TARGETING ADENOSINE RECEPTORS TO IMPROVE VACCINE EFFICACY

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By

Ali Can SAVAŞ

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

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Abstract

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M.S. in Molecular Biology and Genetics
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December, 2016

Vaccination is the major protection method against many diseases caused by pathogens through creating acquired immunity. Vaccines can be classified in two major groups, which are subunit vaccines and attenuated vaccines. Attenuated vaccines can create effective immunity however; they also can induce many different side effects such as fever and allergic reactions. On the contrary, with subunit vaccines side effects are decreased but the efficacy of the vaccines is also decreased and there is a need for repetitive vaccinations to provide long lasting immunity. That is why, there is a need for developing more efficient vaccines and particularly vaccine adjuvants. Adenosine receptors, as part of purinergic signaling, have a regulatory role in immune system. Adenosine and 4 different adenosine receptors have an immunosuppressive role in major immune cells to create acquired immunity such as DCs, macrophages and lymphocytes. That is why, we hypothesize that, the efficacy of vaccines can be decreased by endogenous adenosine and the usage of antagonists in adjuvant formulations can increase this efficacy by inhibiting the suppressive effects caused by endogenous

adenosine. To be able to test this hypothesis, we first determine the specific adenosine receptor and antagonists taking a role in this immunosuppressive effect. For this purpose, we use primary dendritic cells and macrophages. We see that A2A and A2B receptors create most effective immunosuppression and SCH 58261 (A2A antagonist) and PSB 603 (A2B antagonist) are the main antagonists taking a role in the inhibition of this suppression. We then evaluated these two molecules in a vaccine formulation comprising MPL-A and AddaVax. As a result, these antagonists do not significantly change the general initial immune responses significantly however they create more antigen specific response. On the other hand, after antigen re-stimulation, mice taking these antagonists shows more antigen specific response and they also create higher antibody titers. With this study, adenosine receptor antagonists used in adjuvant formulations for the first time and it was shown that, with more study, they can be important in increasing vaccine efficacy created by immunostimulatory adjuvants.

Keywords: Adenosine, Adenosine Receptors, Vaccines, Adjuvants,

Özet

ADENOZİN ALMAÇLARININ HEDEFLENEREK AŞI ETKİNLİĞİNİN ARTTIRILMASI

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Hastalıklara karşı uygulanan en önemli korunma yollarından birisi aşılamadır. Aşılar iki ana grup altında toplanabilir. Bunlar ısı yoluyla öldürülmüş, ya da etkisizleştirilmiş aşılar ile alt birim aşılardır. Isı yolu ile öldürülmüş ya da etkisizleştirilmiş aşılar hastalıklara karşı bağışıklık kazanılmasında etkili olsa da ateş ya da alerjik reaksiyonlar gibi pek çok yan etkileri bulunmaktadır. Bunlara karşılık, alt birim aşılar ile buy an etkiler azaltılmıştır fakat, alt birim aşılar da yeterli bağışıklık gösterememekte ve tekrarlanan aşılama ihtiyacı doğurmaktadır. Bu nedenlerden dolayı etkisi arttırılmış ve yan etkileri az olan aşılara ihtiyaç duyulmaktadır. Pürinerjik adenozin almaçları, bağışıklık sisteminde düzenleyici rol oynamaktadırlar. Adenozin ve 4 adenozin almacı, dendritik hücreler, makrofajlar ve lenfositler gibi edinilmiş bağışıklık kazanılmasında önem gösteren hücrelerin baskılanmasında rol oynamaktadırlar. Bu nedenlerle, biz, iç kaynaklı adenozinin, aşıların etkinliğini azaltabileceği ve adenozin almaçlarının antagonistlerinin kullanılması ile aşı etkinliğinin arttırılabileceği hipotezini kurduk. Bu hipotez doğrultusunda, ilk olarak bağışıklık

