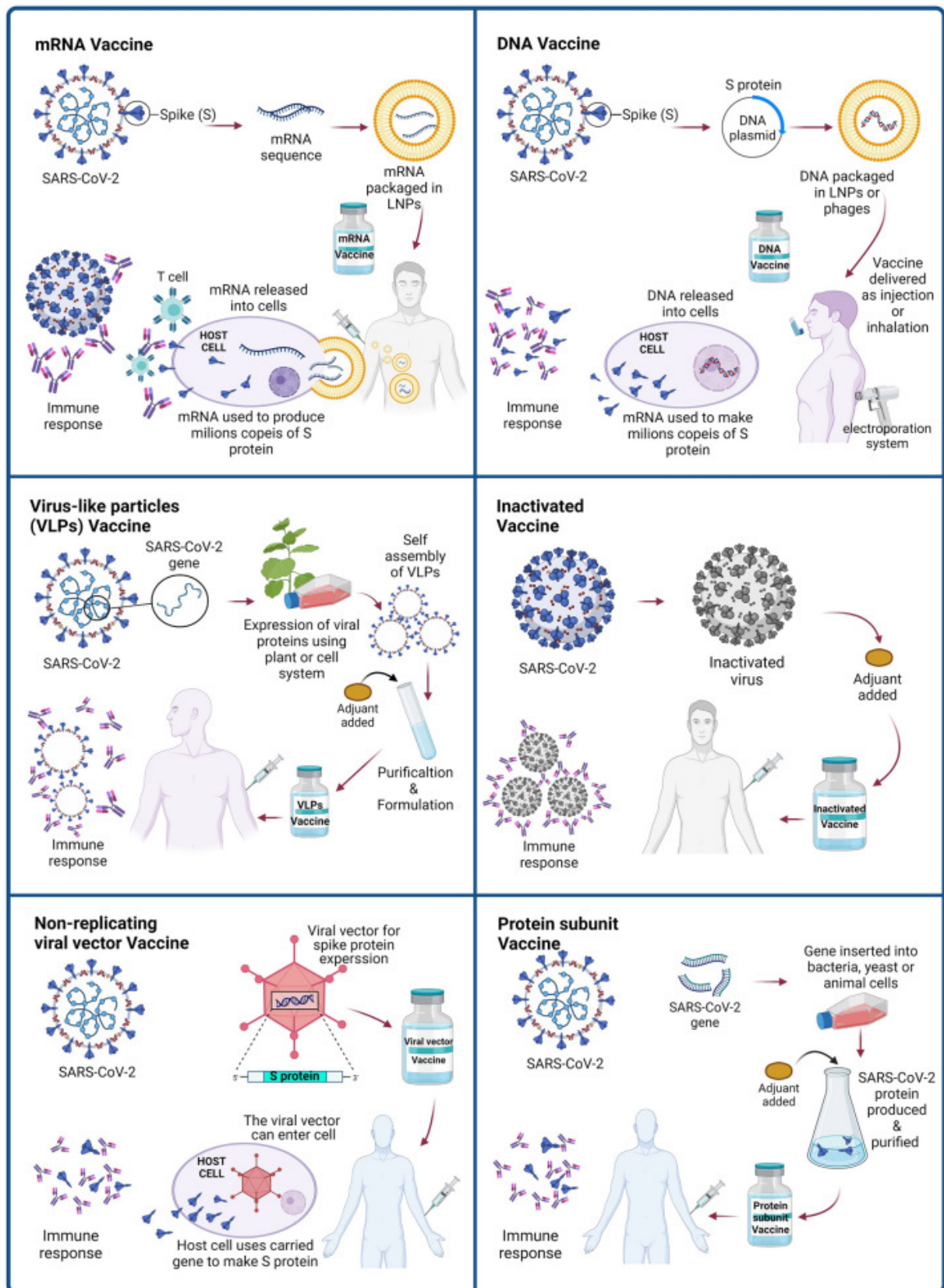


Figure 1.7. Mechanisms of vaccine platforms used for SARS-CoV-2 infection (Adapted from Jafari et. al., 2022)

When producing an inactivated virus vaccine, chemical or physical methods, or both, are used to inactivate the virus. Potent adjuvants must be added to formulations to achieve enhanced immune response as inactivation process decreases the immunogenicity of virus (Savina et al., 2022). Various inactivated virus vaccines against SARS-CoV-2 have been developed and entered phase clinical trials, one of them, CoronaVac, is approved for emergency use (Acosta-Coley et al., 2022). In viral vector vaccines, the attenuated recombinant virus is used as a vector to deliver antigen sequence to host cells. For SARS-CoV-2 vaccines, vectors expressing S or RBD are designed to induce production of these proteins in host cells, and induce robust immune responses (Savina et al., 2022). Although this type of vaccine is successful in getting rid of virus-infected cells through strong cytotoxic T cell response, if the host already has anti-vector immunity, the expected effect from the vaccine will not be observed and, there is a risk of integration of the viral genome into the host genome (Acosta-Coley et al., 2022). For DNA vaccine development, plasmid vectors are used to express viral antigenic parts. Plasmids have to enter the nucleus of the host cells to initiate the production of antigens. However, this type of vaccines need transporting molecules to secure the delivery of plasmid DNA to host cell nucleus. Also, adjuvants and convenient route of administration are required to enhance their immunogenicity. Besides, viral genome integration risk is present in this type of vaccine (Acosta-Coley et al., 2022). The remarkable type of vaccine in COVID-19 pandemic are mRNA vaccines. mRNA vaccines induce viral antigen production by the host cells, causing immune response. mRNA vaccines are easy to adjust against newly emerged diseases, produce in large-scales in short period of time and they have high potency. However, the mRNAs need packaging for their delivery to the cells because they can easily be degraded by extracellular ribonucleases and cannot manage to enter the cells (Acosta-Coley et al., 2022). There are two approved mRNA vaccines against SARS-CoV-2, manufactured by Moderna and Pfizer/BioNTech. Both vaccines utilized lipid nanoparticles for packaging the mRNA encoding spike protein of SARS-CoV-2. Both are the most effective vaccines against SARS-CoV-2 among the others (Prates-Sayed et al., 2021). Still, these vaccines have some weaknesses like the requirement for extra cold chain storage as mRNAs are unstable at high temperatures (Acosta-Coley et al., 2022). Protein subunit vaccines employ purified viral proteins as immunogens to elicit immune response. For SARS-CoV-2 subunit vaccines, spike or RBD were used as the subunits in combination with various adjuvants. Although highly immunogenic subunit of the virus is used in these types of vaccines, they may not elicit immune response as robust as the vaccines that incorporate whole viral antigenic parts (Heidary et al., 2022). VLP vaccines are another popular flexible vaccine platform employed for viral infections.

Immunogenic properties and safety of VLPs make them attractive for vaccine development. VLPs can be produced rapidly and efficiently by different expression systems like bacteria, plant, and mammalian cells. Thanks to their self-assembling property, the final product looks like the original virus. According to WHO, there are 7 candidate VLP vaccines in clinical trials for SARS-CoV-2, including the vaccine developed by our lab in collaboration with METU. The distinguishing feature of our vaccine is that it expresses all four structural proteins of the virus, whereas other candidate VLP vaccines use either RBD or S as antigens (Sharifzadeh et al., 2022).

1.5 Aim and Outline of the Study

The rapid transmission of SARS-CoV-2 to millions has prompted the urgent need to produce a vaccine against the virus. Different strategies and platforms are used to create vaccines such as mRNA and inactivated whole virus vaccines. Our lab used the VLP platform to produce a vaccine incorporating all four virus structural proteins. Alum and K3 CpG ODN were used as adjuvants, and the vaccine has entered human phase clinical trials. This study aimed to increase the immunogenicity of the Delta variant spike expressing VLPs by complexing them with liposomes and different adjuvants. Three injections are carried out (Day 0, Day 15 and Day 73) and animals were bled at regular intervals (Day 14, Day 28 and Day 42, Day 72, Day 90). Enzyme Linked Immunosorbent Assay (ELISA) was applied to all animal sera to determine the immunogenicity of the vaccine formulations. Overall, all animals produced varying levels of antibodies against the viral proteins S and RBD.

2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffers and Solutions

Homemade 10X phosphate-buffered saline (PBS) was prepared by using 80 grams sodium chloride (NaCl) (Isolab, Germany), 2 g potassium Chloride (KCl) (Merck, Germany), 15.2 g sodium phosphate dibasic dehydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) (Sigma Aldrich, USA), 2 g potassium phosphate monobasic (KH_2PO_4) (Sigma Aldrich, USA), completed into 1 L with ddH₂O, pH was adjusted to 6.8, and autoclaved. Commercial 1X PBS was purchased from Biological Industries, Israel. 5% blocking buffer was prepared by using 100 g Bovine Serum Albumin (Capricorn, Germany), 1 mL Tween20 (VWR, USA), and completed into 2 L with 1X PBS. Wash buffer was prepared by using 2 L homemade 10X PBS, 10 mL Tween20, and 18 L dH₂O. Coating solution for nanopore used in qNano was prepared according to the manufacturer's instructions (TRPS Reagent Kit, IZON, UK). PNPP (p-Nitrophenyl Phosphate) solution was prepared according to the manufacturer's instructions; one tablet is dissolved in 4 mL ddH₂O and 1mL 5X concentrate diethanolamine substrate buffer (Thermo Scientific, USA)

2.1.2 Phospholipids and Lipids Used in Liposome Preparation

Cholesterol (Chol) was purchased from Sigma Aldrich (USA). -[N-(N',N'-Dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol), 1,2- Dioleoyl-sn-Glycero-3-phosphoethanolamine-N-[methoxy(Polyethylene glycol)- 2000] (Ammonium Salt) (PEG-PE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (USA).

2.1.3 Enzyme Linked Immunosorbent Assay (ELISA) Reagents

Recombinant Spike protein was produced homemade in collaboration with METU, Prof. Dr. Mayda Gürsel's laboratory. Delta Plus RBD was kindly provided by İzmir Biomedicine and Genome Institute. Other recombinant proteins, antibodies and ELISA reagents with their working concentrations are provided in Table 2.1.

Table 2.1 Recombinant proteins, antibodies, and ELISA reagents used throughout this study.

Product	Catalog Number	Clone	Company	Working Concentration
Recombinant Spike	-	-	Home-made	0.5µg/mL
Delta RBD	-	-	Home-made	1µg/mL
Goat Anti-Mouse IgG, Human ads-AP	1030-04	Polyclonal	Southern Biotech	1:1000 dilution
Goat Anti-Mouse IgG1, Human ads-AP	1070-04	Polyclonal	Southern Biotech	1:1000 dilution
Goat Anti-Mouse IgG2c, Human ads-AP	1079-04	Polyclonal	Southern Biotech	1:1000 dilution
PNPP Tablets	34047	-	Thermo Scientific	1 tablet/5 mL

2.1.4 ODN and Ligands Used Throughout the Study

TLR9 targeting CpG ODN sequence is indicated in Table 2.2. Working concentrations and signaling pathways of other ligands are listed in Table 2.3. 2'3'-cGAMP is the cyclic dinucleotide used in this study.

Table 2.2 Oligodeoxynucleotide (ODN) used throughout this study.

Oligodeoxynucleotide (ODN)	Sequence	Company
K3	ATCGACTCTCGAGCGTTCTC	RiboBio

Table 2.3 Ligands used throughout this study.

Ligand Name	Company	Catalog Number	Working Concentration	Signaling Pathway
p(I:C)	Invivogen	tlr-picw	50 µg/ml	TLR3
2'3'-cGAMP	Invivogen	tlrl-nacga23	5 µg/ml	STING

2.2 Methods

2.2.1 Liposome Preparation

Various phospholipid stocks were prepared in chloroform at 10-20 mg/ml stock solutions and stored at -20°C until further use. Lipids were mixed according to the ratios indicated in Table 2.5 in a round bottom flask to prepare liposomes via the dehydration-rehydration method. Mixtures in chloroform were evaporated using a rotary evaporator (ILMVAC, Germany). The solvent-free dry lipid film was purged with nitrogen to eliminate residual chloroform and oxygen, to prevent lipid peroxidation. Empty multi-lamellar vesicles (MLV) were generated by adding glass beads and 1 ml PBS into 10 micromolar dried lipid film and were swirled until all lipid layers were dissolved into solution. Sonication was performed to MLVs with ten cycles of 30 s ON and 10 s OFF using a Vibra Cell Sonicator (Sonics and Materials, Danbury, CT, USA) to generate the small unilamellar vesicles (SUV). The whole liposome preparation process is shown in Figure 2.1. Lipid components and their corresponding ratios are shown in Table 2.4.

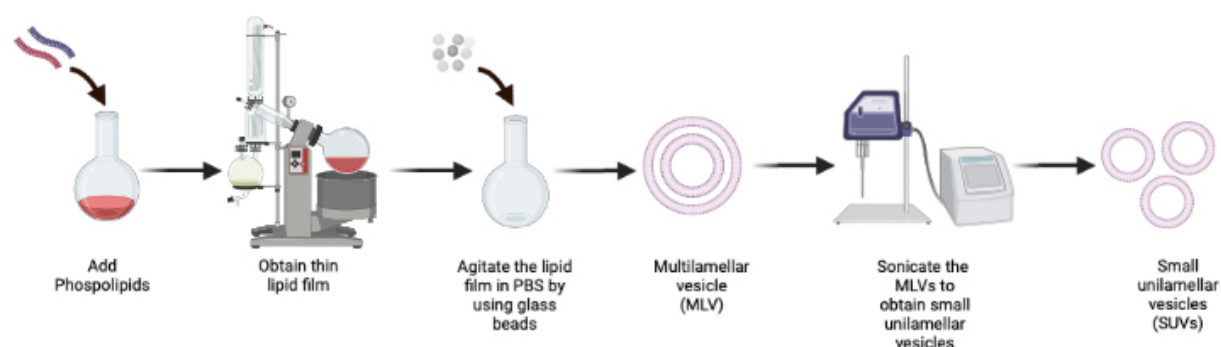


Figure 2.1. Process of liposome preparation (Created with BioRender).

Table 2.4. Lipid Composition (molar ratios) and charges

Liposome Type	Charge	Lipid Ratios		
		DC-Chol	DOPE	PEG-PE
SSCL	+	4	6	0.06

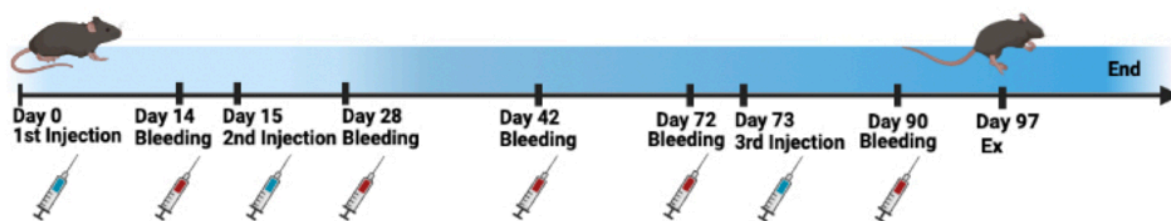
DC-Chol: dimethylaminoethanecarbamol-cholesterol; DOPE: dioleoylphosphatidylethanolamine; PEG-PE: polyethylene glycol-phosphatidylethanolamine; SSCL: sterically stabilized cationic liposomes.

2.2.1.1 Size Distribution and Concentration Analysis with Tunable Resistive Pulse Sensing

Size distribution and concentration of liposomes were analyzed with qNano Gold (#S/N 601A 1812 0592, Izon Science, New Zealand) via TRPS method. NP400 Nanopore was used throughout analysis and nanopore coating was performed with coating solution (#TK5-091, IZON Training kit). Nanopore's stretch was adjusted to 47 mm and the current was stabilized according to the manufacturer's instructions. Calibration was performed with 35 μ l of 1:1000 diluted CPC400 calibration particles (#RK3-167, IZON Reagent Kit). 35 μ l sample was loaded on the upper fluid cell to carry out the measurement. For analysis, at least 500 particles were counted and particle rate was exceeded 100 particles/min. IZON Control Suite 3.4.2.48 software (IZON Science LTD) was used to obtain the results and the plots.

2.2.2 *In vivo* Experiments with Mice

6-8 weeks-old female C57BL/6 mice were used throughout the study. Timeline of the experiment is depicted in Figure 2.1.

**Figure 2.2.** *In vivo* experiment timeline (Created with BioRender).

2.2.2.1 Preparation of vaccine formulations

SUVs were mixed with Delta variant spike expressing VLP, ODN, and ligands alone or with different combinations intended for encapsulation. Then, the mixture was snap-frozen in liquid nitrogen and freeze-dried overnight (VirTis Benchtop K, Germany). Encapsulation was achieved during the rehydration step: i) sterile DNase/RNase free dH₂O (1/10 of total initial snap-frozen volume) was added to the dehydrated liposome powder and vigorously vortexed for 15 s every 5 min for a total of 30 min at room temperature (RT), ii) equal volume of PBS (4.5/10 of total volume) was added to the mixture at the end of 30 mins and incubated for 10 mins at RT to allow equilibration of the tonicity. iii) PBS (4.5/10 of total volume) was added to the mixture, yielding a liposome concentration of 10µM lipid/mg encapsulated molecule(s). Liposome formulations were stored at 4°C for further use.

Table 2.5. Amounts of reagents used in vaccine formulations

Component	Concentration
VLP	5 µg/mouse
CpG	30 µg/mouse
poly(I:C)	50 µg/mouse
2'3'-cGAMP	5 µg/mouse
SSCL	1 µmol/mouse

2.2.2.2 Immunization Studies

Different combinations of VLP, CpG, 2'3'-cGAMP (denoted as CDN hereafter) and poly(I:C) formulations were injected intraperitoneally (IP) into animals in 200 µl. Injection groups are listed in Table 2.6. Booster injection was performed two weeks after the primary injection. The second booster was performed two months after the first booster injection.

Table 2.6. Injection groups of the study

1	Placebo
2	(VLP)SSCL
3	(VLP+CpG)SSCL
4	(VLP+poly(I:C))SSCL
5	(VLP+CDN)SSCL

6	(VLP+CpG+poly(I:C))SSCL
7	(VLP+CpG+CDN)SSCL
8	(VLP+CDN+poly(I:C))SSCL
9	(VLP+CpG+CDN+ poly(I:C))SSCL

2.2.2.3 Bleeding of Animals

Animals were bled at specific times, as indicated in Figure 2.1. All bleedings were carried out from the tail vein of the animals and approximately 300 µl of blood was collected in blood collection tubes (BD Microtainer SST Tubes). Blood samples were centrifuged at 2500g for 10 minutes to obtain sera. Serum samples were stored at -20°C until further use.

2.2.3 Vaccine Specific IgG Titer Determination with ELISA

2HB Maxi-binding 96-well immunoplates (SPL Life Sciences, Korea) were coated overnight at 4°C with 50µl/well of Recombinant Spike and RBD in DPBS (Biological Industries, USA). Coating concentrations are shown in Table 2.1. Plates were blocked for 4 hours with 200 µl/well blocking buffer at room temperature. After blocking step, plates were washed 3 times with wash buffer, then 2 times with distilled water, and then the plates were dried to remove excess water. Master plates were prepared by diluting the serum of each animal in blocking buffer by 1/50 in the first row, then 4-fold serially for the rest of the 8 rows. 50µl of diluted sera were transferred to immunoplates and incubated overnight at 4°C. The plates were washed with the same protocol and 1:1000 diluted ALP-conjugated anti-mouse IgG, anti-mouse IgG1, and anti-mouse IgG2c (all Southern Biotech) were added as 50µl/well and the plates were incubated for 2 hours at room temperature. Plates were washed and PNPP substrate solution (Thermo Scientific, USA) was added as 50µl/well. Optical density (OD) values were measured at 405nm using a plate reader (Molecular Devices, USA). Log10 titers were calculated by using GraphPad Prism 9 software.

2.2.4 Statistical Analyses

GraphPad Prism 9 software was used to analyze the statistical differences. Kruskal-Wallis Test with Dunn's multiple comparisons test were used to analyze the titers of the groups in each bleeding. Mixed-effects analysis with Tukey's test were used to analyze the titers of each group between bleedings. P values below 0.05 were determined as statistically significant.

3 RESULTS

3.1 Characterization of liposomes with TRPS measurement

Tunable resistive pulse sensing (TRPS) method was employed to characterize the VLPs, liposomes and vaccine formulations in terms of their size distribution, and also to count the number of particles. qNano Gold instrument was used for measurements.

Table 3.1. TRPS measurements of VLP, empty liposome and vaccine formulations.

Groups	Mean (nm)	Standard Deviation	Mode (nm)	Maximum (nm)	Minimum (nm)	Concentration (particles/ml)
Empty SSCL	371	98.4	321	1024	238	1.22e+11
VLP	91	19.2	84	268	62	1.02e+10
(VLP)SSCL	305	75.2	273	870	200	5.59e+10
(VLP+CpG)SSCL	297	104.8	244	1241	175	2.47e+10
(VLP+p(I:C))SSCL	278	72.8	239	876	181	2.18e+10
(VLP+CDN)SSCL	288	74.8	261	739	183	5.21e+10
(VLP+CpG+p(I:C)) SSCL	320	87.4	284	1220	210	1.56e+10
(VLP+CpG+CDN) SSCL	287	74.7	234	891	195	2.69e+10
(VLP+CDN+ p(I:C)) SSCL	291	78.8	252	1183	188	2.66e+10
(VLP+CpG+CDN+ p(I:C)) SSCL	286	84.1	247	976	190	1.97e+10

Table 3.1 shows the TRPS measurement results. According to the results of VLPs, they are similar to original virus in terms of their particle size, as the size of SARS-CoV-2 ranges from 60nm to 140nm (Laue et al., 2021). Sizes of liposomes remained similar after lyophilisation, which are indicated by similar mean values of liposomes in different vaccine formulations.

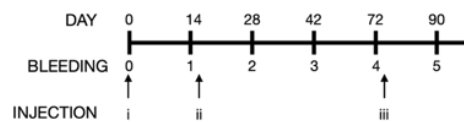
3.2 Assessment of the Humoral Immune Response Generated After Vaccination

6-8 weeks old 7 female C57BL/6 mice were used for each vaccine. Mice were injected intraperitoneally with 200µl of vaccine formulation. First injection was performed at Day 0, the booster injection was performed two weeks after the first injection, and the second booster was performed two months after the booster injection. Blood samples from the tail vein of mice were collected two weeks after the first injection, two weeks after the booster injection, four weeks after the booster injection, eight weeks after the booster injection and two weeks after the second booster injection to obtain their sera. To interpret the immunogenicity of the vaccines, sera were used in the Enzyme-linked Immunosorbent Assay (ELISA), which is the test used to evaluate the humoral immune responses of mice.

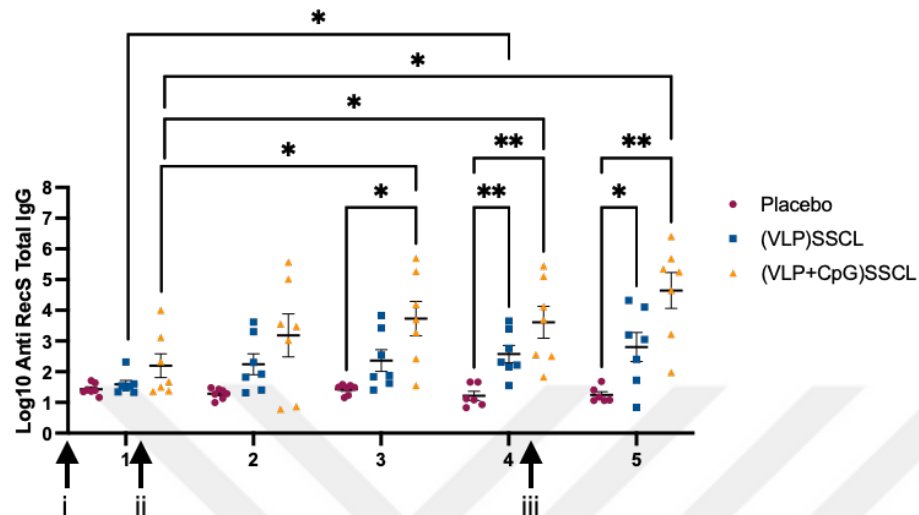
3.2.1 IgG Responses against Spike

Spike of the SARS-CoV-2 is the primary target of antibodies because of its role in recognizing host cells and mediating viral entry into host cells (Chen et al., 2023). IgG responses to the spike were examined by analyzing Total IgG, IgG1 and IgG2c secretions using ELISA. Homemade recombinant spike (RecS) was used to coat the plates.

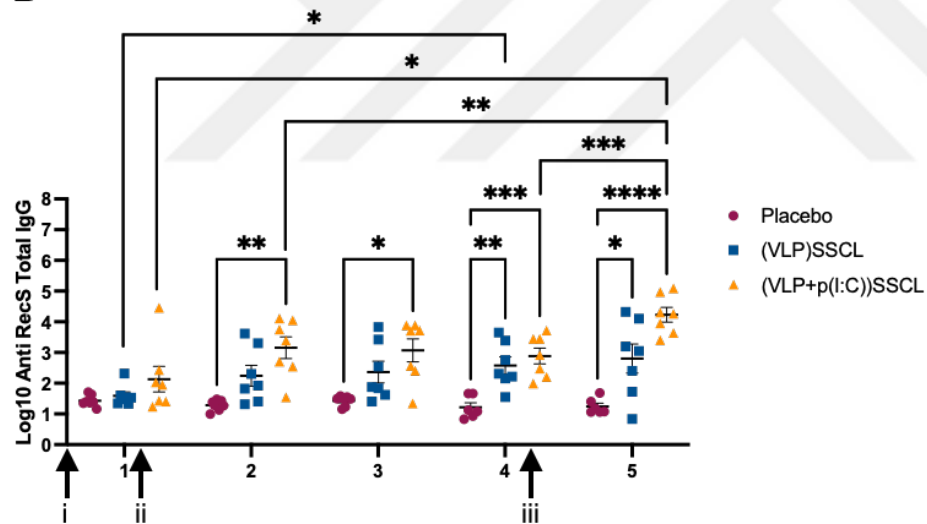
Figure 3.1 shows Total IgG titers of all vaccine formulation groups against RecS together with placebo and only VLP encapsulated SSCL group to see the effect of addition of adjuvants on Total IgG production in all bleedings. Figures 3.1 A, B and C show the impacts of VLP encapsulated with single adjuvant, CpG, poly(I:C) and CDN, respectively, on Total IgG secretion. VLP encapsulation with CpG or poly(I:C) resulted in higher Total IgG titers than VLP encapsulation alone. On the other hand, adding CDN did not bring out the same effect, resulting in lower Total IgG titer levels than VLP alone. Figure 3.1 D, E and F show Total IgG responses of groups injected with liposome-encapsulated VLP and dual adjuvant combinations CpG and poly(I:C), CpG and CDN, and CDN and poly(I:C), respectively. In general, these formulations did not cause a significant increase in Total IgG titers compared to VLP encapsulation alone, except for the CpG and poly(I:C) group, which resulted in a slight increase in Total IgG production after the third injection. Figure 3.1 G shows the Total IgG titers of the group that received VLP with all three adjuvants encapsulated within the liposomes. Total IgG titers of this group were similar to that of the VLP alone group in the first four bleedings whereas there was a little difference between the groups in the fifth bleeding that the group with adjuvants showed slightly better Total IgG response than the VLP alone group.



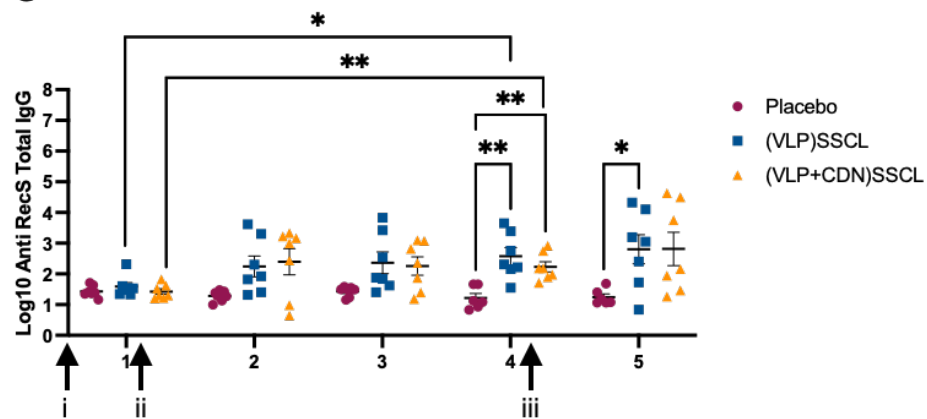
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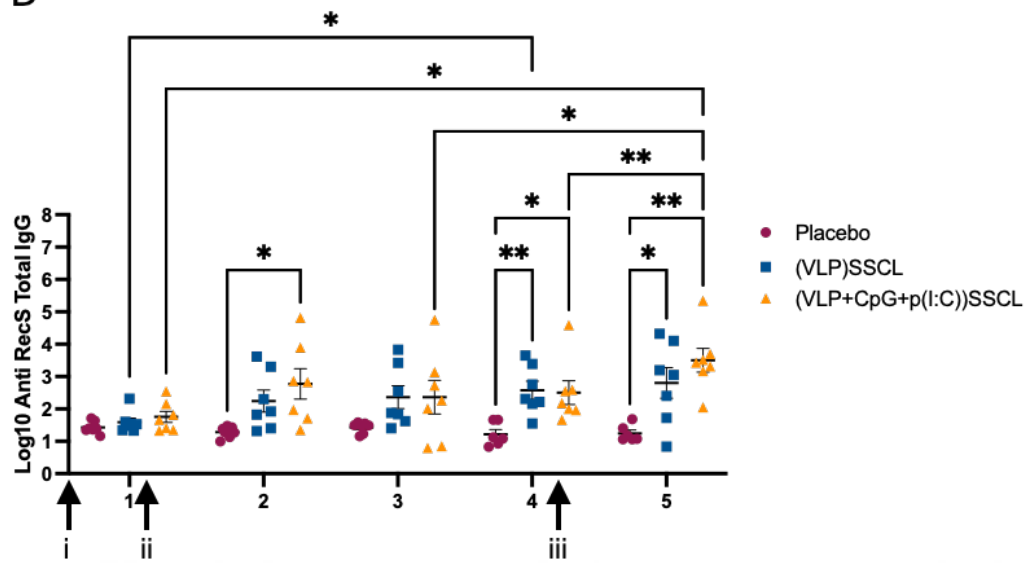
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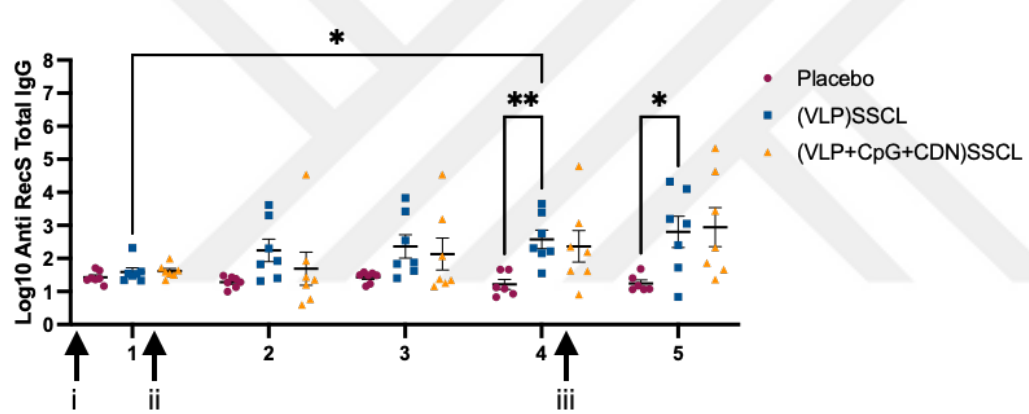
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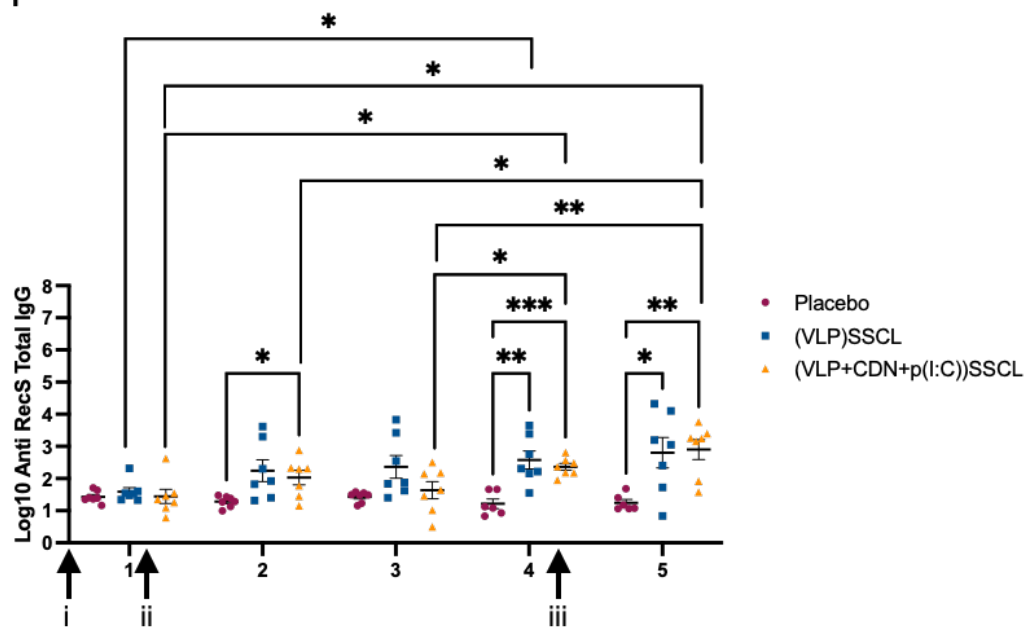
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E



F



G

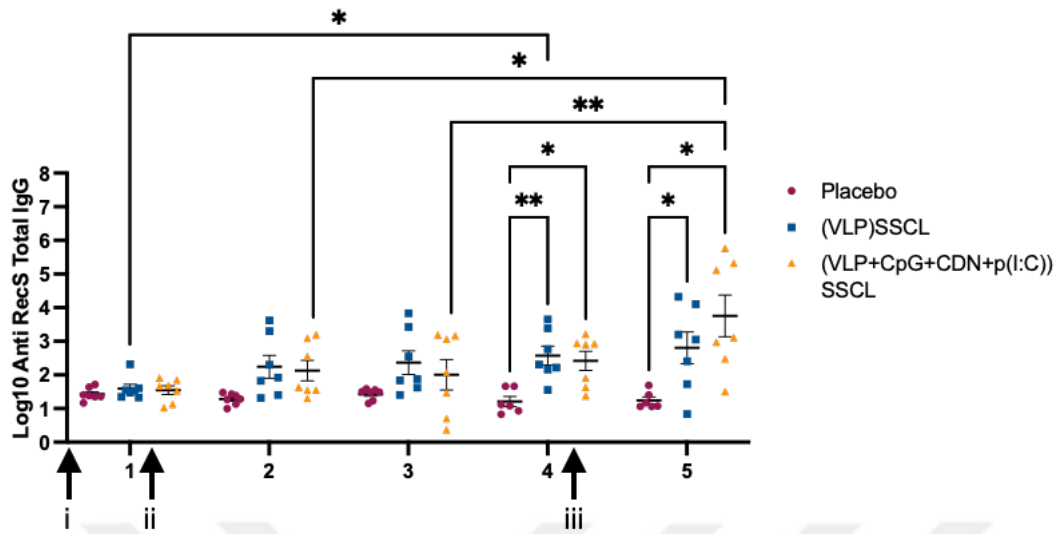
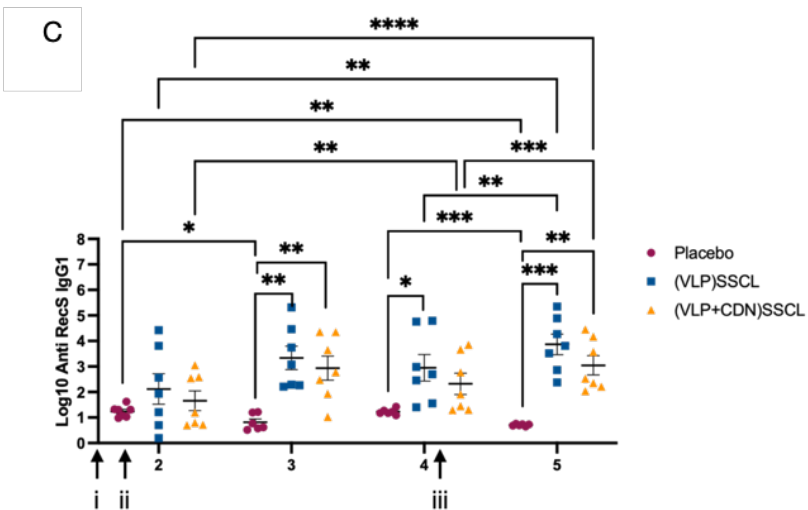
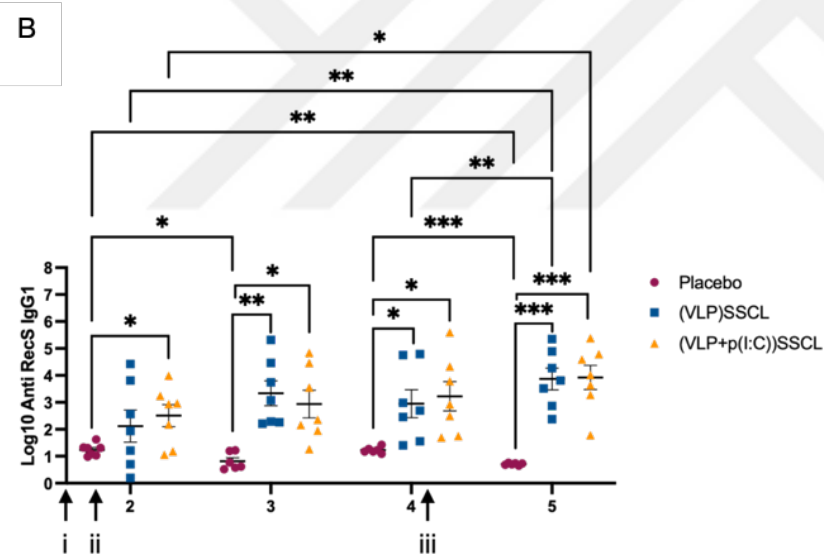
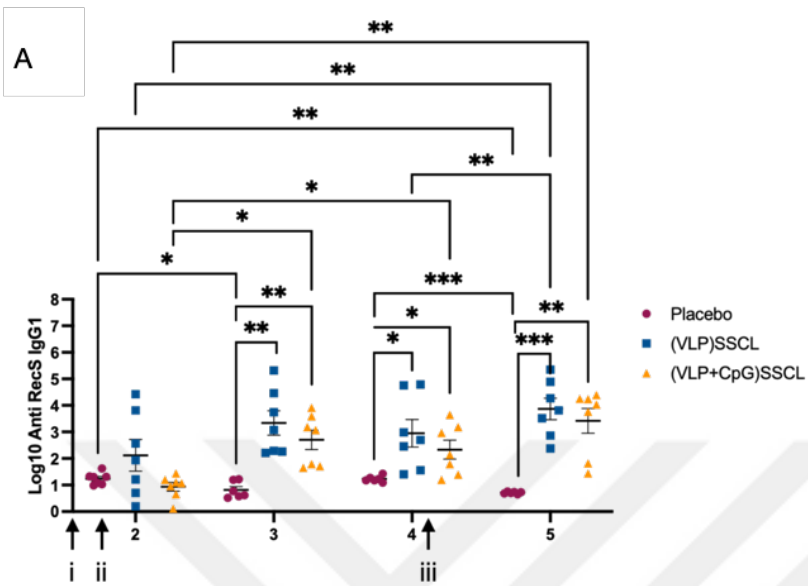
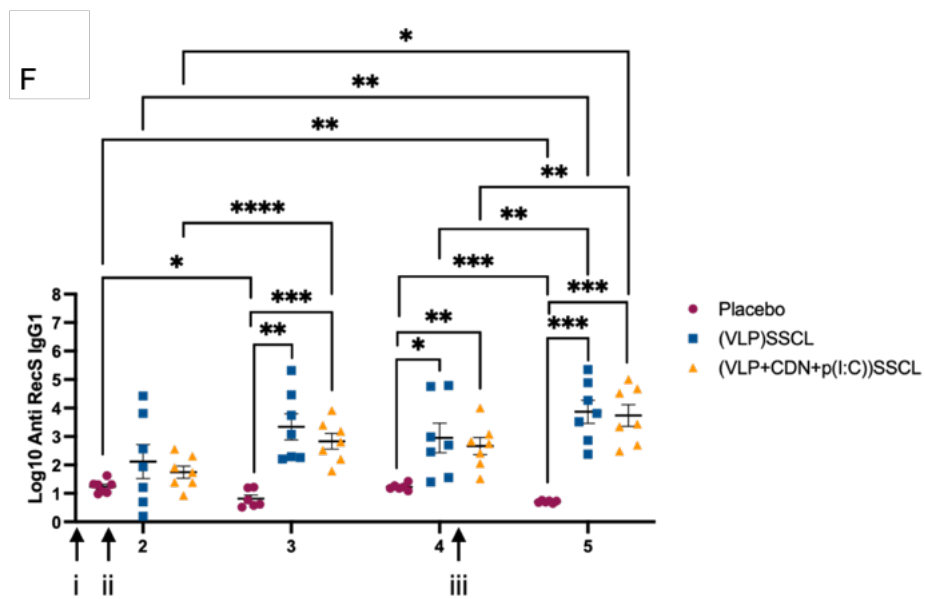
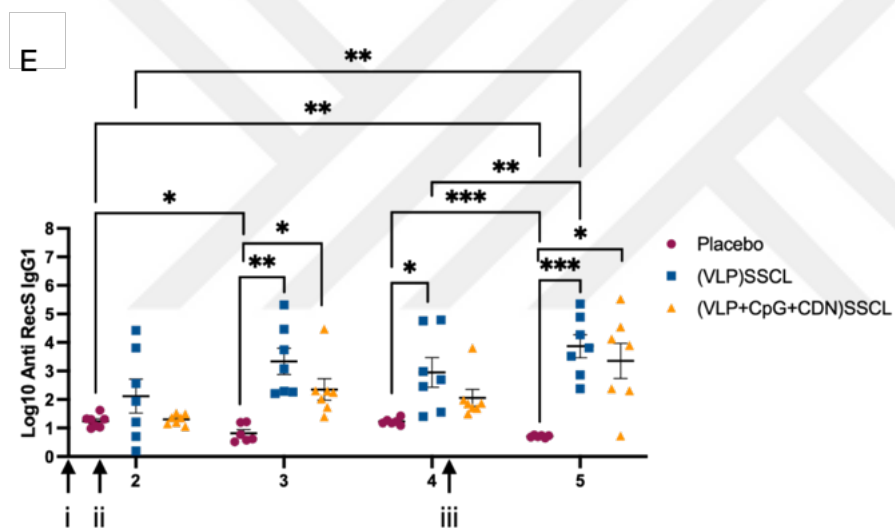
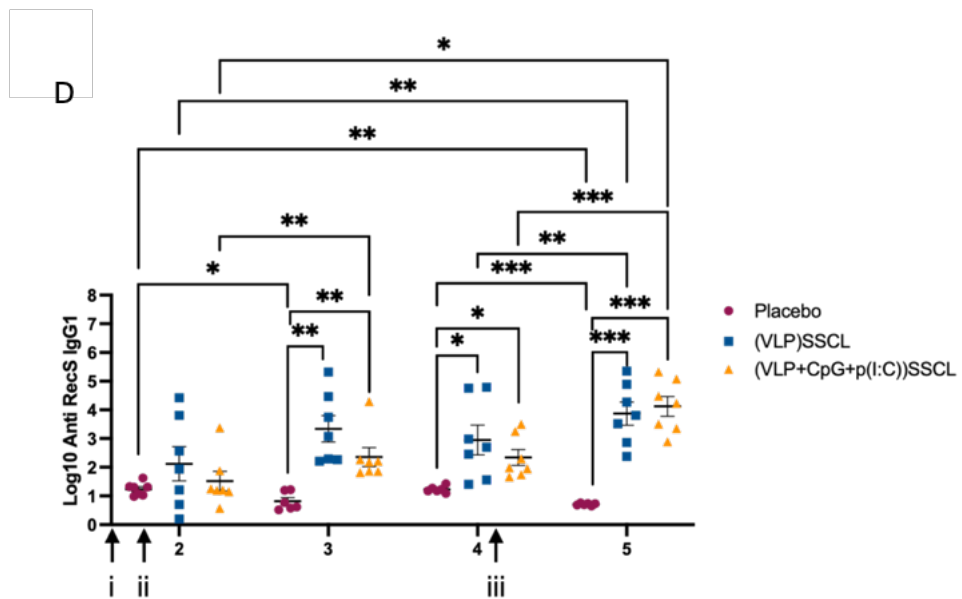


Figure 3.1. Evaluation of Total IgG responses of mice injected with liposomal vaccines against RecS by ELISA. The timeline above the graphs shows bleeding and injection days. Anti-spike Total IgG responses of 7 female C57Bl/6 mice in groups of Placebo, only VLP (5 μ g) encapsulated in liposome, (A) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) (B) VLP (5 μ g) encapsulated in liposome with poly(I:C) (50 μ g) (C) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) (D) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and poly(I:C) (50 μ g) (E) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and 2'3'-cGAMP (5 μ g) (F) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) (G) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g), 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) in all 5 bleedings. The data indicate the mean $\pm$ SEM. Comparisons of the IgG responses were done by Mixed-effects analysis with Tukey's Test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3.2 shows the IgG1 titers of groups against RecS in the last four bleedings. Figures 3.2 A and C show that VLP with either CpG or CDN encapsulated in SSCL caused less IgG1 production than VLP alone encapsulation. Figure 3.2 B shows injection of VLP encapsulated with poly(I:C) in liposome resulted in similar IgG1 production as VLP encapsulation alone. Figures 3.2 D, E, F show that injection with VLP encapsulated in SSCL with binary combination of adjuvants resulted in lower IgG1 levels than only VLP encapsulated liposome, except the fifth bleeding where third injection made them reach similar levels. Lastly, in Figure 3.2 G, the injection of VLP encapsulated in SSCL with all three adjuvants resulted in lower IgG1 levels than the injection with only VLP encapsulated liposome in all bleedings.





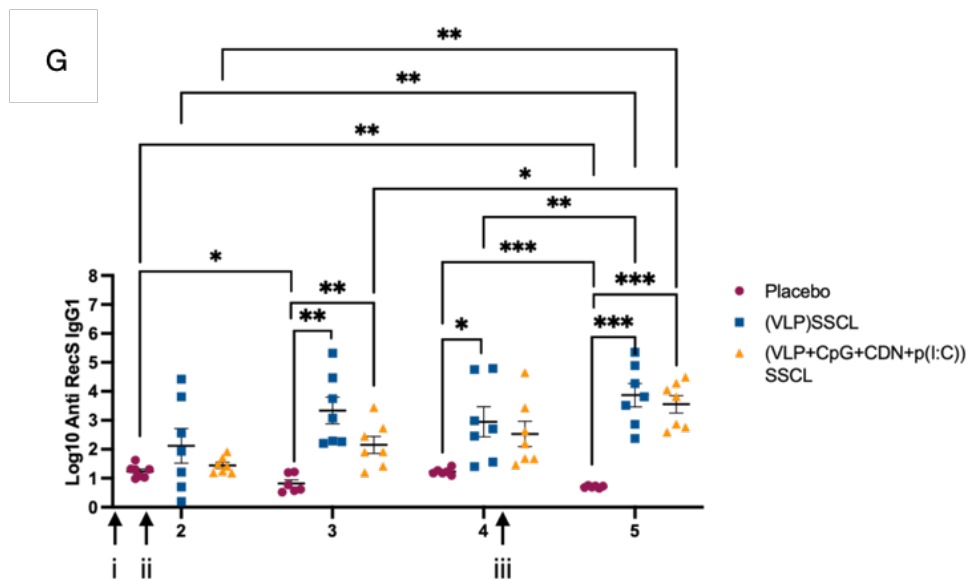
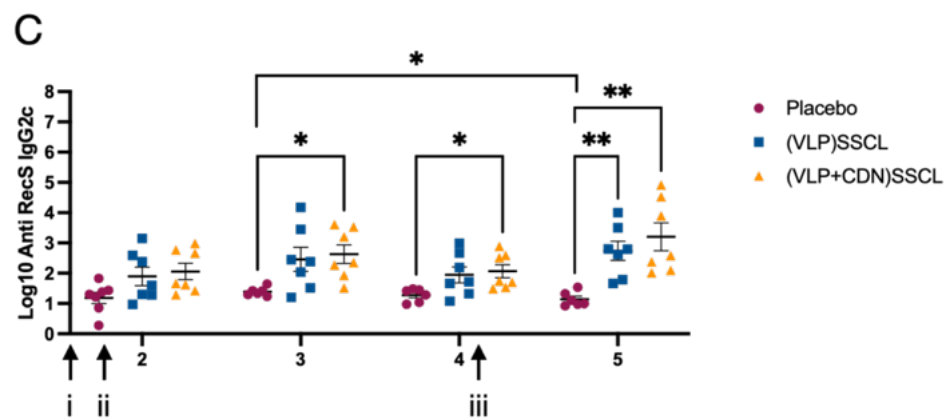
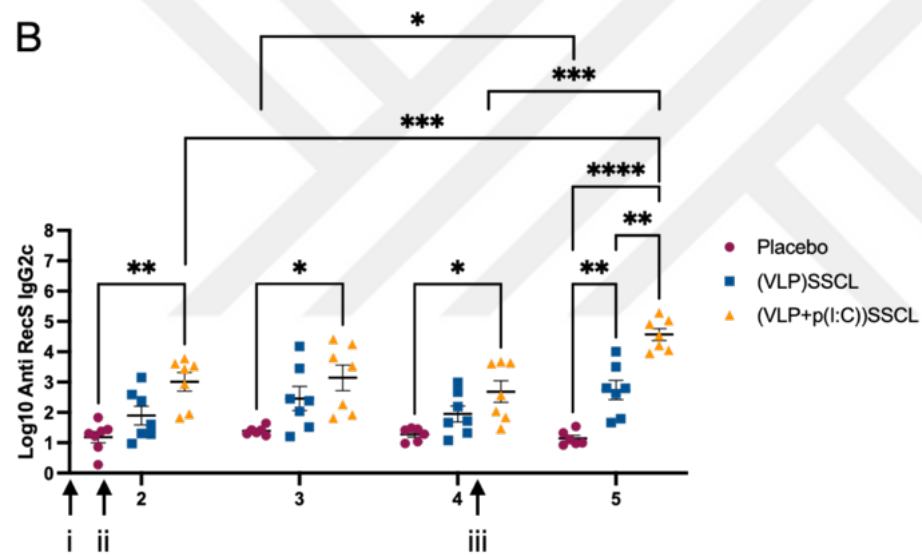
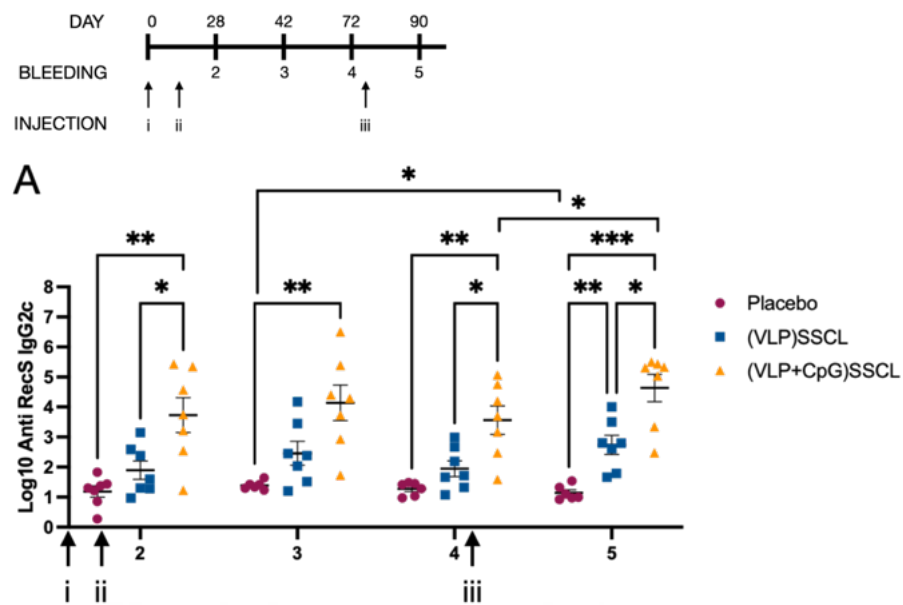
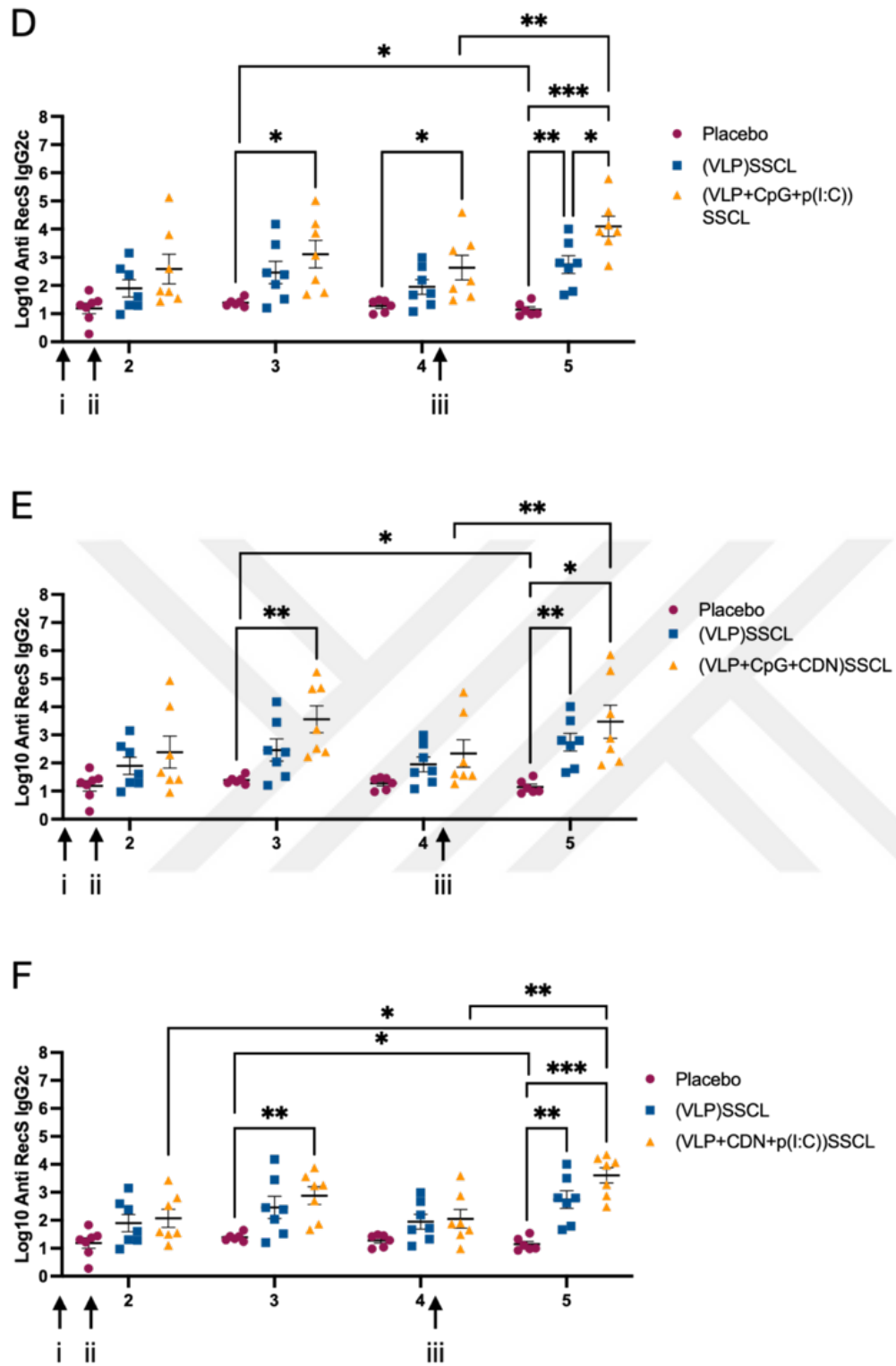


Figure 3.2. Evaluation of IgG1 responses of mice injected with liposomal vaccines against RecS by ELISA. The timeline above the graphs shows bleeding and injection days. Anti-spike IgG1 responses of 7 female C57Bl/6 mice in groups of Placebo, only VLP (5 μ g) encapsulated in liposome, (A) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) (B) VLP (5 μ g) encapsulated in liposome with poly(I:C) (50 μ g) (C) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) (D) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and poly(I:C) (50 μ g) (E) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and 2'3'-cGAMP (5 μ g) (F) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) (G) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g), 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) in 4 bleedings. The data indicate the mean $\pm$ SEM. Comparisons of the IgG1 responses were done by Mixed-effects analysis with Tukey's Test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

IgG2c responses of groups against RecS are presented in Figure 3.3. Except the group injected with VLP and CDN encapsulated vaccine (Figure 3.3 C), which produced similar levels of IgG2c as the group injected the only VLP encapsulated vaccine, all other groups produced more IgG2c than the group injected with the only VLP encapsulated vaccine (Figure 3.3 A, B, D, E, F, G). In general, a decrease in IgG2c titers of all groups was observed in the fourth bleeding, but the second booster injection made the titers rise again.





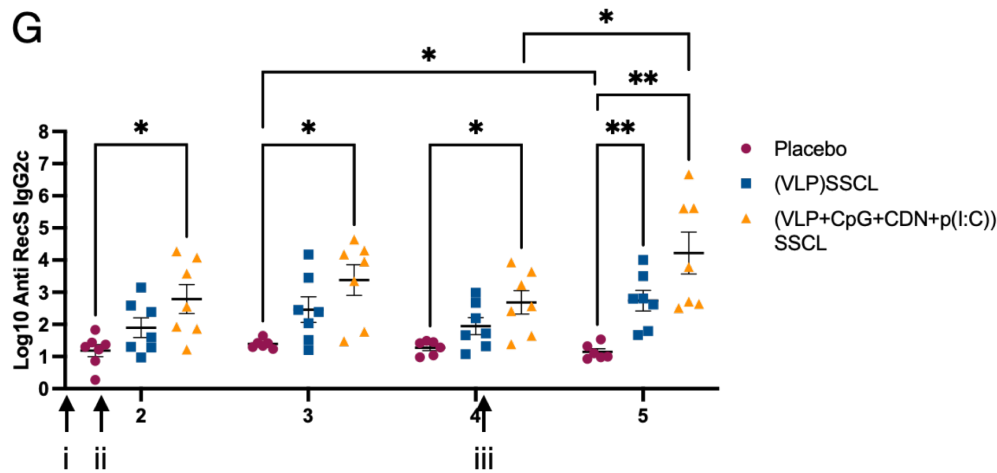


Figure 3.3. Evaluation of IgG2c responses of mice injected with liposomal vaccines against RecS by ELISA. The timeline above the graphs shows bleeding and injection days. Anti-spike IgG2c responses of 7 female C57Bl/6 mice in groups of Placebo, only VLP (5 μ g) encapsulated in liposome, (A) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) (B) VLP (5 μ g) encapsulated in liposome with poly(I:C) (50 μ g) (C) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) (D) VLP (5 μ g) encapsulated in liposome with CpG(30 μ g) and poly(I:C) (50 μ g) (E) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and 2'3'-cGAMP (5 μ g) (F) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) (G) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g), 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) in 4 bleedings. The data indicate the mean $\pm$ SEM. Comparisons of the IgG2c responses were done by Mixed-effects analysis with Tukey's Test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To predict the cellular immune responses of animals elicited after injections, the IgG2c/IgG1 ratios of each animal in the groups other than the placebo group were plotted, as presented in Figure 3.4. The results were obtained by dividing the IgG2c titer of each animal by its IgG1 titer. Except for the Day 28 data, IgG2c/IgG1 ratio of the only VLP encapsulated liposomal vaccine injected group did not breach the threshold where the titers of the two IgG subtypes are almost equal. Since the animals' IgG1 responses were more robust than their IgG2c responses, they elicited anti-RecS specific Th2-biased immunity. The group VLP plus CpG encapsulated within liposome maintained its highest IgG2c/IgG1 ratio in the last four bleedings, meaning that this formulation elicited strong anti-RecS specific Th1-biased immunity. IgG2c/IgG1 ratios of the group injected with the vaccine including adjuvants CpG plus CDN, and the group injected with the vaccine, including all three adjuvants, exceeded the threshold. The ratios of the remaining groups were almost at the threshold level.

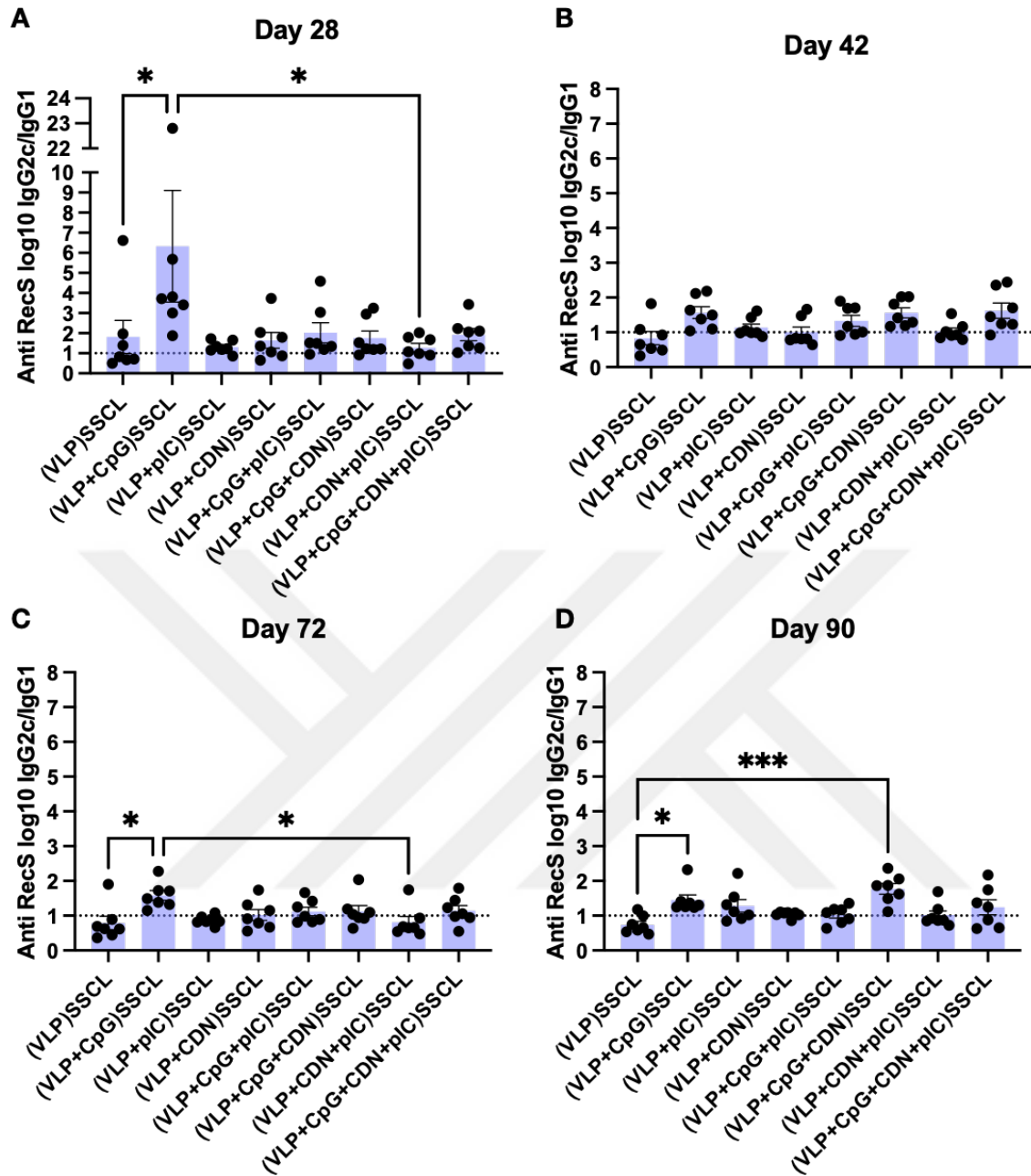
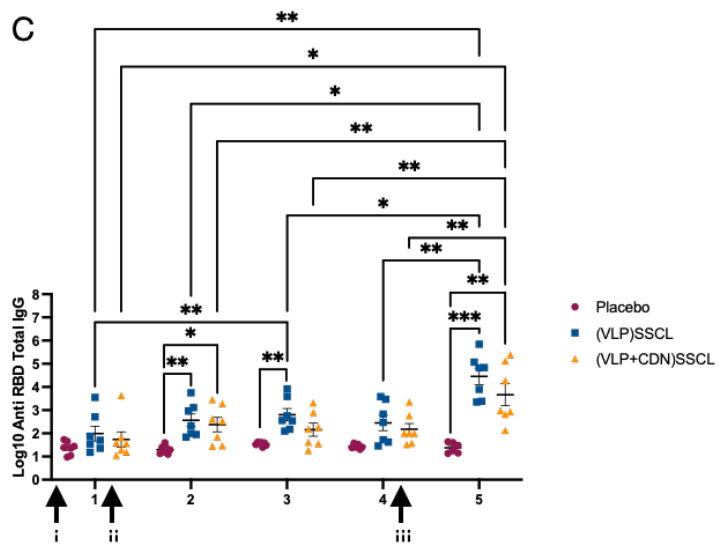
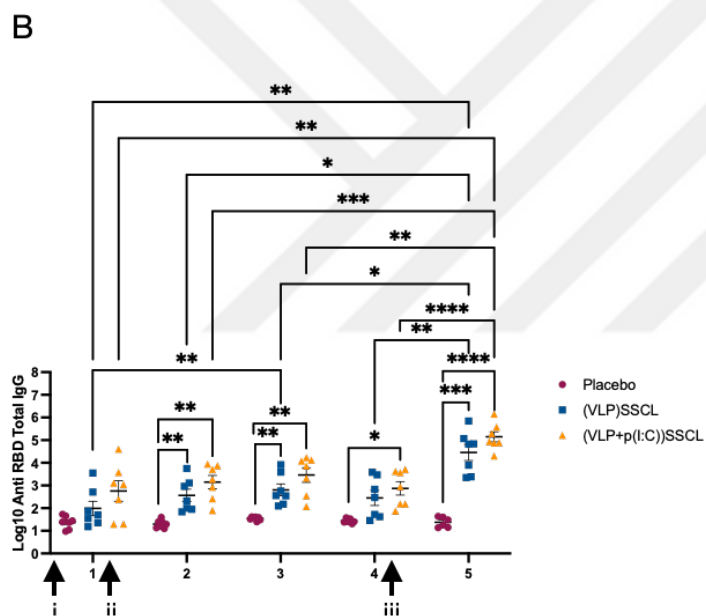
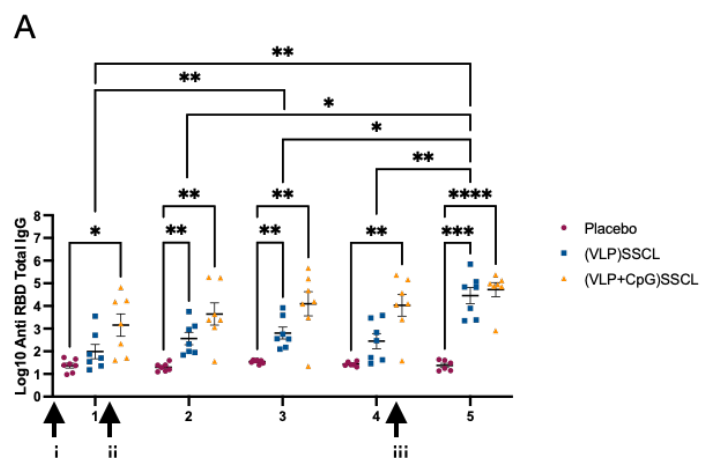


Figure 3.4. IgG2c/IgG1 ratios against RecS. Anti-RecS IgG2c titers of each animal in each group are divided into anti-RecS IgG1 titers for the last four bleedings. Groups of only VLP (5 μ g) encapsulated in liposome, VLP (5 μ g) encapsulated in liposome with CpG (30 μ g), VLP (5 μ g) encapsulated in liposome with poly(I:C) (50 μ g), VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g), VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and poly(I:C) (50 μ g), VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and 2'3'-cGAMP (5 μ g), VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g), VLP (5 μ g) encapsulated in liposome with CpG (30 μ g), 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) are used in the graphs. The data indicate the mean $\pm$ SEM. Comparisons of the groups were done by Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

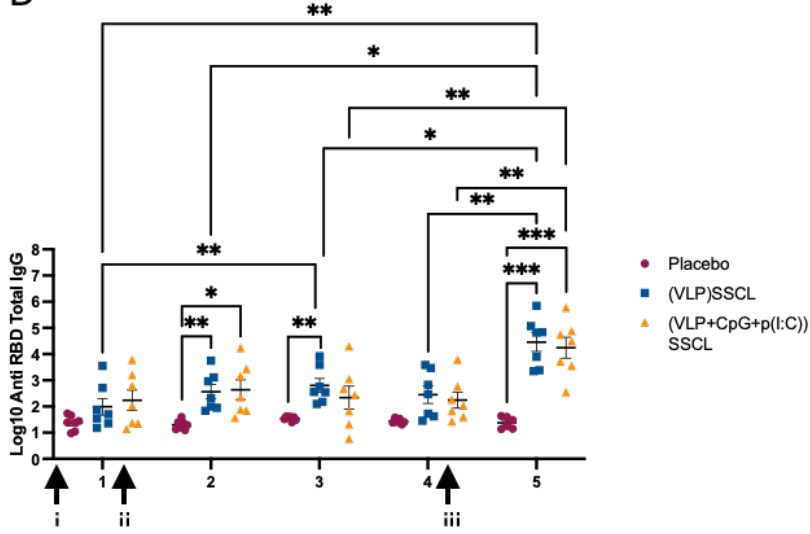
3.2.2 IgG Responses against Receptor Binding Domain

Receptor binding domain (RBD) is the essential part of the spike that enables the entry of SARS-CoV-2 to the host cells. Among the anti-SARS-CoV-2 antibodies discovered, anti-RBD antibodies were found to be the most common (Chen et al., 2023). Therefore, IgG responses of mice injected with the liposomal vaccine formulations against RBD were investigated by analysing Total IgG, IgG1 and IgG2c levels by using ELISA. Homemade Delta RBD was used for coating the plates (1 µg/ml).

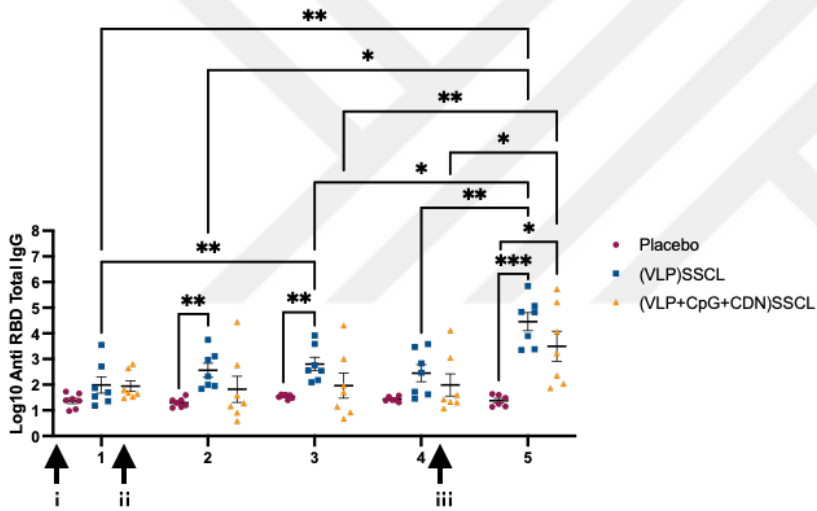
Figures 3.5, 3.6 and 3.7 show mice's total IgG, IgG1 and IgG2c responses against RBD, respectively. Graphs display IgG titers of groups injected with VLP encapsulated in liposomes with different adjuvant combinations. Titers of Placebo and the group injected with only VLP encapsulated liposomal vaccine are included in all graphs to compare the effect of adjuvants on titers. Figure 3.5 shows Total IgG titers of all five bleedings. In Figure 3.5 A and B, adding a single adjuvant, either CpG or poly(I:C), resulted in better Total IgG responses in all bleedings than in the group without any adjuvant. However, Figure 3.5 C shows this is not the case for the group with CDN; their titers were lower than that of the group with VLP alone. Figures 3.5 D, E and F show the Total IgG titers of dual adjuvant combinations. VLP with CpG and poly(I:C) resulted in a similar Total IgG response as the VLP alone. However, CpG plus CDN and CDN plus poly(I:C) resulted in a lower Total IgG response than VLP alone. Figure 3.5 G shows that combining VLP with all three adjuvants resulted in lower Total IgG titers than the VLP alone group in the first four bleedings. Still, in the fifth bleeding, the group with three adjuvants caught the titers of the group without adjuvants.



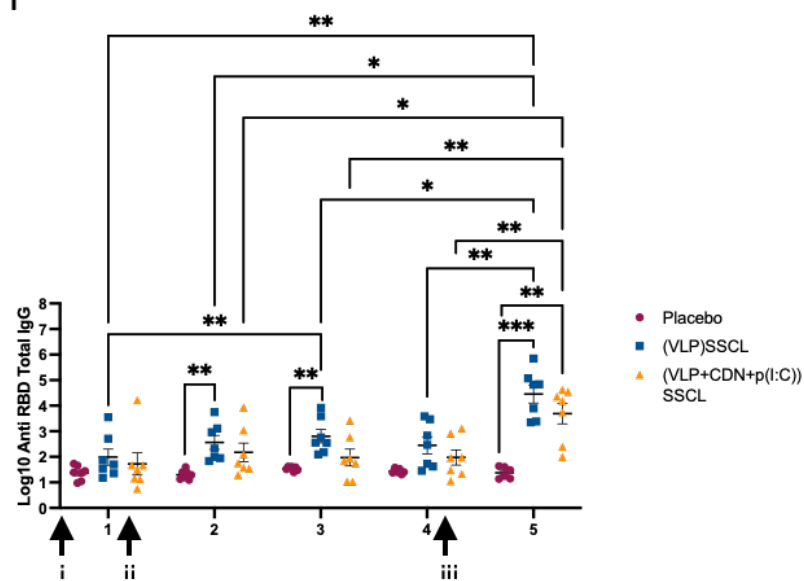
D



E



F



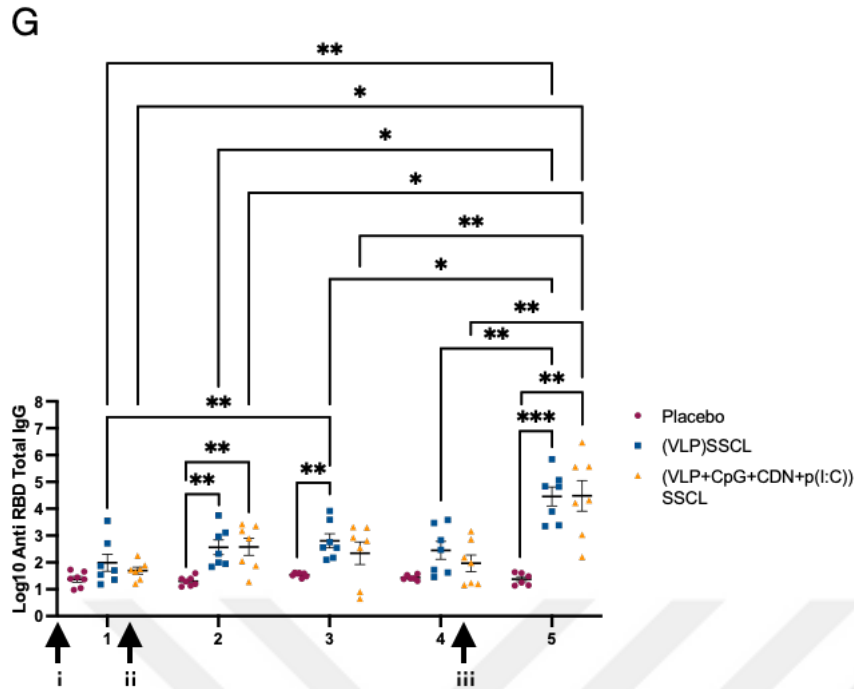
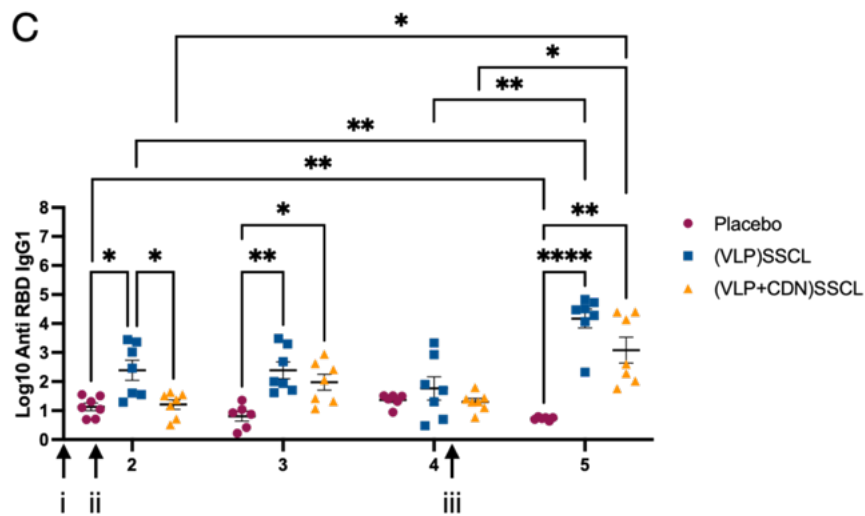
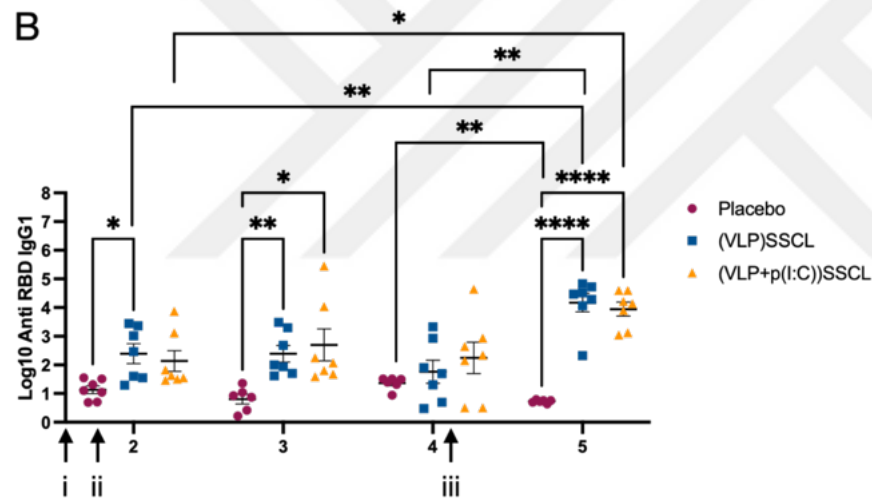
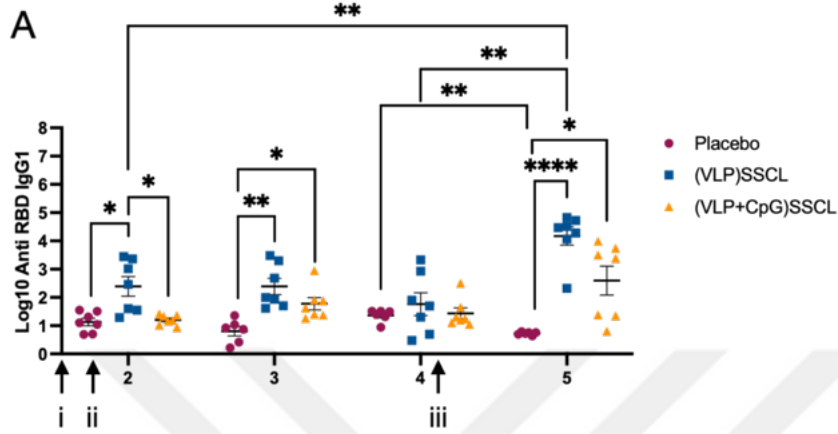
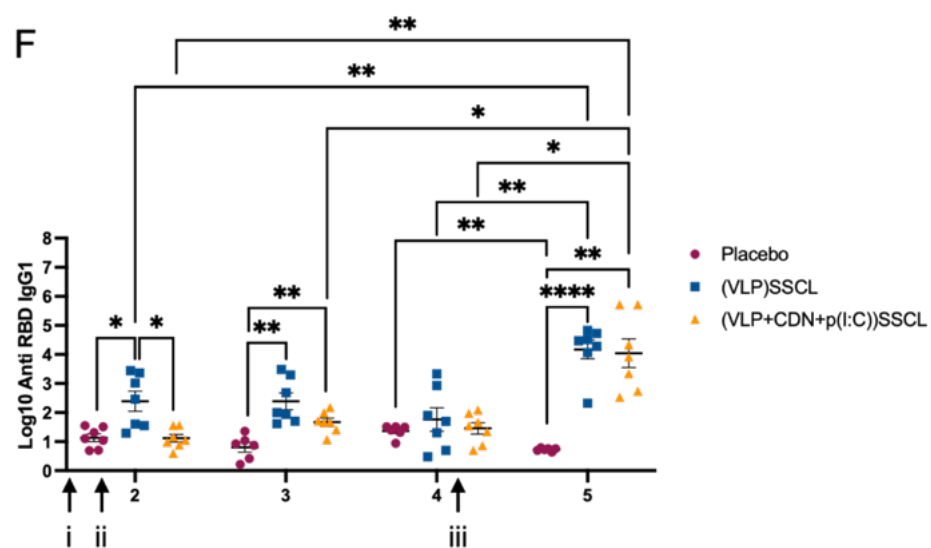
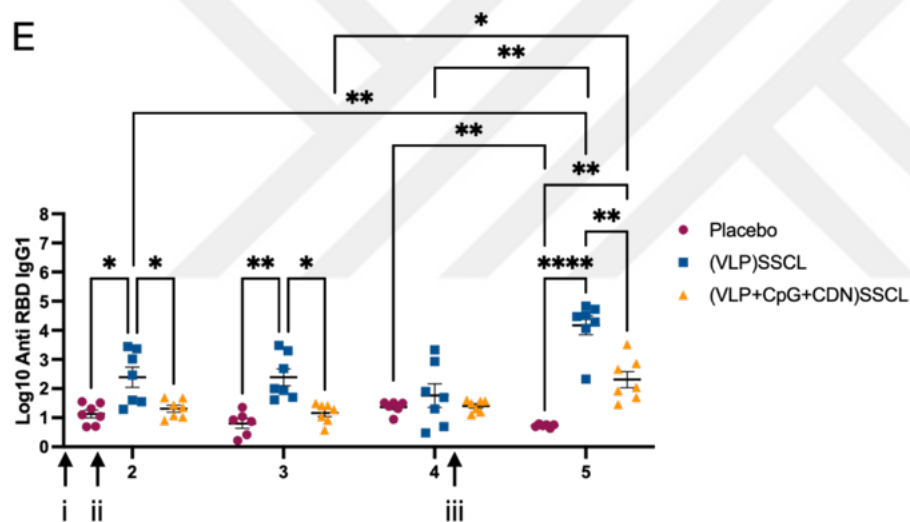
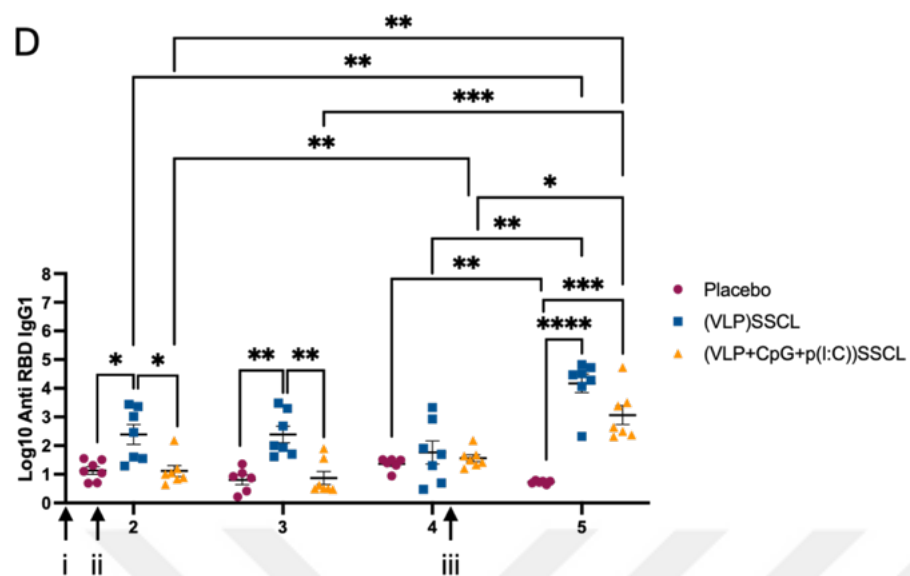


Figure 3.5. Evaluation of Total IgG responses of mice injected with liposomal vaccines against Delta Plus RBD by ELISA. The timeline above the graphs shows bleeding and injection days. Anti-RBD Total IgG responses of 7 female C57Bl/6 mice in groups of Placebo, only VLP (5 μ g) encapsulated in liposome, (A) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) (B) VLP (5 μ g) encapsulated in liposome with poly(I:C) (50 μ g) (C) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) (D) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and poly(I:C) (50 μ g) (E) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and 2'3'-cGAMP (5 μ g) (F) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) (G) VLP (5 μ g) encapsulated in liposome with CpG(30 μ g), 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) in all 5 bleedings. The data indicate the mean $\pm$ SEM. Comparisons of the IgG responses were done by Mixed-effects analysis with Tukey's Test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3.6 shows the IgG1 titers against RBD in the last four bleedings. Addition of either CpG or CDN to the liposomal VLP vaccine formulation resulted in lower IgG1 titers than the group with VLP alone, as depicted in Figures 3.6 A and 3.6 C. Figure 3.6 B shows that IgG1 responses of the group, where poly(I:C) was used as the only adjuvant, were generally similar to the group without adjuvant. Figure 3.6 D, E, F, G shows that groups injected with liposomal vaccines containing double and triple adjuvant combinations with VLP elicited lower IgG1 responses than the group injected the liposomal vaccine containing only VLP.





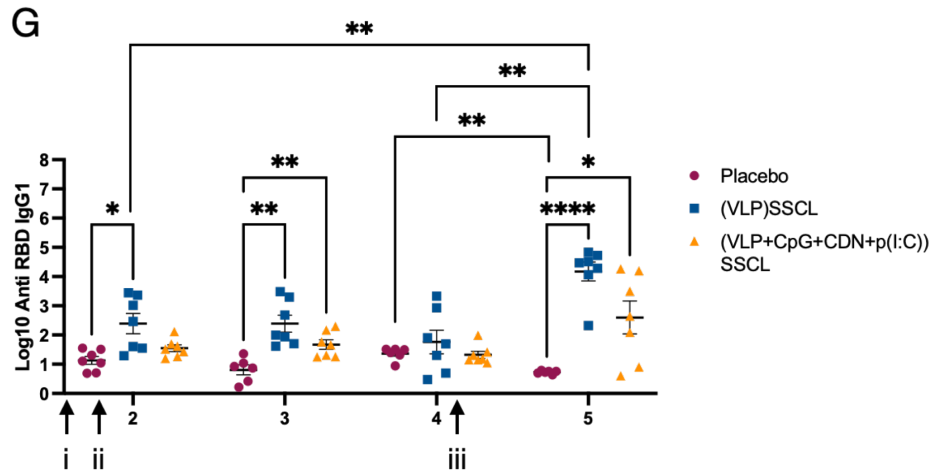
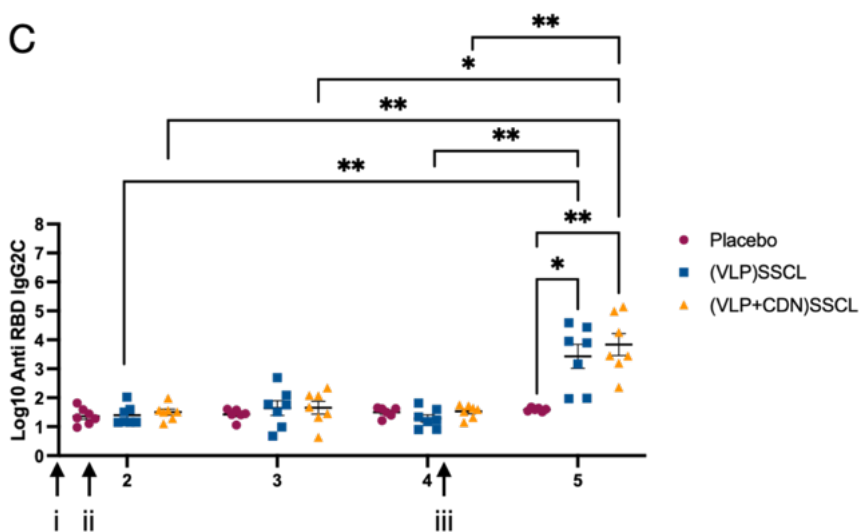
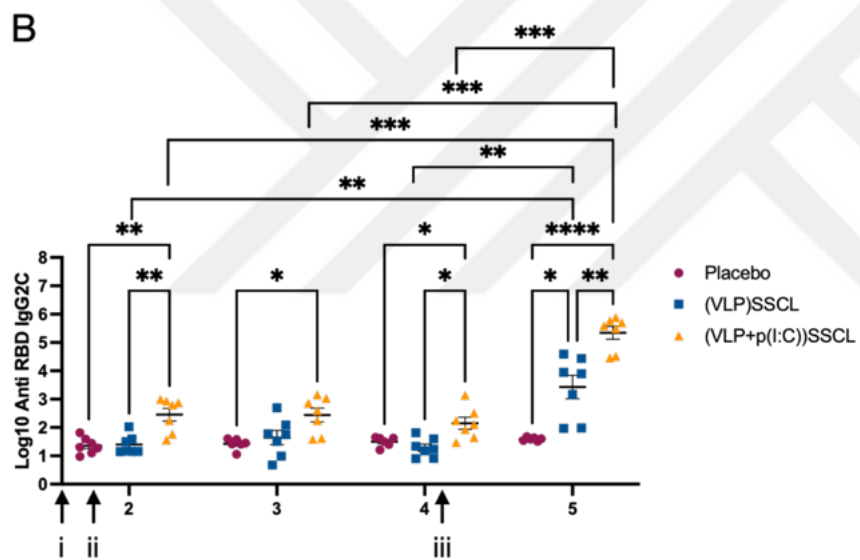
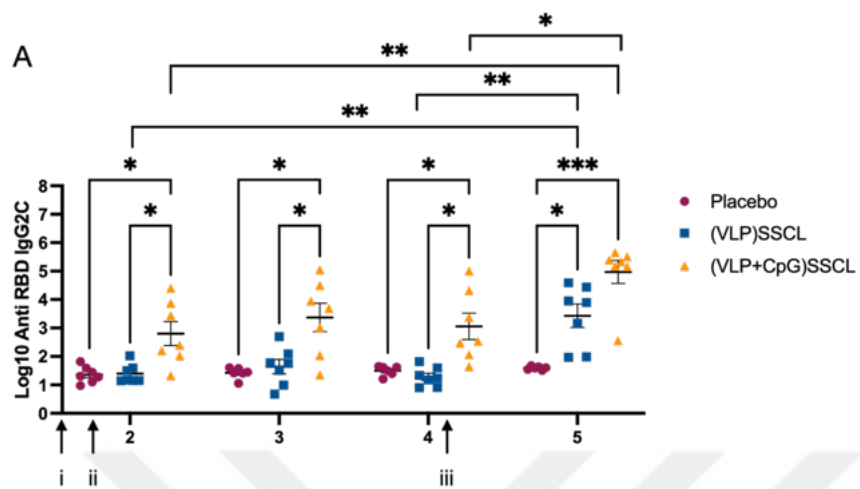
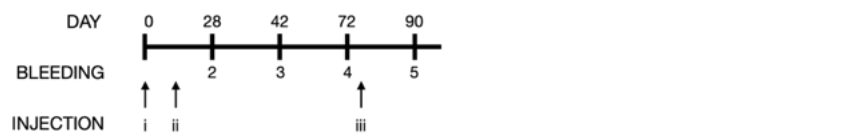
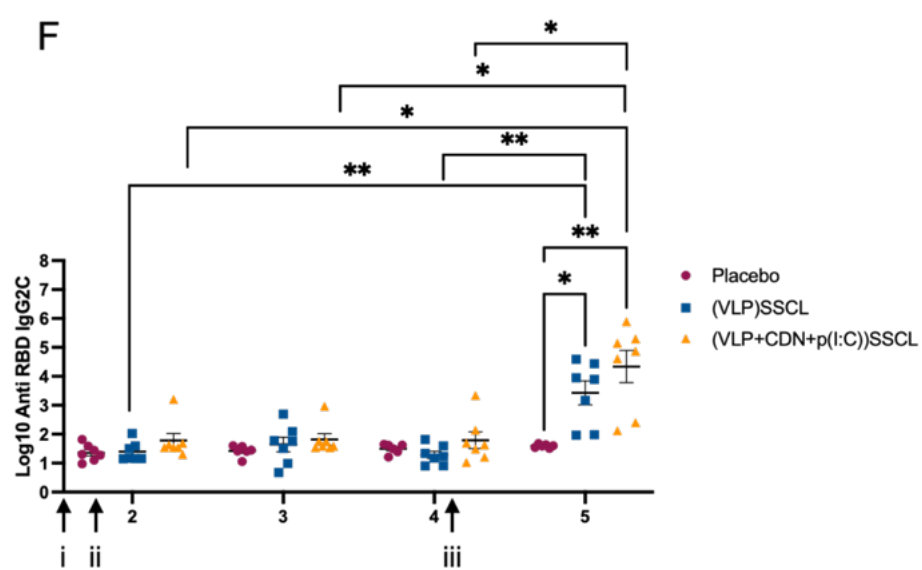
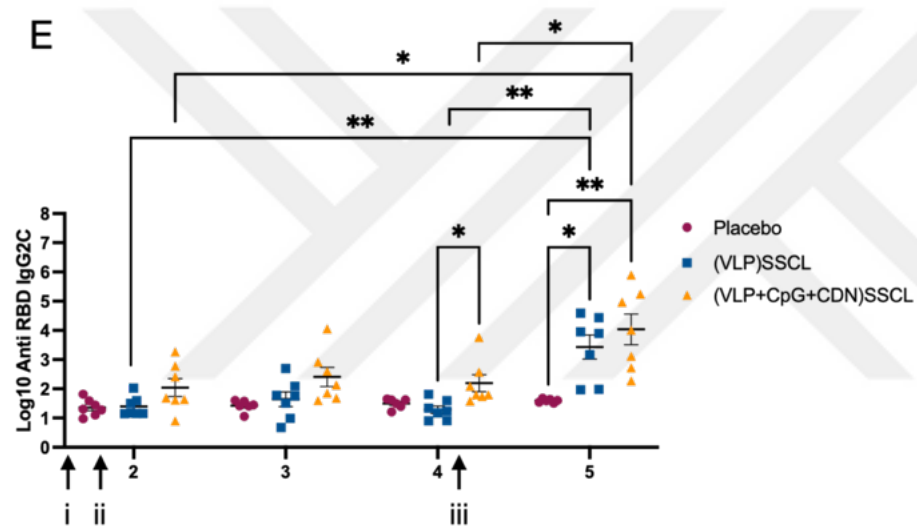
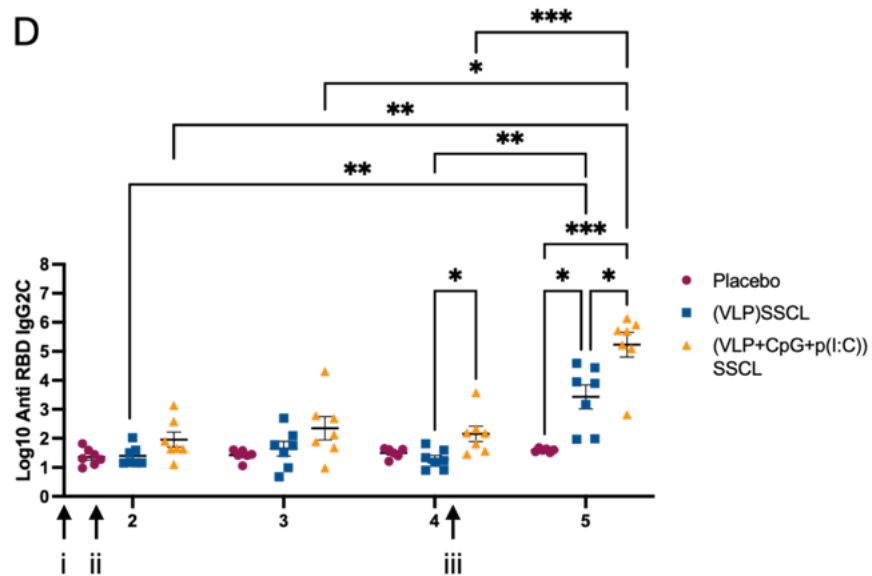


Figure 3.6. Evaluation of IgG1 responses of mice injected with liposomal vaccines against Delta Plus RBD by ELISA. The timeline above the graphs shows bleeding and injection days. Anti-RBD IgG1 responses of 7 female C57Bl/6 mice in groups of Placebo, only VLP (5 μ g) encapsulated in liposome, (A) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) (B) VLP (5 μ g) encapsulated in liposome with poly(I:C) (50 μ g) (C) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) (D) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and poly(I:C) (50 μ g) (E) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and 2'3'-cGAMP (5 μ g) (F) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) (G) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g), 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) in 4 bleedings. The data indicate the mean $\pm$ SEM. Comparisons of the IgG1 responses were done by Mixed-effects analysis with Tukey's Test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3.7 shows IgG2c titers of groups against RBD in the last four bleedings. Except the fifth bleeding, mice injected with VLP encapsulated with CpG within the liposomes produced significantly more IgG2c antibodies than mice injected with only VLP encapsulated within liposomes, as depicted in Figure 3.7 A. Figure 3.7 B shows that use of only poly(I:C) as an adjuvant also resulted in higher IgG2c titers than VLP alone. On the other hand, use of only CDN with VLP gave similar IgG2c titer results as VLP alone, as shown in Figure 3.7 C. Addition of two different adjuvant combinations or all of the adjuvants to the formulation resulted in slightly higher IgG2c titers than the vaccine consisting of VLP alone in liposome.





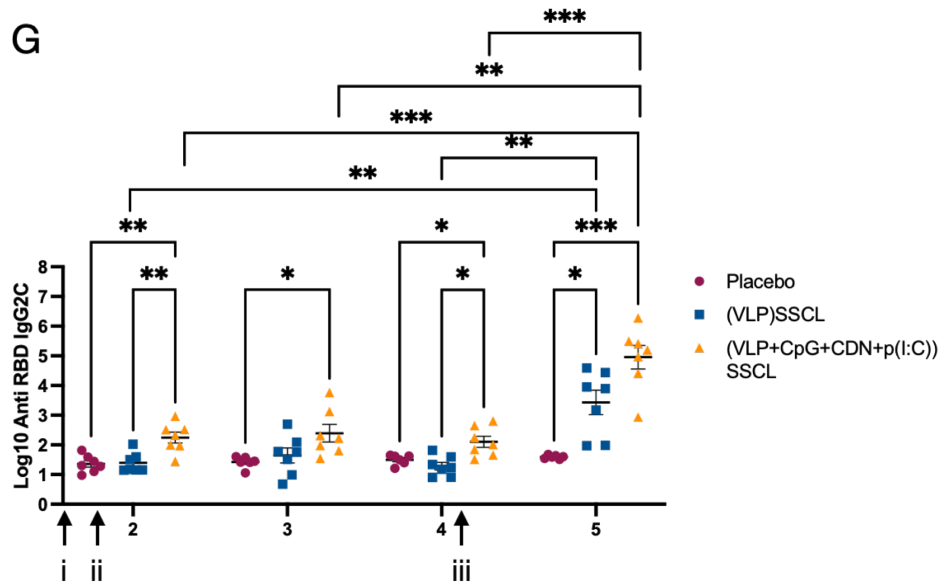


Figure 3.7. Evaluation of IgG2c responses of mice injected with liposomal vaccines against Delta Plus RBD by ELISA. The timeline above the graphs shows bleeding and injection days. Anti-RBD IgG2c responses of 7 female C57Bl/6 mice in groups of Placebo, only VLP (5 μ g) encapsulated in liposome, (A) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) (B) VLP (5 μ g) encapsulated in liposome with poly(I:C) (50 μ g) (C) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) (D) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and poly(I:C) (50 μ g) (E) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and 2'3'-cGAMP (5 μ g) (F) VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) (G) VLP (5 μ g) encapsulated in liposome with CpG (30 μ g), 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) in 4 bleedings. The data indicate the mean $\pm$ SEM. Comparisons of the IgG2c responses were done by Mixed-effects analysis with Tukey's Test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To predict the T cell responses induced by the vaccines against RBD, IgG2c/IgG1 ratios of the last four bleedings are plotted in Figure 3.8. Injection of VLP with CpG in liposome resulted in persistent Th1 biased immunity, as indicated by significantly elevated IgG2c/IgG1 ratio in all bleedings. According to Day 90 results (Figure 3.8 D), it can be interpreted that addition of adjuvant combinations CpG plus p(I:C), CpG plus CDN and all three in liposome with VLP also shifted the cellular immune response towards Th1, as their IgG2c/IgG1 ratios are higher than the ratio of only VLP encapsulating liposome injected group.

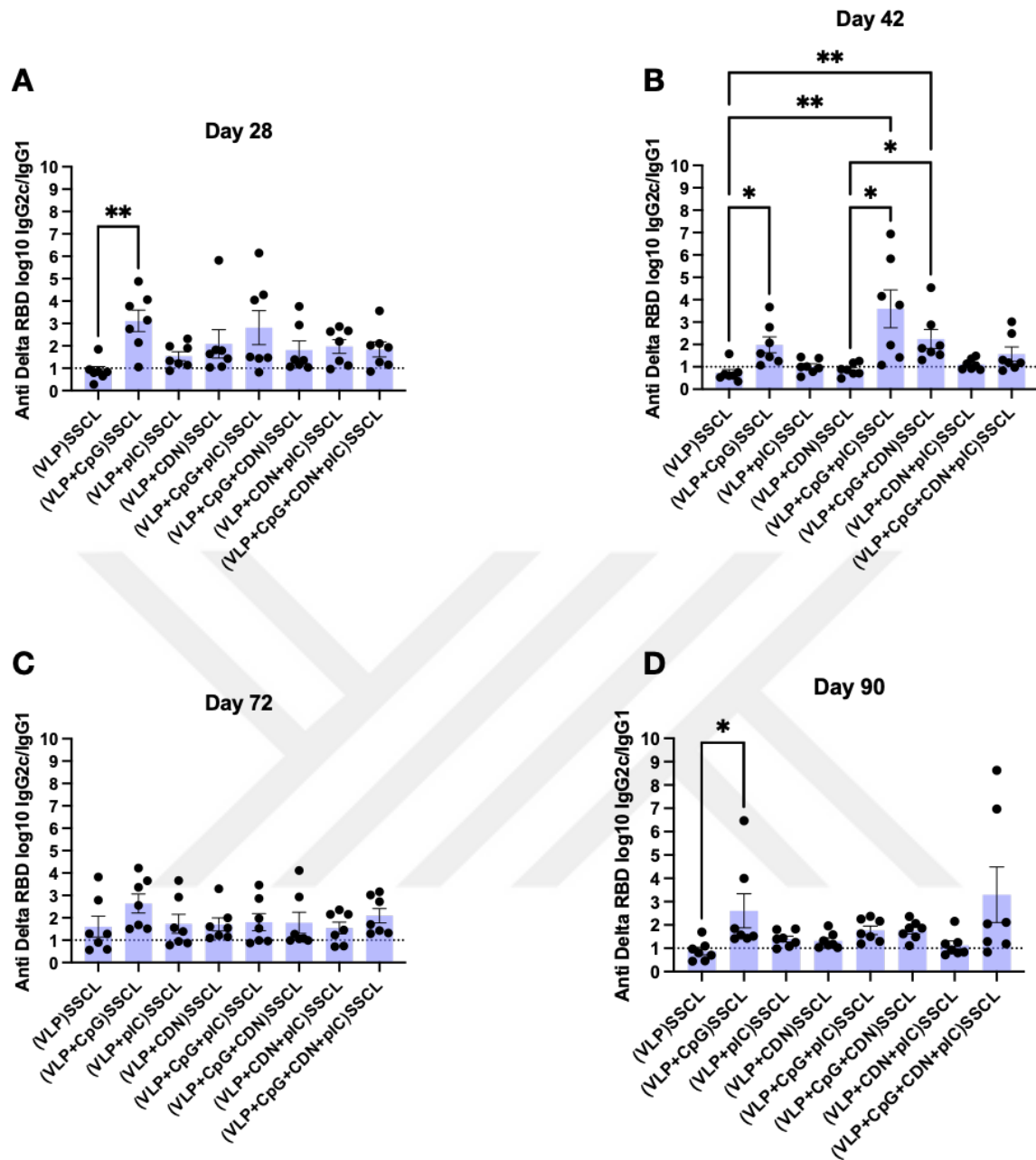


Figure 3.8. IgG2c/IgG1 ratios against Delta Plus RBD. Anti-RBD IgG2c titers of each animal in each group are divided to anti-RBD IgG1 titers for the last four bleedings. Groups of only VLP (5 μ g) encapsulated in liposome, VLP (5 μ g) encapsulated in liposome with CpG (30 μ g), VLP (5 μ g) encapsulated in liposome with poly(I:C) (50 μ g), VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g), VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and poly(I:C) (50 μ g), VLP (5 μ g) encapsulated in liposome with CpG (30 μ g) and 2'3'-cGAMP (5 μ g), VLP (5 μ g) encapsulated in liposome with 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g), VLP (5 μ g) encapsulated in liposome with CpG (30 μ g), 2'3'-cGAMP (5 μ g) and poly(I:C) (50 μ g) are used in the graphs. The data indicate the mean $\pm$ SEM. Comparisons of the groups were done by Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The outbreak of SARS-CoV-2 in late 2019 suddenly changed the lifestyle of people worldwide. Rapid virus transmission and high mortality rate prompted scientists to develop vaccines against SARS-CoV-2. Vaccine development is a challenging and time-consuming procedure. During the COVID-19 pandemic, thousands of vaccines from different platforms have been created. According to the recent data, 199 candidate vaccines are in the pre-clinical development stage, while 183 candidate vaccines are in the clinical development stage. As of late 2022, WHO granted 11 vaccines from protein subunit, mRNA, inactivated virus and viral vector platforms for emergency use. 4% of the candidate vaccines are from the VLP platform, but no VLP vaccine is approved for use (COVID-19 Vaccine Tracker and Landscape, 2023).

The principal aim of most vaccines developed against SARS-CoV-2 is to elicit an immune response against the virus by producing neutralizing antibodies that target the highly antigenic spike proteins of the virus. Spike has two conformational states; metastable prefusion and postfusion states. Since it is hard to produce recombinant metastable prefusion spike glycoprotein and prefusion stabilized proteins are better in immunogenicity, prefusion stabilization was indispensable. Spike variants with proline substitutions S-2p and S-6p were created, of which latter had higher stability and immunogenicity. Moreover, S-6p showed a better expression profile and binding affinity to human ACE2 receptors (Hsieh et al., 2020). The virus has evolved, creating different variants of concern (VOC). Mutations on spike confer resistance to the virus against antibodies generated after previous vaccinations (Harvey et al., 2021). Delta variant was the dominant VOC when this project started. Collectively, these are the reasons that Delta variant S-6p expressing VLPs are used in our vaccine. In addition to S; M, N and E proteins are expressed in our VLPs. The N is the most abundant protein in the virus and has high immunogenicity. Although M and E proteins of the SARS-CoV-2 are less immunogenic than S and N, their inclusion to the vaccine could broaden the T cell responses to the virus, as their genomic sequences are highly similar to those of SARS-CoV and MERS-CoV. (Currier et al., 2021). Even though VLPs are nanocarriers themselves, we hypothesized that using an additional carrier system could improve the efficacy of the vaccines. Therefore, liposomes have been used in vaccine formulations to protect VLPs, as they are known as potent immune response stimulators. This study uses K3 CpG ODN, poly(I:C), and 2'3'-cGAMP as

adjuvants. They were used together with the VLPs alone or in different combinations to see their effects on eliciting immune response against SARS-CoV-2.

It has previously been shown that CpG ODN uptake is improved when encapsulated within SSCL (Gursel et al., 2001). In addition to this, SSCL encapsulating antigen with CpG ODN and poly(I:C) or CpG ODN and 2'3'-cGAMP successfully regressed tumor growth (Bayyurt B., 2017). These achievements contributed to our selection of SSCL as the type of liposome in this study to encapsulate the VLPs and adjuvants.

Liposomes were prepared by dehydration-rehydration method. This method yields MLVs, which were sonicated ten times to obtain unilamellar vesicles. TRPS method was employed to characterize the liposomes by measuring their size distribution and concentration. According to the literature, although we intended to produce SUVs, our results show that our liposomes are large unilamellar vesicles (LUVs), as their sizes are in the range of LUVs, 100nm to 1µm (Lombardo and Kiselev, 2022). After the lyophilization step, although all vaccine formulation groups showed similar size distributions, their sizes were slightly lower than the size of the empty SSCL. This shows that the process of preparation of vaccine formulations is not so detrimental that it did not cause significant change in particle sizes and concentrations of liposomes. According to the literature, this difference could be explained by the absence of cryoprotectants. (El-Nesr et al., 2010). Overall, liposomes were of such size that immune cells, especially phagocytes, had no difficulty recognizing and engulfing them. (Hoshyar et al., 2016).

C57BL/6 mice were used in *in vivo* studies. There were nine experiment groups, and seven mice were present in each group, as shown in Table 2.6. SSCLs are drug delivery vehicles used in these vaccines and are used as 1µmol per animal. VLPs are the antigens in these vaccines, which are used as 5µg per animal. CpG ODN, poly(I:C) and 2'3'-cGAMP are the adjuvants, which are used as 30µg, 50µg and 5µg per animal, respectively, in the formulations that they are included. The first injection is done on Day 0, and the first bleeding of animals is carried out 14 days after the first injection, followed by the second injection on Day 15. Second, third, and fourth bleedings are carried out two, four, and eight weeks after the second injection, respectively. The final injection is done on the day after the fourth bleeding, and the fifth bleeding is carried out two weeks after that injection. Using the serum samples of animals, Total IgG, IgG1 and IgG2c ELISAs were carried out against S and RBD proteins of the virus to assess the humoral immune responses elicited in mice after the injections. RBD is located on the S1 subunit of the S that recognizes receptors on the host cell so it can induce potent antibody

production. Antibodies targeting the RBD are most likely neutralizing antibodies as they inhibit viral binding to host cells (Premkumar et al., 2020). Overall, the spike protein of the virus is responsible for host cell entry, so robust antibody response can be induced against S by the vaccines. In-house recombinant spike and Delta Plus RBD proteins were used for the ELISAs.

Total IgG responses against S and RBD were checked for all bleedings (Figures 3.1 and 3.4). According to the results of the first bleeding, all groups showed low IgG levels, except the (VLP+CpG)SSCL group, which produced significantly more Total IgGs than the Placebo against both S and Delta Plus RBD. Total IgG responses of all groups against both proteins showed an increasing trend until the third bleeding and levelled off or slightly decreased at the fourth bleeding, except the fourth bleeding titers of (VLP+CDN+p(I:C))SSCL group against RecS. The results demonstrate that high levels of IgG antibodies persisted two months after the booster injection. After the second booster injection, Total IgG titers rose significantly, implying that the third injection boosted the humoral immune response against both virus proteins.

B cells of the vertebrates initially produce IgM when they encounter an antigen. For long-term protection against the antigen, B cells switch to IgG production. Th cells influence which IgG subtype is produced by the B cells through the cytokines they secrete. Mice express four different IgG subtypes that differ in seroprevalence and functions. These are IgG1, IgG2a/c, IgG2b and IgG3 (Weber et al., 2014). IgG1 and IgG2c levels of mice were analyzed in this study against RecS and Delta Plus RBD. Graphs in Figure 3.2 compare IgG1 responses of each group received vaccine formulations with adjuvants against RecS with Placebo and (VLP)SSCL groups. Except for the (VLP+p(I:C))SSCL group, which showed similar IgG1 titers as the (VLP)SSCL group, other groups showed lower IgG1 titers than the (VLP)SSCL group with varying levels. In Figure 3.3, IgG2c responses of groups against RecS are plotted. It can be seen that all groups with adjuvants had higher IgG2c titers than the (VLP)SSCL group with varying levels. IgG1 and IgG2c responses of mice against Delta Plus RBD, as shown in Figures 3.6 and 3.7, are similar to those against RecS. (VLP+p(I:C))SSCL group had similar IgG1 titers as the (VLP)SSCL group, and all other groups had lower IgG1 titers than the (VLP)SSCL group. IgG2c titers of (VLP+CDN)SSCL and (VLP+CDN+p(I:C))SSCL groups were similar to the titers of (VLP)SSCL group. The remaining groups with adjuvants had higher IgG2c levels than the (VLP)SSCL group with varying levels.

Th1 cells release cytokines, of which IFN γ is the most prominent, which promotes switching B cells to IgG2c-secreting cells. Th2 cells secrete cytokines, like IL-4, that promote B cells switching towards IgG1 secretion (Weber et al., 2014). Th1 response is vital for fighting against intracellular pathogens. Th1 cells encourage the development of cell-mediated immunity, primarily by activating macrophages and cytotoxic T cells. Th2 response plays a role in eliminating extracellular pathogens by promoting the development of humoral immunity (Aleebrahim-Dehkordi et al., 2021). As with viral infections, SARS-CoV-2 infection can be attentively eliminated if a proper Th1 response is developed (Gil-Etayo et al., 2021). Otherwise, the Th2 response is associated with a poor prognosis of COVID-19 (Aleebrahim-Dehkordi et al., 2021). The IgG2c/IgG1 ratio is used to evaluate which Th cells became dominant after the vaccinations. Figure 3.4 shows the ratios (IgG2c/IgG1) against RecS for the last four bleedings. Generally, adjuvant addition resulted in balanced Th1 and Th2 response or Th1 biased response, as the ratios are at or above the threshold, respectively. The ratio of the VLP alone is below the threshold, correlating with the Th2 response. Figure 3.8 shows the ratios against Delta RBD; generally, the results are similar to those against RecS. Among all groups, for anti-RecS (VLP+CpG)SSCL showed a persistent Th1-biased profile, as its ratios were significantly higher than the (VLP)SSCL in almost all bleedings. For anti-RBD, (VLP+CpG)SSCL and (VLP+CpG+CDN+p(I:C))SSCL induced strongest Th1 biased response.

As mentioned earlier, CpG ODN favors strong Th1 response and antibody production that persist for a long period (Facciola et al., 2022). The efficacy of CpG ODN can be advanced by using delivery systems, and liposome encapsulation is one of the proven ways. In this way, CpG ODN is not only protected from early degradation by nucleases but also its targeted delivery to immune cells is ensured (Gursel et al., 2001). Poly(I:C) is a strong anti-viral immune response inducer, but it can result in undesirable outcomes like overstimulation of the immune response and autoimmunity. To avoid these issues, the dosage of poly(I:C) without side effects should be determined, and an appropriate delivery system should be used. Cationic liposomes are one of the delivery systems used for poly(I:C), and their combination has been proven to be efficient inducers of immune response (Hafner et al., 2013). In this research, when Total IgG responses against RecS and RBD are considered, CpG and poly(I:C) successfully stimulated the immune system. Titer graphs in Figure A.1 and Figure A.4 show that the (VLP+CpG)SSCL group has the highest mean in all bleedings, showing an increasing trend over time except Day 72. (VLP+p(I:C))SSCL is the second group in the ranking. 2'3'-cGAMP is another adjuvant that stimulates immune response through the STING pathway. As shown in the titers graphs in

Figure A.1 and Figure A.4, 2'3'-cGAMP was not as successful as CpG and poly(I:C) in inducing immune responses in this research, and it failed to induce synergic activity. Overall, titers of all groups increased over time up to Day 42, then levelled off or slightly decreased on Day 72 but rose considerably on Day 90 due to the second booster injection. Dual and triple adjuvant combinations resulted in lower titers than the groups where CpG or poly(I:C) were used as the only adjuvant. The results indicate that there is no need to combine more than one adjuvant with the VLP to induce a strong humoral immune response; either CpG or poly(I:C) is sufficient to generate high levels of IgG. For better cellular immune response, CpG should be used as the (VLP+CpG)SSCL group had a better Th1-biased profile over the timeline.

Previous studies have shown that three doses of mRNA and protein subunit vaccines provide potent protection against SARS-CoV-2 infection. (Herzberg et al., 2022 & Dai et al., 2022). In line with these findings, this study supported the importance of the second booster injection against SARS-CoV-2. Among the groups, CpG, p(I:C) and CpG+p(I:C) formulated vaccines showed sustained antibody response profile over time and the antibody responses of other groups started to wane off two months after the second injection. However, the second booster injection augmented the antibody titers.

VLPs are promising candidates in vaccine platform as they are versatile and easy to produce quickly. Our lab has developed a VLP vaccine where VLPs express hexaprolin stabilized WT or Alpha variant spike and other structural proteins of the SARS-CoV-2, which entered human clinical trials (NCT04818281). In this vaccine, VLPs were formulated with alum and CpG ODN (Yılmaz et al., 2021). In this thesis study, the VLPs are modified with hexaprolin stabilized Delta variant Spike and formulated with adjuvants. The results build on the existing evidence of enhanced immunogenicity of VLPs by CpG ODN addition (Yılmaz et al., 2021). Most importantly, formulations were encapsulated in liposomes to take advantage of the depot effect of the liposomes and enhance cellular uptake of the contents. The encapsulation process requires lyophilization, eliminating the need for cold chain storage until the vaccine reconstitution.

Overall, the vaccine formulations proved successful in eliciting a robust immune response against SARS-CoV-2. Despite the presence of intragroup differences in IgG responses and the small sample size, this study lays the foundation for future investigations. In vitro studies should be conducted to confirm the cellular uptake of liposomes and assess the cytokine secretion profiles of T cells. Furthermore, it is important to challenge immunized ACE2 transgenic mice

with SARS-CoV-2 to evaluate the level of protection conferred by the vaccines. Virus neutralization assays should be performed to determine the presence of neutralizing antibodies.

Combination vaccines enable the simultaneous administration of multiple different vaccines in a single injection. Traditionally, combination vaccines are used to simplify individuals' adherence to their immunization schedules, and the Diphtheria-Tetanus-Pertussis vaccine serves as an example of this (Ellis and Brown, 1997). Influenza A virus causes respiratory infections and is so prevalent that it leads to seasonal epidemics each year. It has been reported that the risk of transmission and death increased due to the high incidence of flu and SARS-CoV-2 coinfections during the COVID-19 pandemic (Shi et al., 2022). There are few published studies regarding the SARS-CoV-2 and Influenza combination vaccine. In one study, the Receptor-Binding Domain (RBD) of SARS-CoV-2 was conjugated onto inactivated influenza A virus (Wang et al., 2023). In another study, subunits of both viruses, which are the most common targets of neutralizing antibodies, were combined to create the combination vaccine (Shi et al., 2022). The results of both studies demonstrated the strong immunogenicity of the combination vaccines against SARS-CoV-2 and Influenza A (Shi et al., 2022 & Wang et al., 2023). The concept of a combination vaccine against SARS-CoV-2 and Influenza A can be applied within the scope of this study, where SARS-COV-2 VLPs can be encapsulated within liposomes along with Influenza antigens.

Prior to advancing to human trials, additional research will entail testing the vaccine formulations that demonstrated superior results in this study on higher animals. Specifically, these formulations should undergo evaluation in various disease models, including those related to diabetes and Alzheimer's, as the effects of infection and vaccines may differ among individuals with diverse pre-existing health conditions.

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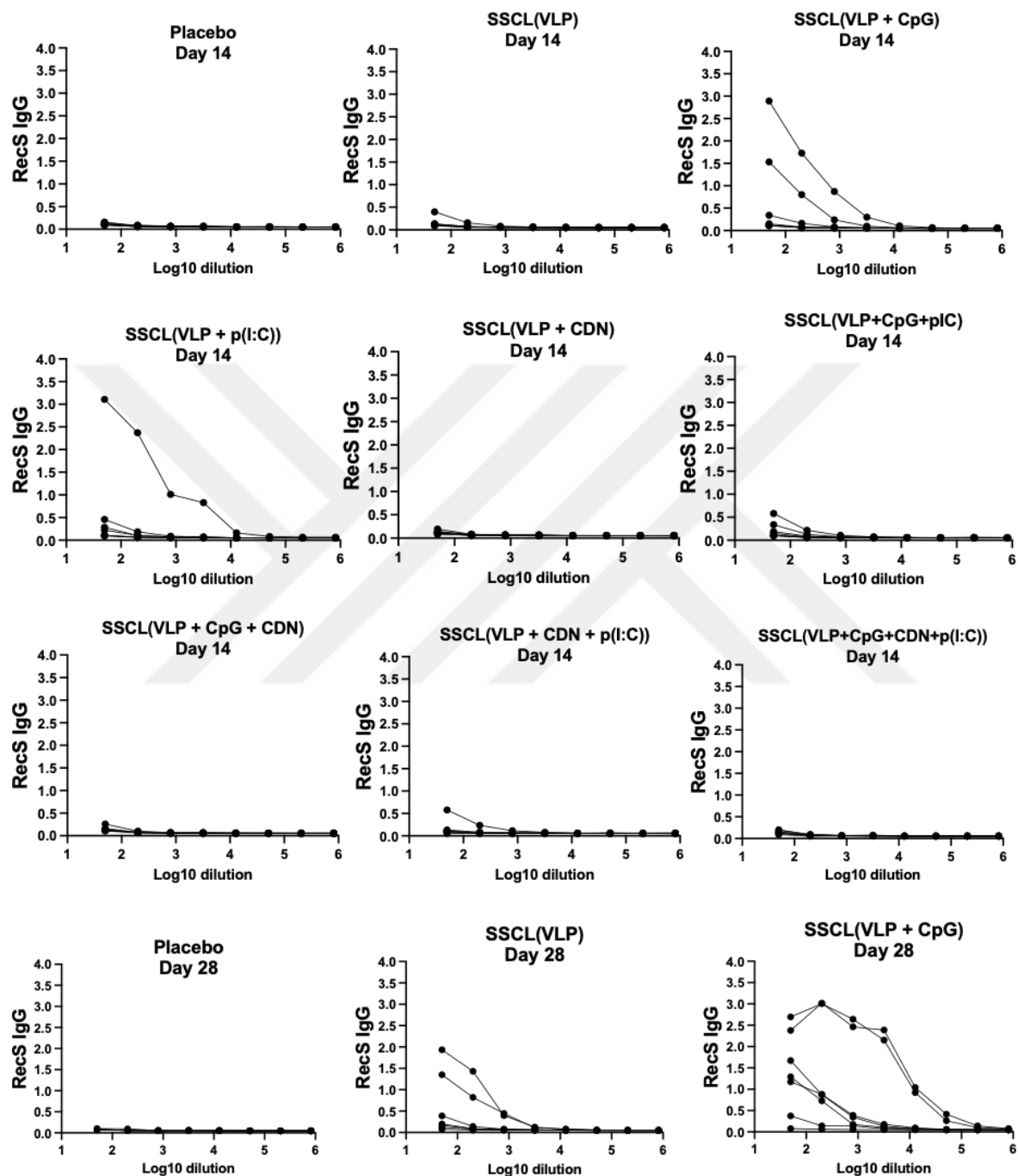
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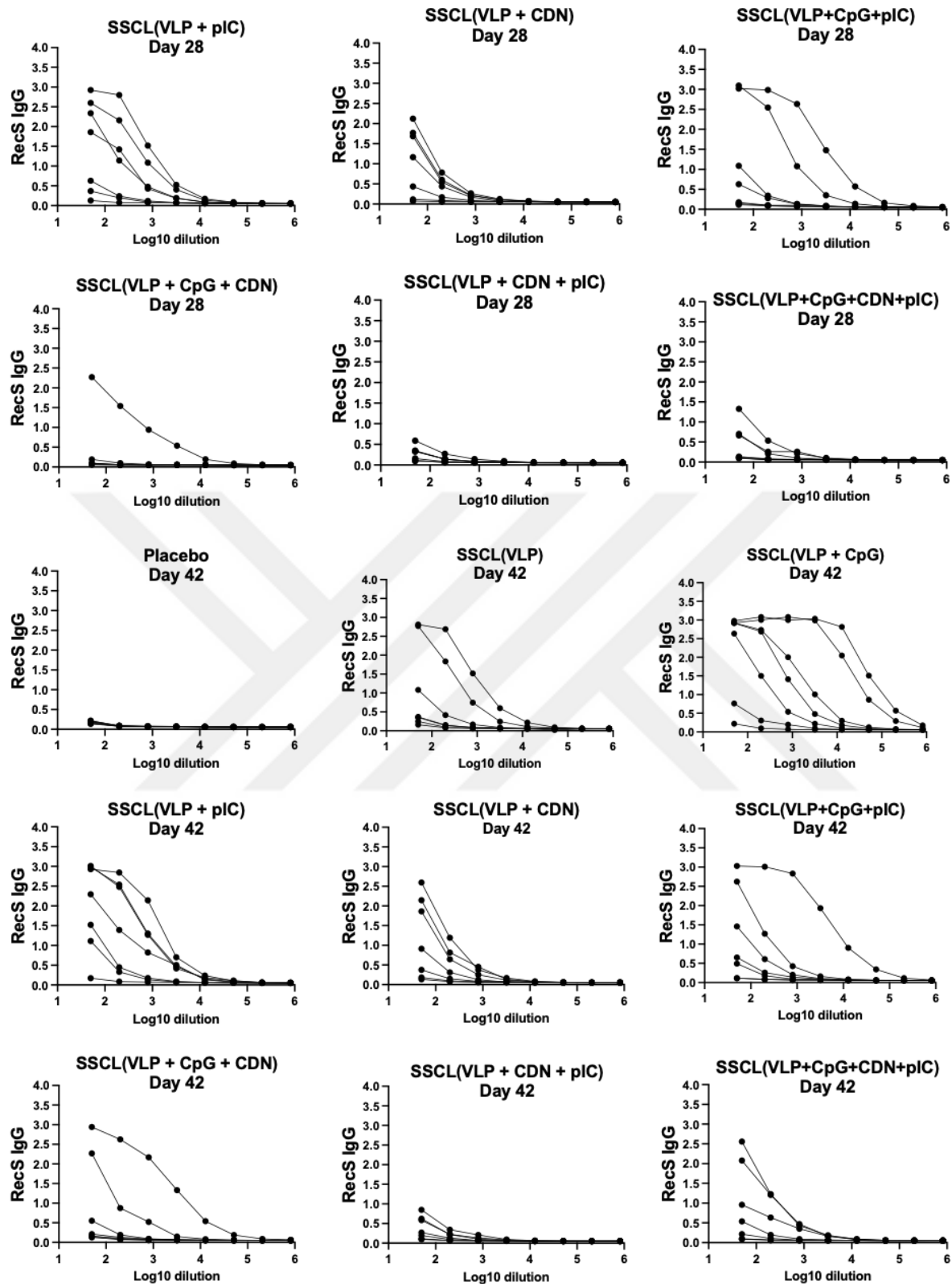
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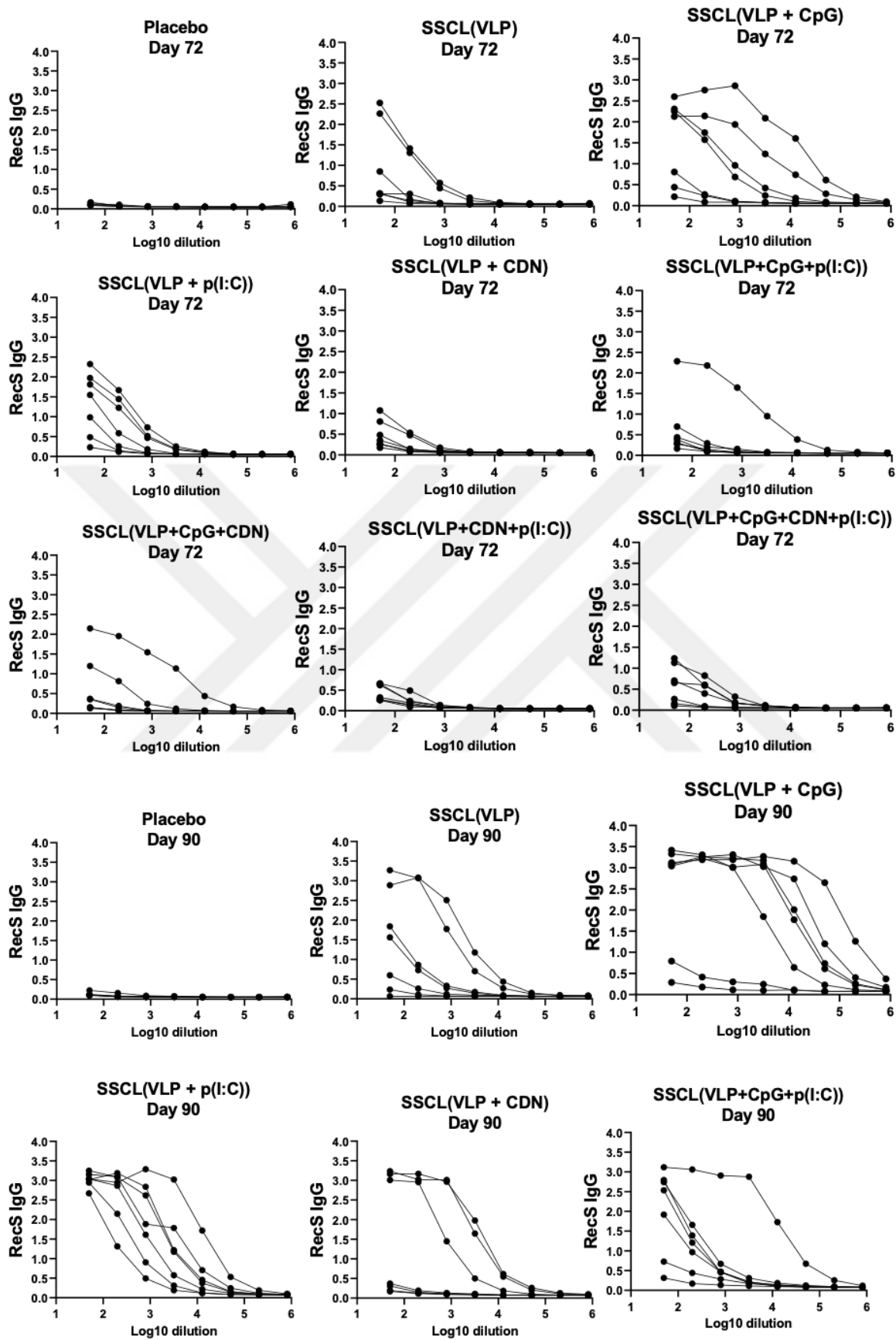
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APPENDIX







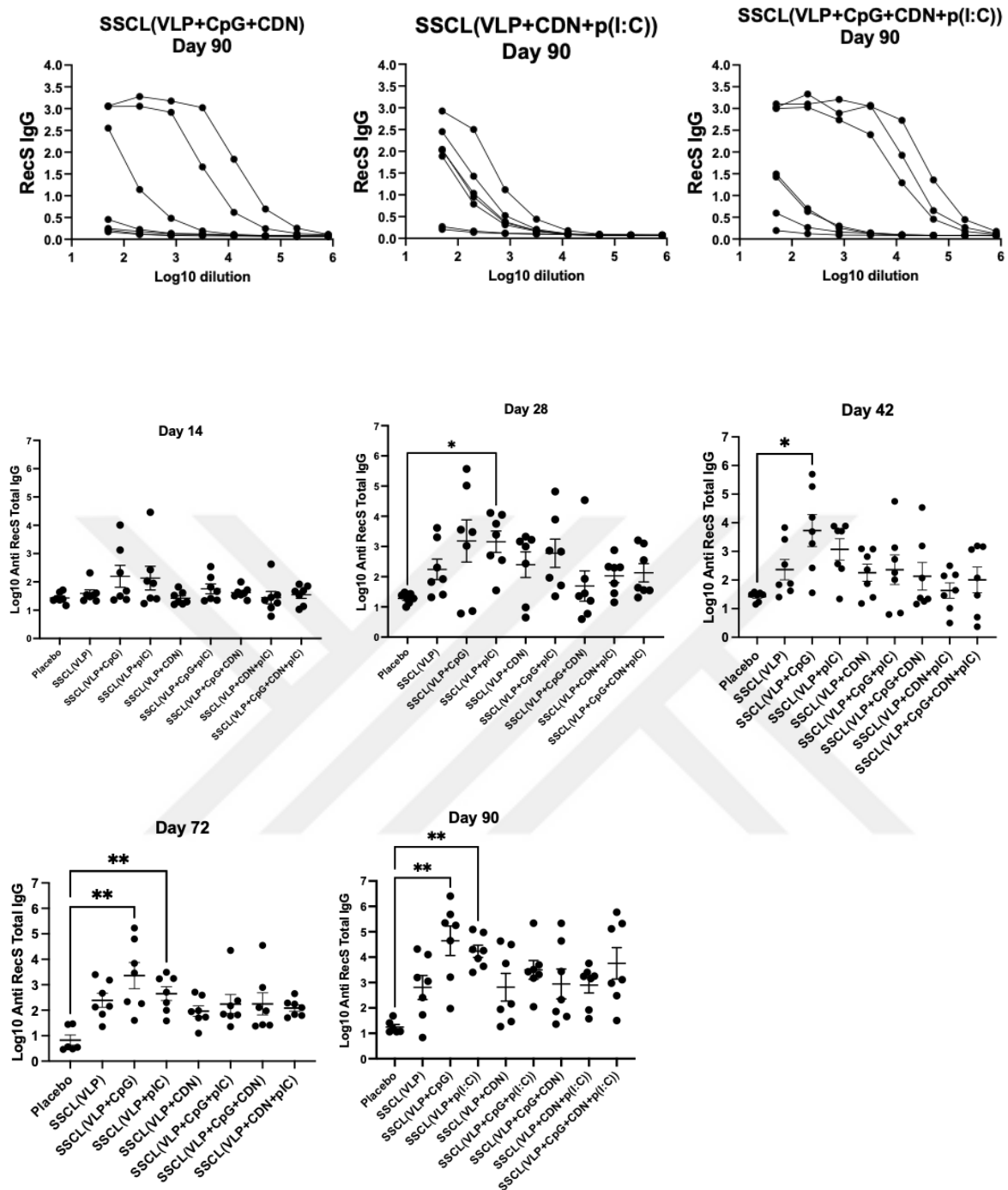
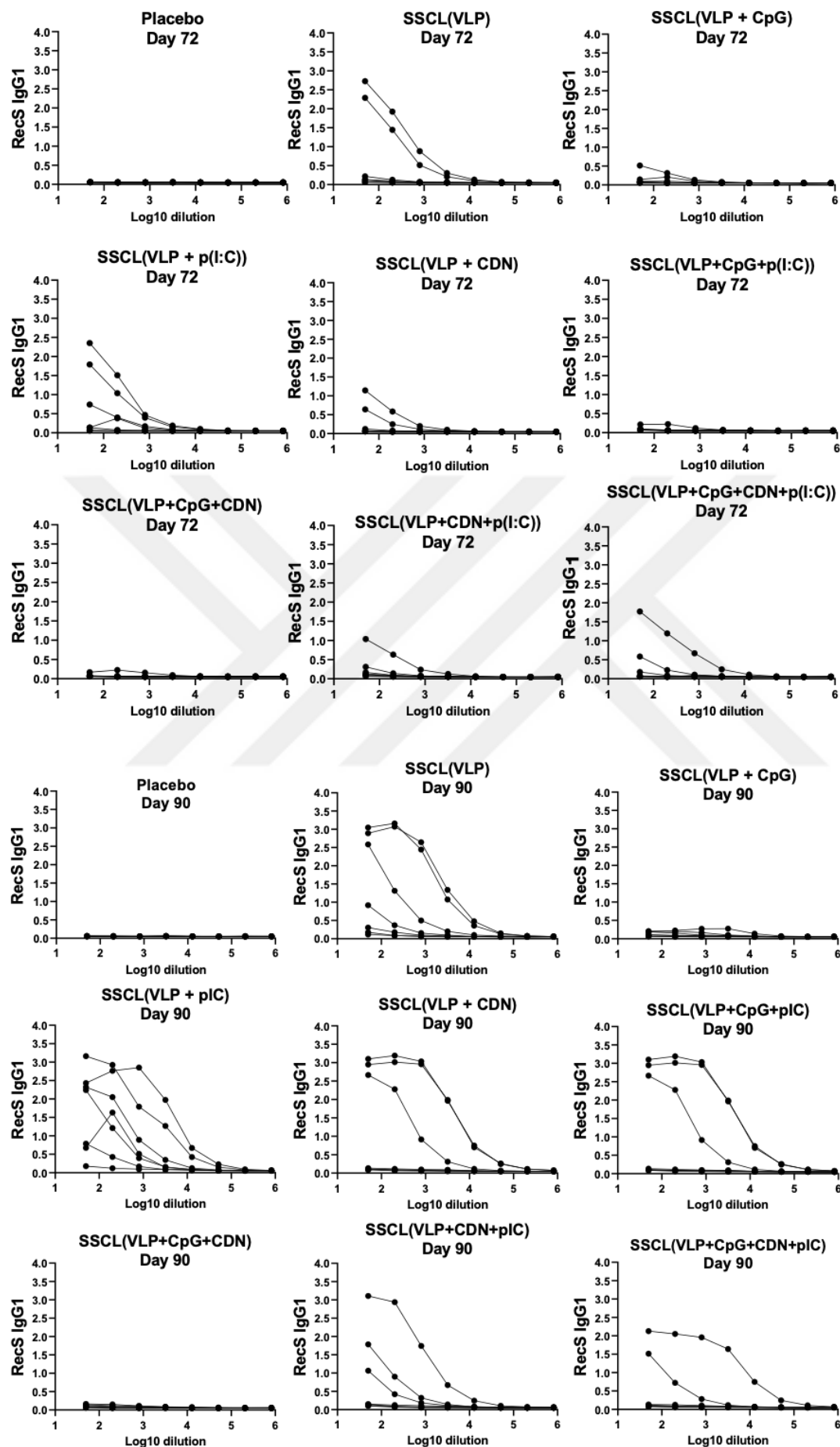


Figure A.1. The Total IgG responses of injection groups against recombinant spike (RecS) were evaluated by ELISA. 2HB immunoplates were coated overnight with 0.5 $\mu\text{g}/\text{ml}$ of RecS. After the blocking step, animal sera collected on Day 14, Day 28, Day 42, Day 72 and Day 90 were diluted in 1:50, then serially diluted fourfold seven times and incubated overnight. The next day, plates were incubated with anti-mouse Total IgG, diluted in 1:1000, for two hours. Lastly, plates were treated with PNPP substrate solution, and OD405 values were recorded, as displayed on graphs. Last five graphs show Total IgG titers of groups in all bleedings. The groups were compared by Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



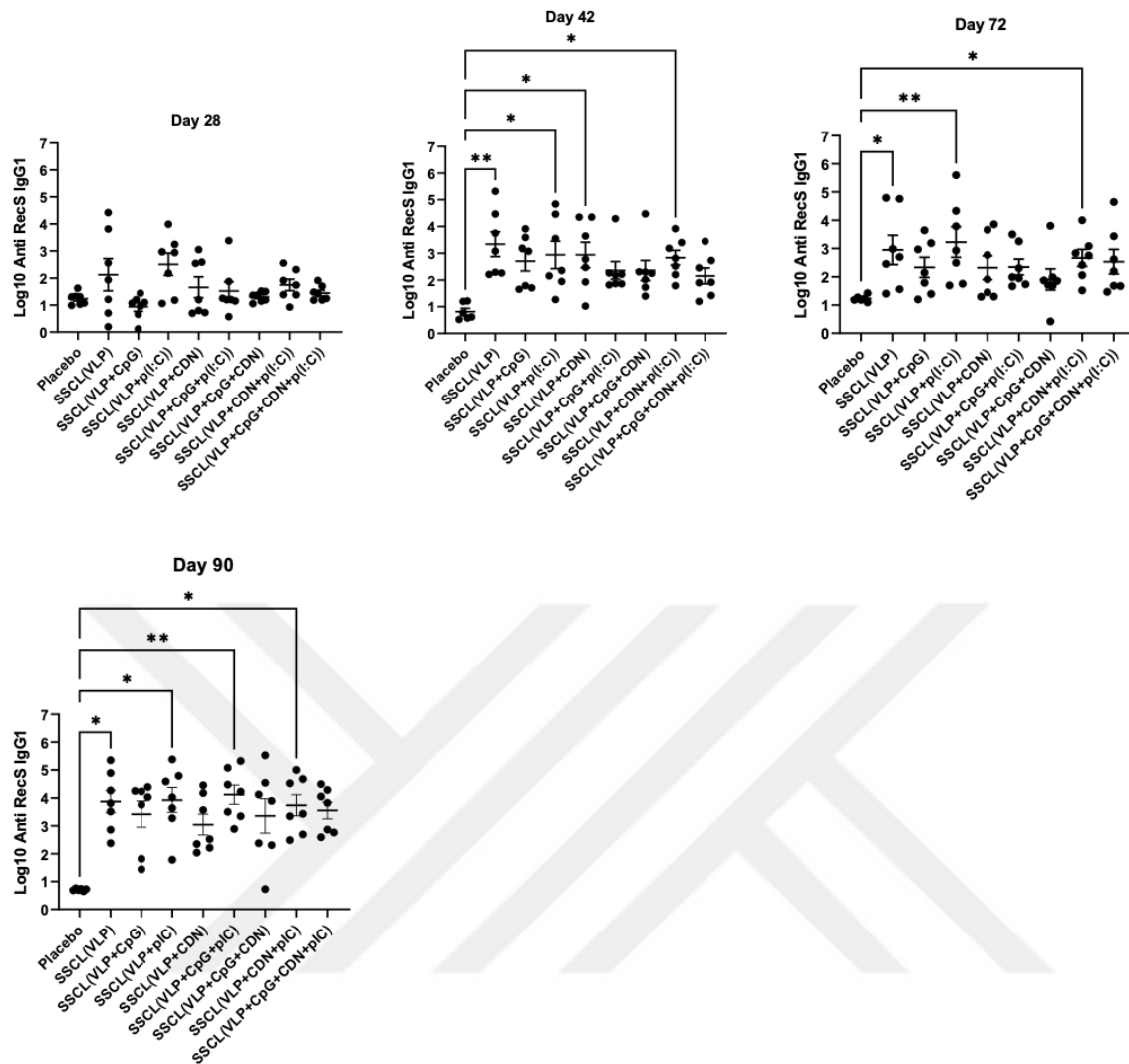
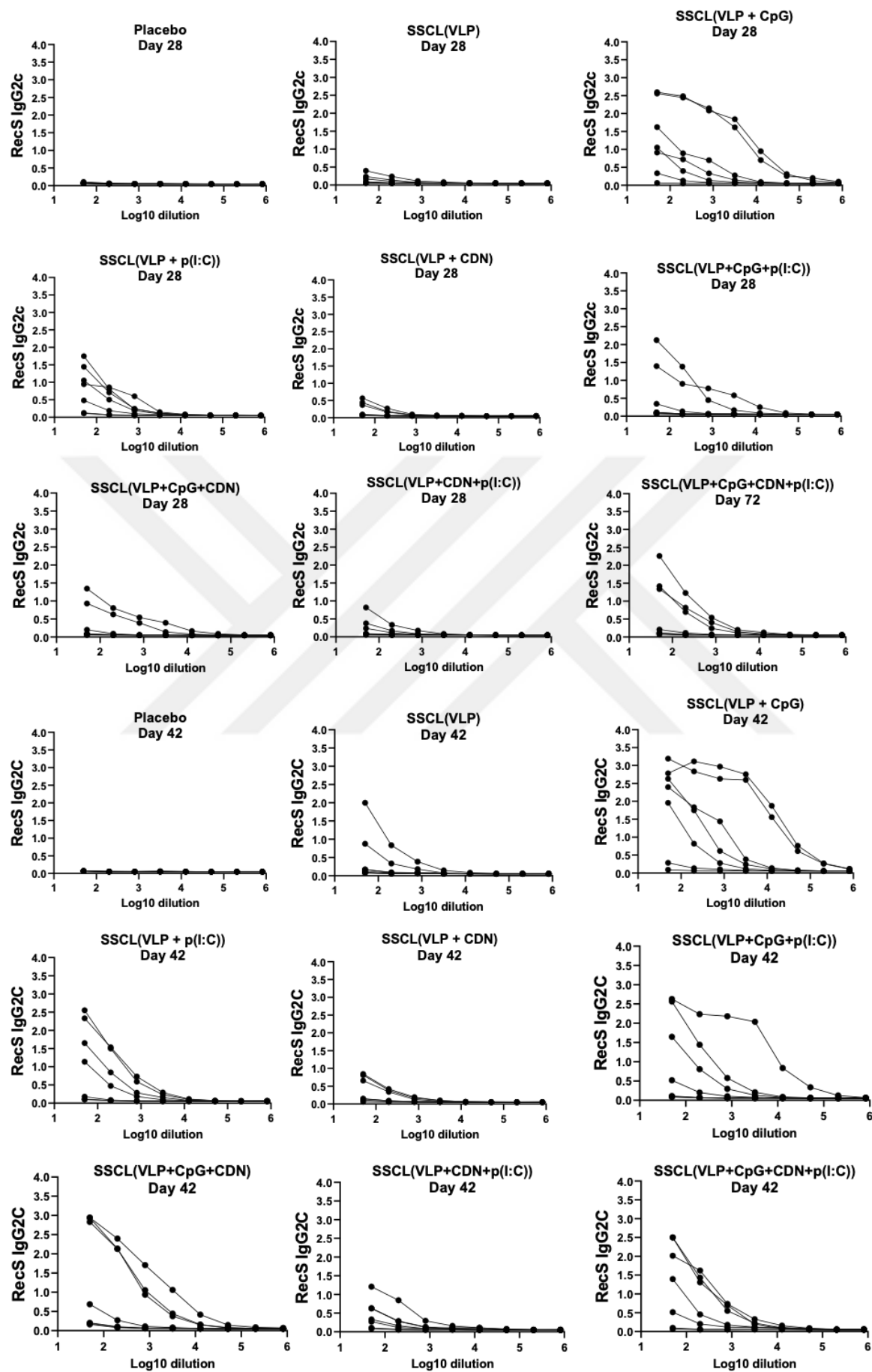
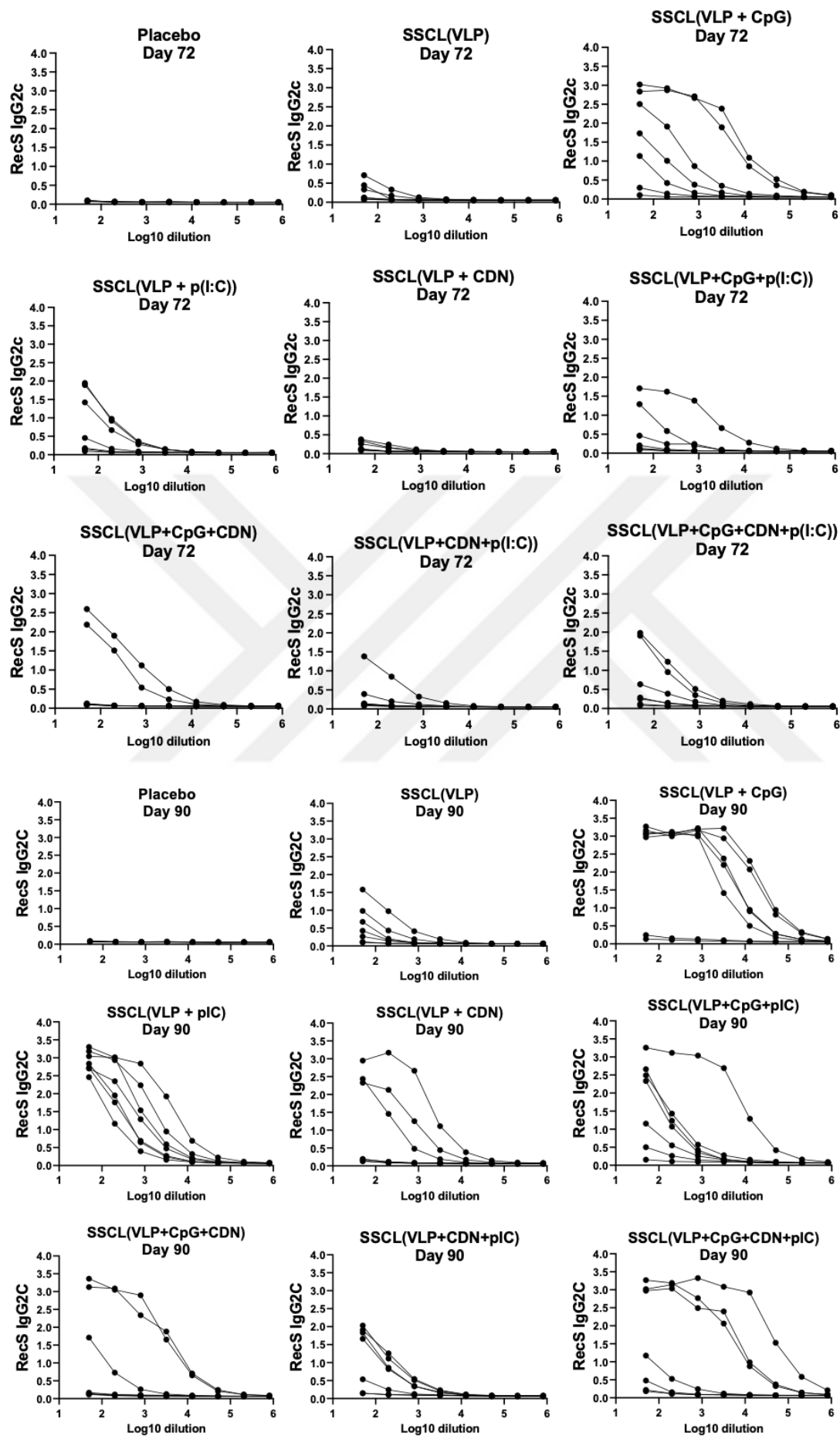
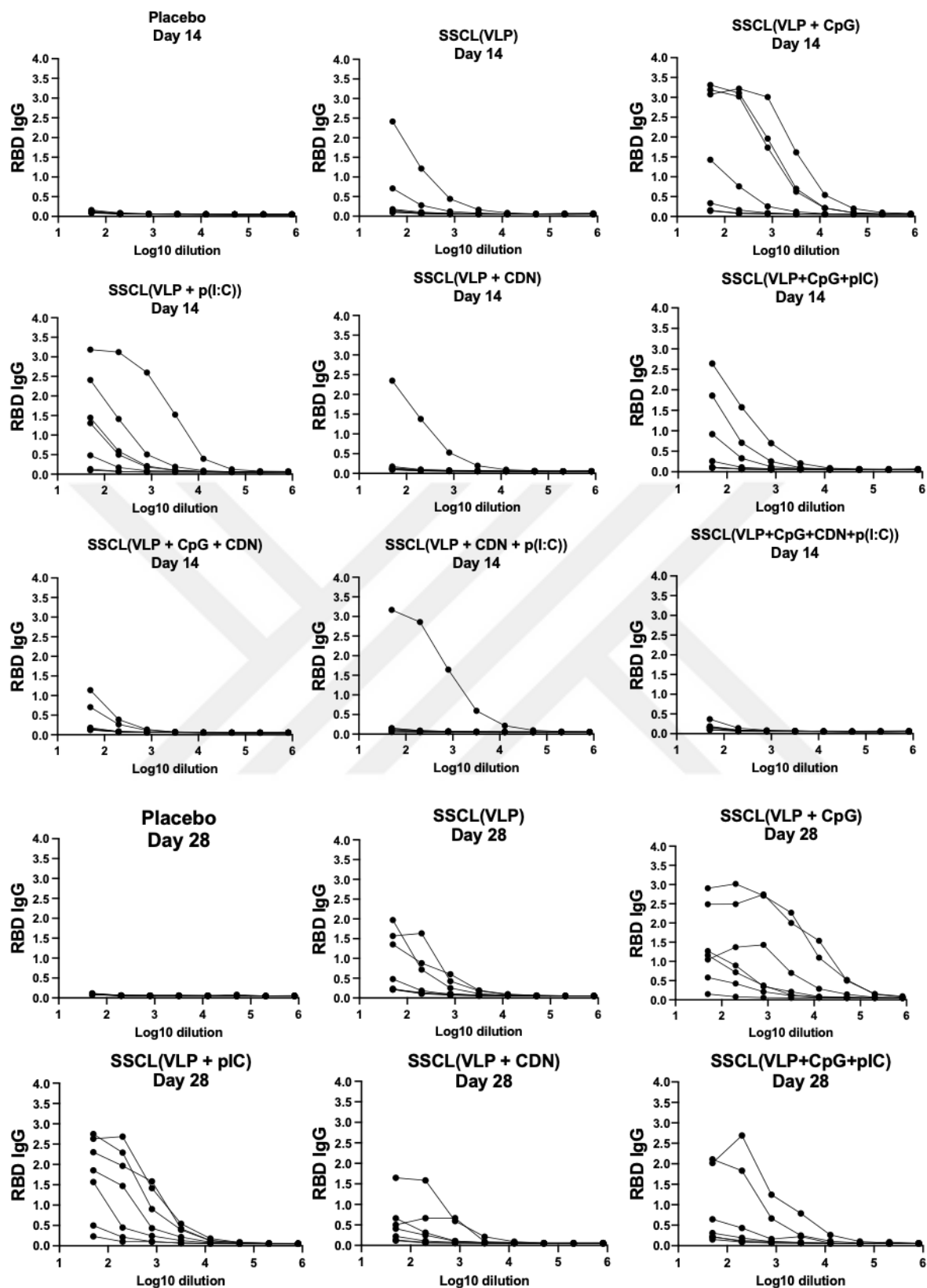
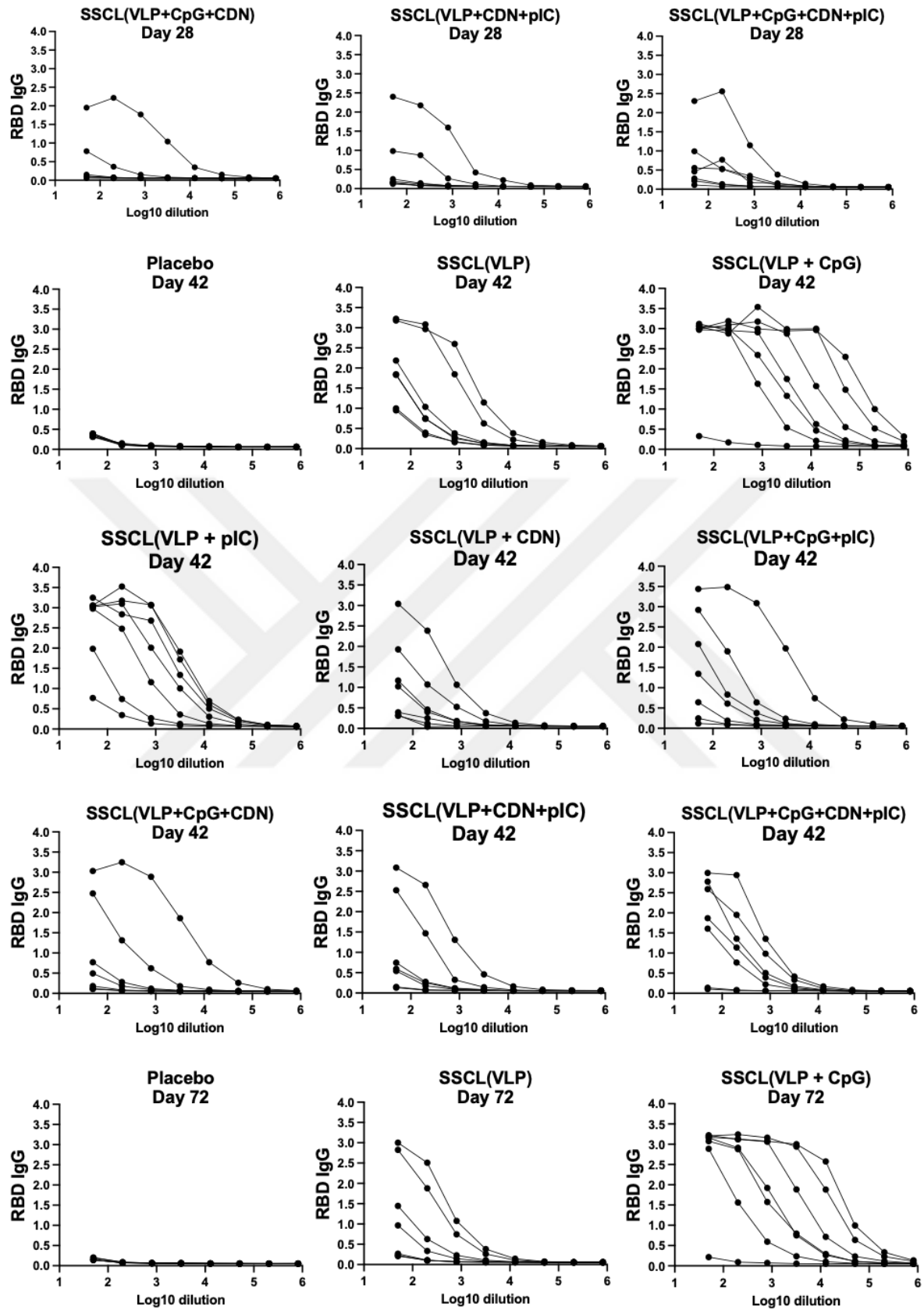


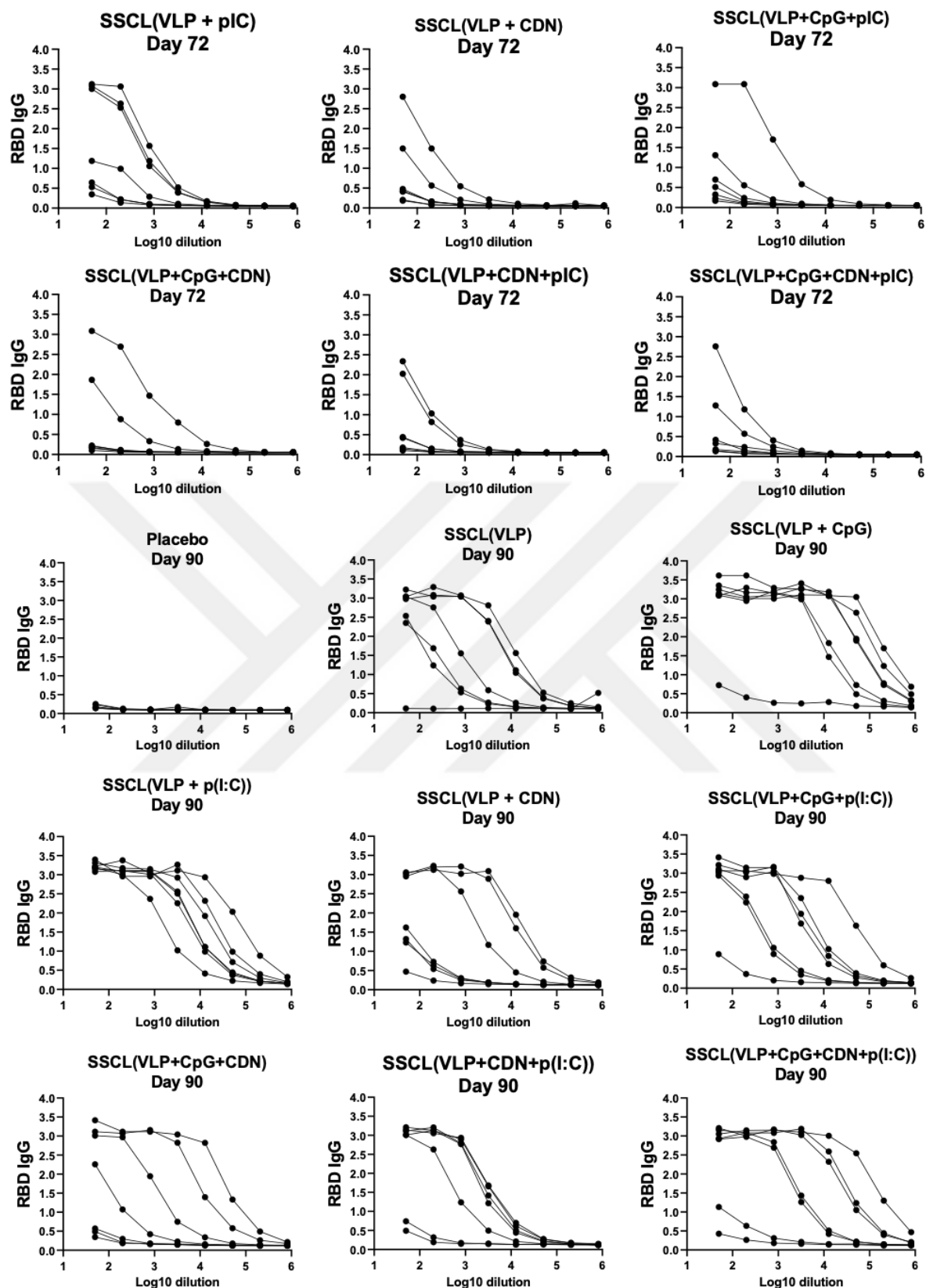
Figure A.2. The IgG1 responses of injection groups against recombinant spike (RecS) were evaluated by ELISA. 2HB immunoplates were coated overnight with 0.5 μ g/ml of RecS. After the blocking step, animal sera collected on Day 14, Day 28, Day 42, Day 72 and Day 90 were diluted in 1:50, then serially diluted fourfold seven times and incubated overnight. Next day, plates were incubated with anti-mouse IgG1, diluted in 1:1000, for two hours. Lastly, plates were treated with PNPP substrate solution, and OD405 values were recorded, as displayed on graphs. Last four graphs show IgG1 titers of groups in four bleedings. The groups were compared by Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.











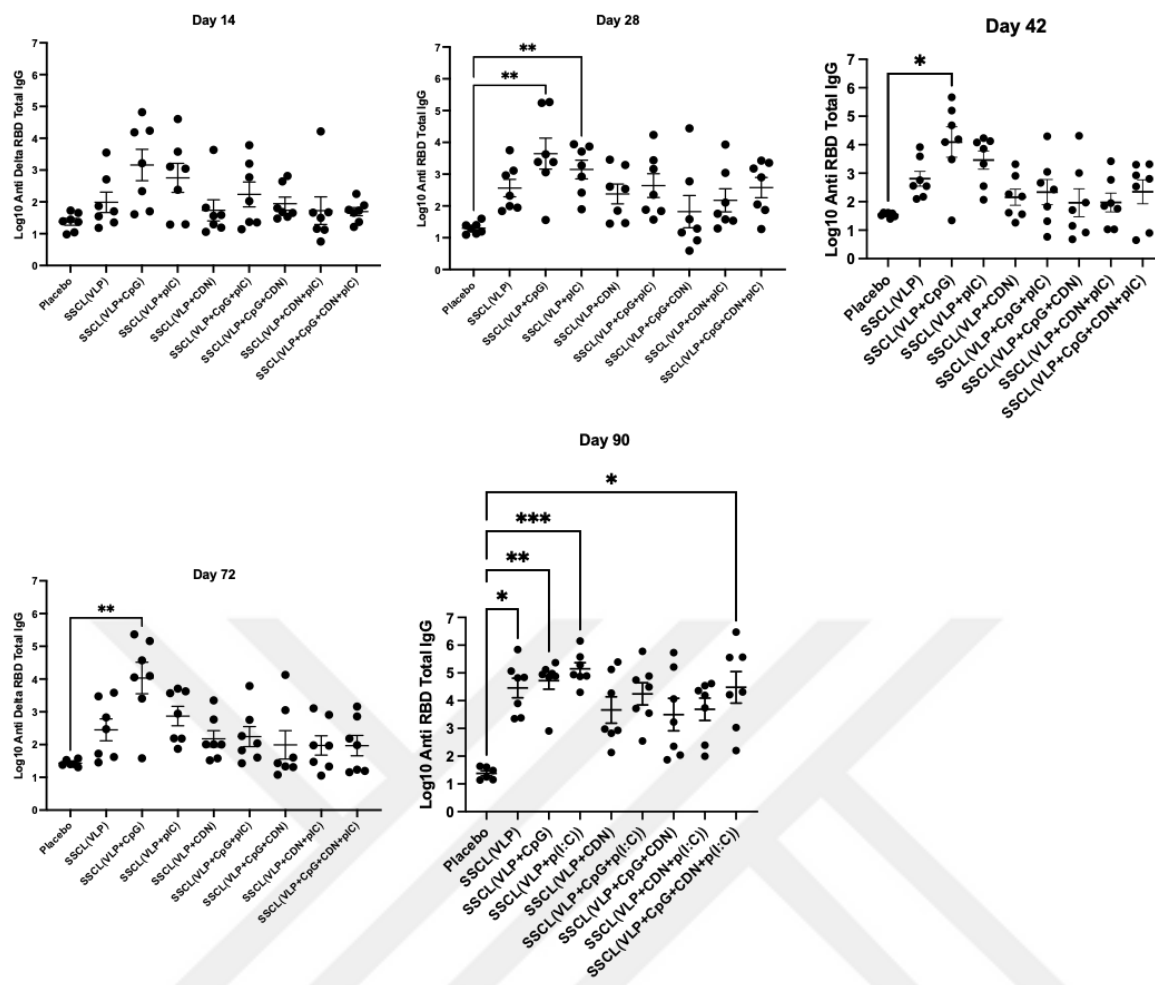
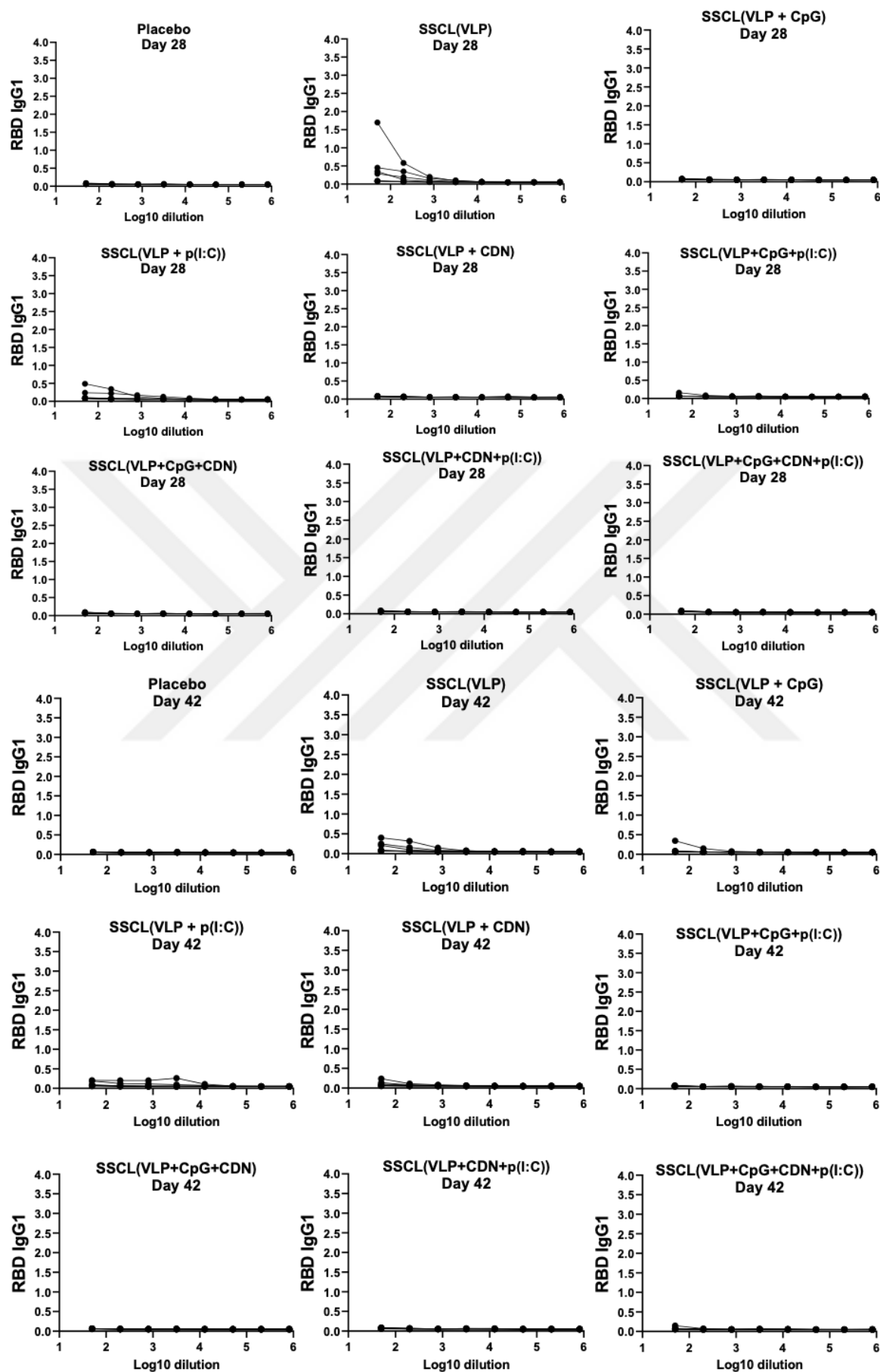
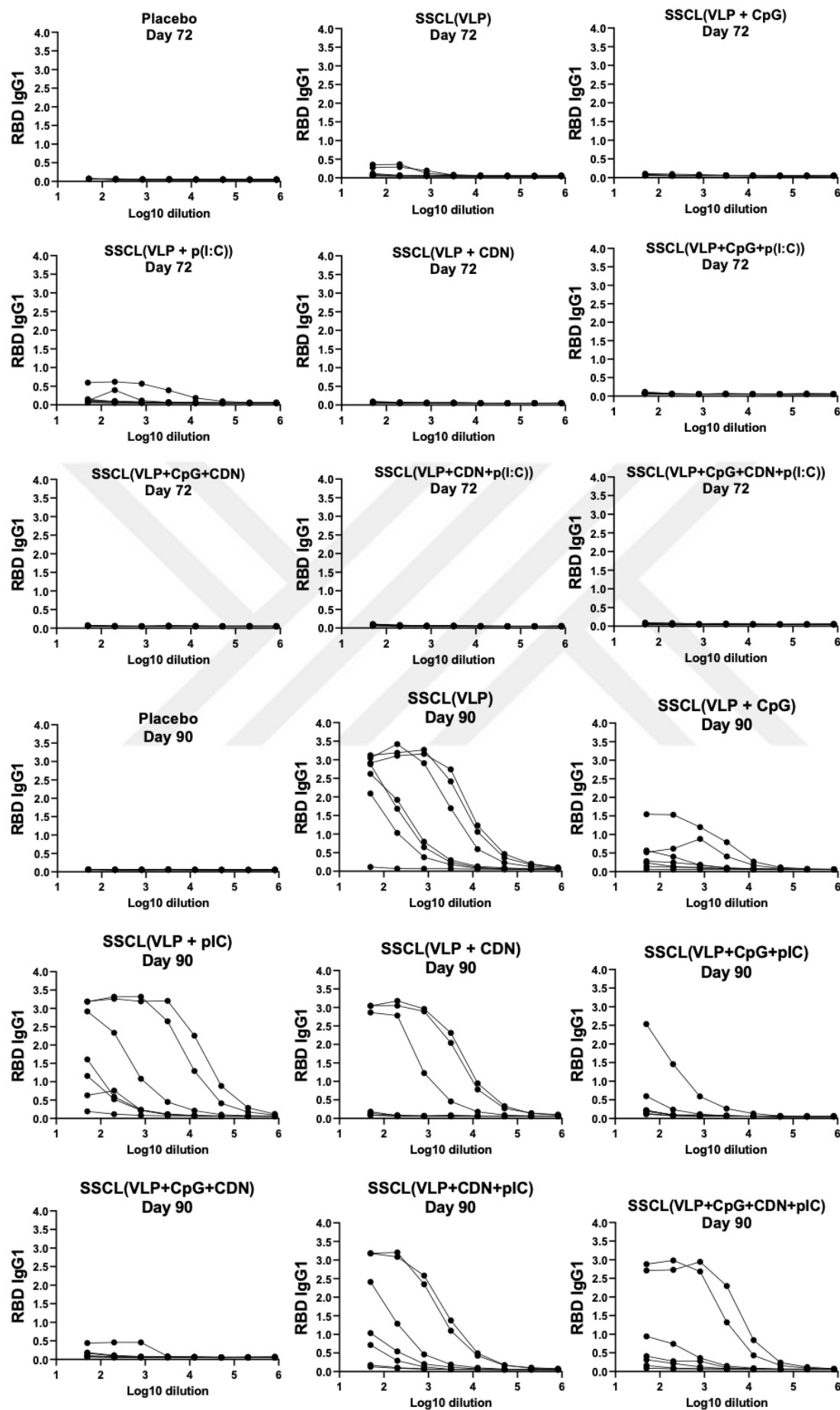


Figure A.4. The Total IgG responses of injection groups against Delta RBD were evaluated by ELISA. 2HB immunoplates were coated overnight with 1 μ g/ml of Delta RBD. After the blocking step, animal sera collected on Day 14, Day 28, Day 42, Day 72 and Day 90 were diluted in 1:50, then serially diluted fourfold seven times and incubated overnight. Next day, plates were incubated with anti-mouse Total IgG, diluted in 1:1000, for two hours. Lastly, plates were treated with PNPP substrate solution, and OD405 values were recorded, as displayed on graphs. Last five graphs show Total IgG titers of groups in all bleedings. The groups were done compared by Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.





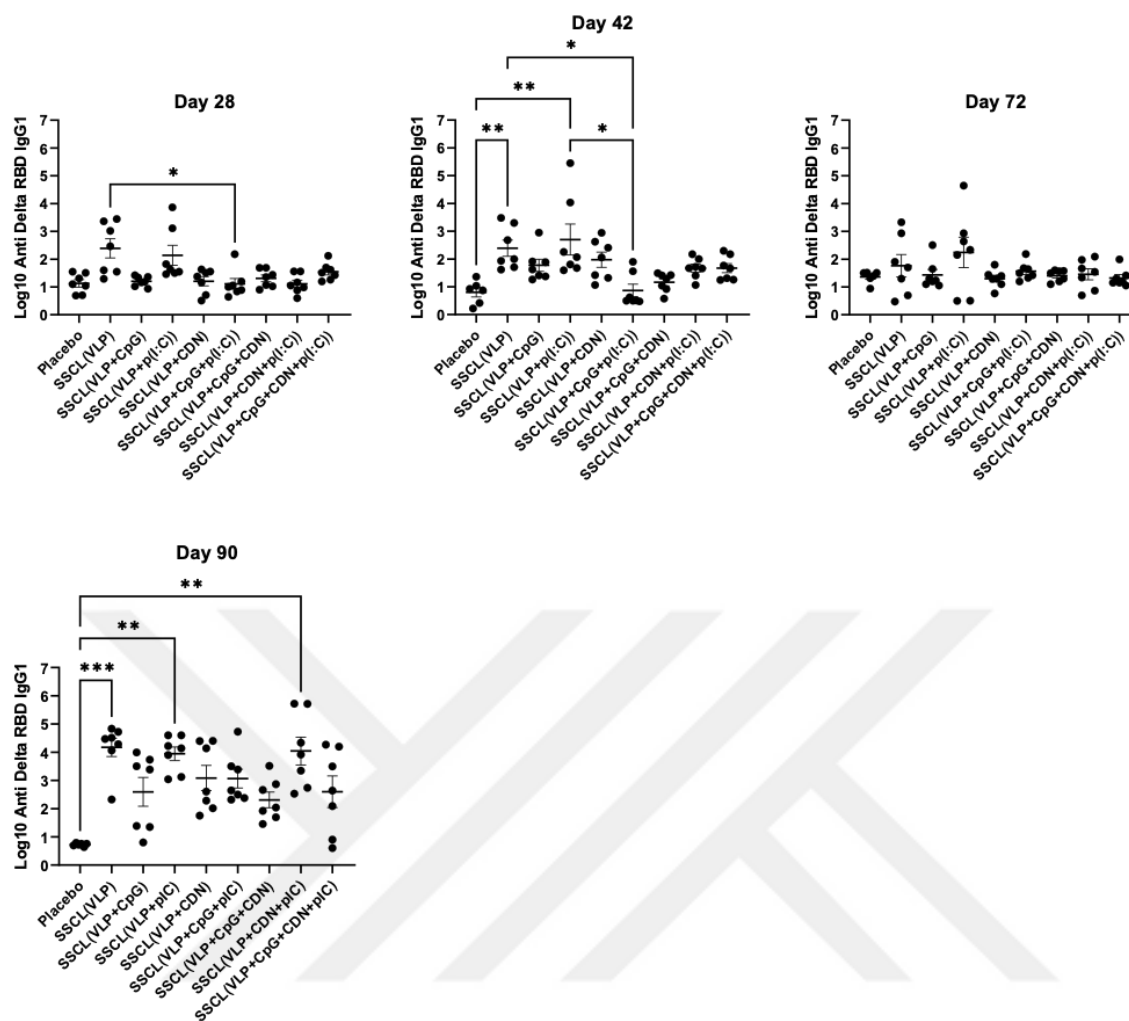
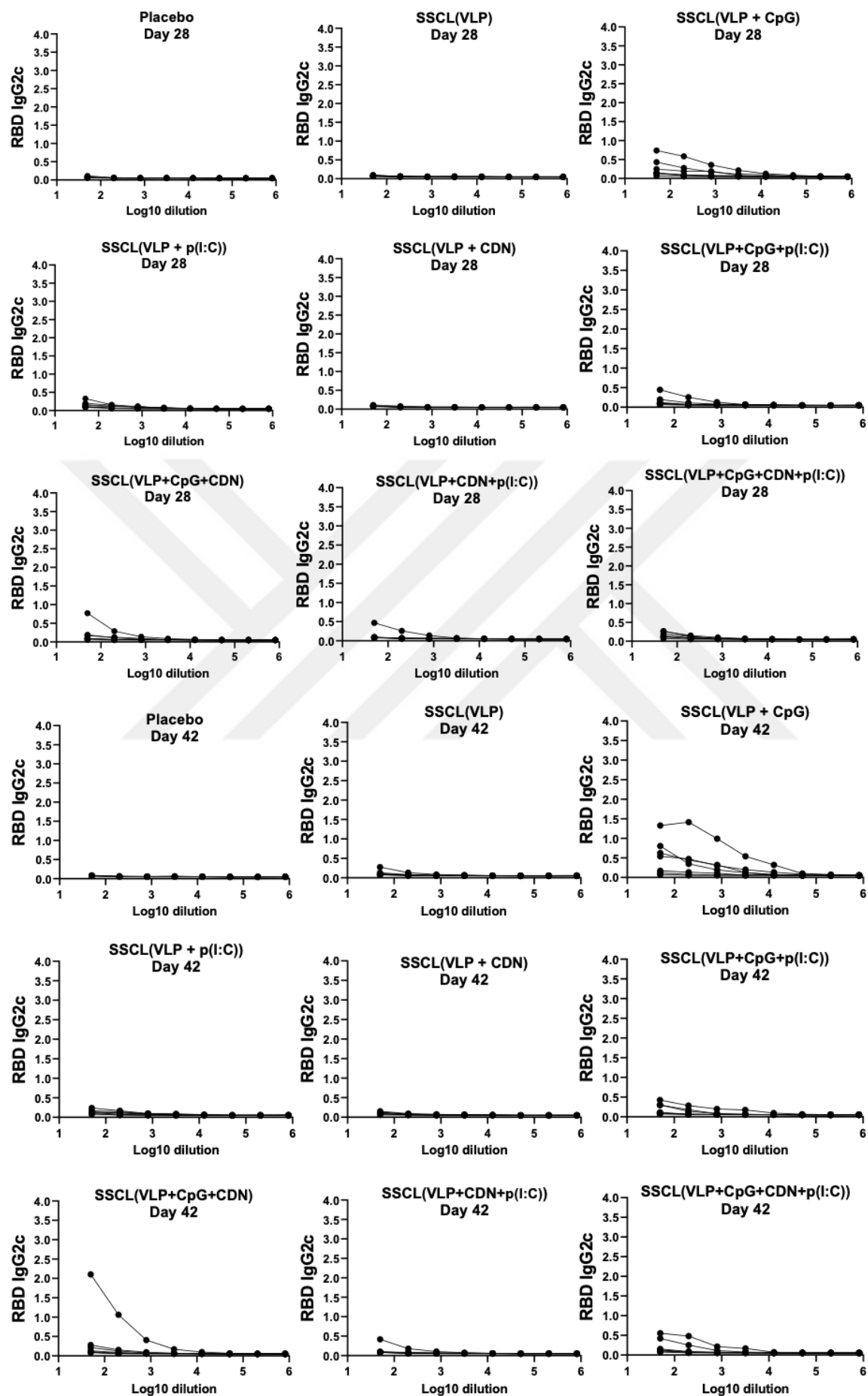
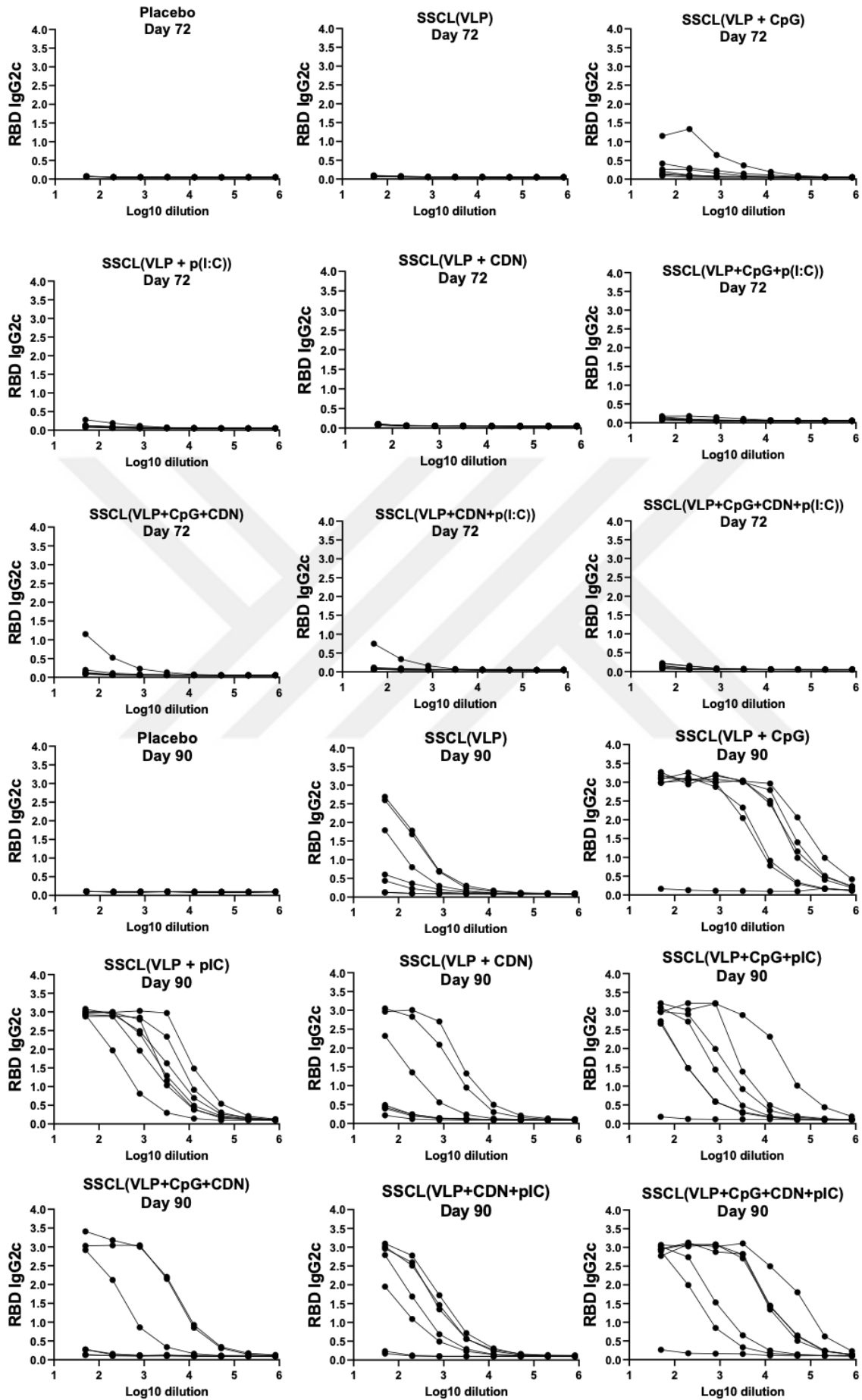


Figure A.5. The IgG1 responses of injection groups against Delta RBD were evaluated by ELISA. 2HB immunoplates were coated overnight with 1 $\mu\text{g}/\text{ml}$ of Delta RBD. After blocking step, animal sera collected on Day 14, Day 28, Day 42, Day 72 and Day 90 were diluted in 1:50, then serially diluted fourfold seven times and incubated overnight. Next day, plates were incubated with anti-mouse IgG1, which was diluted in 1:1000, for two hours. Lastly, plates were treated with PNPP substrate solution, and OD405 values were recorded, as displayed on graphs. Last four graphs show IgG1 titers of groups in four bleedings. The groups were compared by Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.





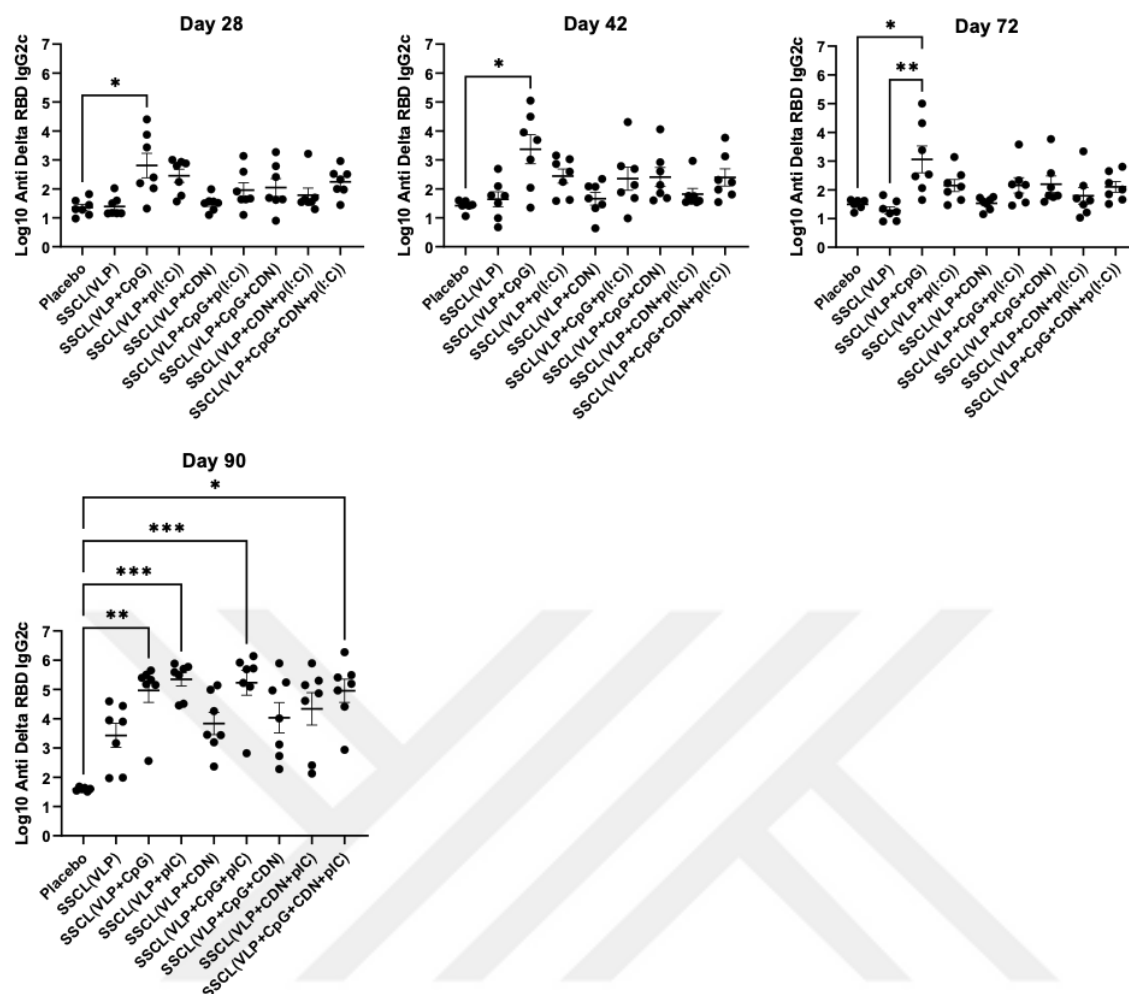


Figure A.6. The IgG2c responses of injection groups against Delta RBD were evaluated by ELISA. 2HB immunoplates were coated overnight with 1 μ g/ml of Delta RBD. After the blocking step, animal sera collected on Day 14, Day 28, Day 42, Day 72 and Day 90 were diluted in 1:50, then serially diluted fourfold seven times and incubated overnight. Next day, plates were incubated with anti-mouse Ig2c, diluted in 1:1000, for two hours. Lastly, plates were treated with PNPP substrate solution, and OD405 values were recorded, as displayed on graphs. Last four graphs show IgG2c titers of groups in four bleedings. The groups were compared by Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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