sistemi baskılamasında hangi adenozin almaçlarının daha fazla rol oynadığını ve hangi antagonistlerin bu baskılamayı geri döndürebileceğini araştırdık. A2A ve A2B almaçlarının en çok baskılama sağladığını ve SCH 58261 (A2A antagonist) ile PSB 603 (A2B antagonist) moleküllerinin bu baskılamayı en fazla durduran moleküller olduğunu gördük. Daha sonra ise bu moleküllerin MPL-A ve Addavax içeren aşı formülasyonlarındaki etkilerini inceledik. SCH 58261 ile PSB 603 ilk bir ay içerisindeki genel bağışıklık cevaplarında fazla bir değişikliğe yol açmasa da antijene yönelik cevaplarda artışa yol açtıkları gözlemlendi. Fakat, bir ay sonra, tekrar antijen ile karşılaşıldığında, bu iki molekül alan farelerin bağışıklık cevaplarında ve üretilen antijene özel antikorlarda artış gözlemlenmiştir. Bu çalışma ile adenozin almaç antagonistleri ilk defa aşı bileşeni olarak değerlendirilmiş olup, uzun süreli bağışıklık yaratmada önemli rol oynayabilecekleri gösterilmiştir.

Anahtar sözcükler: Adenozin, Adenozin Almaçları, Aşı, Adjuvan.

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Abbreviations

Ab	Antibody
ACK	Ammonium-Chloride-Potassium
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AIM2	Absent in melanoma 2
APC	Antigen presenting cell
AR	Adenosine receptor
ATP	Adenosine triphosphate
BCR	B cell receptor
BM	Bone marrow
cAMP	cyclic Adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLR	C-type lectin receptor
CNT	Concentrative nucleoside transporter
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
ddH2O	Double-distilled water
dH2O	Distilled water
DLN	Draining lymph node
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive

ENT	Equilibrative nucleoside transporter
FBS	Fetal bovine serum
FDA	Food and Drug Administration
g	gram
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	G protein coupled receptor
HBSS	Hank's balanced salt solution
HPV	Human papillomavirus
HRP	Horse radish peroxidase
IC-50	Half-maximal inhibitory concentration
IFN	Interferon
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IRF	IFN-regulatory factor
KIR	Killer inhibitory receptor
l	Liter
LPS	Lipopolysaccharide
M	Molar
MAP	Mitogen-activated protein
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
ml	Milliliter
mM	Micromolar
MPL-A	Monophosphoryl Lipid A
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NDLN	Non-draining lymph node
NECA	5' -*N*-Ethylcarboxamidoadenosine

NF- κB	Nuclear factor- κB
NFDM	Non-fat dry milk
ng	Nano gram
NK	Natural killer
NLR	NOD-leucine rich repeat receptors
NOD	Nucleotide-binding oligomerization domain
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
PRR	Pattern recognition receptor
Q-RT-PCR	Quantitative real time polymerase chain reaction
RLR	RIG-I like receptor
RNA	Ribonucleic acid
Rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute
SA	Streptavidin
TCR	T-cell receptor
Tfh	Follicular helper T cells
Th	T helper
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TMB	Tetramethylbenzidin
TNFα	Tumor necrosis factor α
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
UTP	Uracil triphosphate

Chapter 1

Introduction

1.1 The Immune System

The defense mechanisms of the body can be divided into 3 main groups: physical and chemical barriers, innate and adaptive immune system. Skin as the physical barrier and the mucosal surfaces with antimicrobial proteins as the chemical barriers create the first line of defense. When pathogens try to invade the body, they should first breach these barriers. After these barriers evaded, more specialized and cellular responses of the body come into play which are innate and adaptive immune responses. With all these three main branches, immune system is an interactive network of lymphoid organs, cells, humoral factors and cytokines [1]. It is highly complicated and well-organized network to discriminate between self and non-self or even altered self. Specificity of innate immune responses are limited to a broad recognition molecular patterns of danger. Despite its limited specificity innate immune recognition and reactions occur faster and also include a prelude of events leading to the activation of adaptive immune system [2]. Adaptive immune responses are slower but more specific responses and result in the formation of immunological memory to prevent repetition of infections by microbes carrying similar antigenic determinants. Innate and adaptive immune system works in harmony to create fully responsive and functional protection against infections.

1.1.1 Innate Immunity

Innate immunity is the rapid and non-specific arm of the immune system as described above. After physical and chemical barriers, pathogens face the innate immune system which clears most of the infections by itself without the need of adaptive immunity [3]. Innate immunity is also needed to contain the infection in a conserved area until the adaptive immune cells come into play. Antimicrobial peptides, complement system, and cells such as macrophages, granulocytes and dendritic cells are the key players of innate immune system [4]. Complement system composed of many soluble plasma proteins and it works with antibodies. There are two main mechanisms for the complement system. The first one is to create pores on the invading microbes, which leads to their cell lysis. The other mechanism is called opsonization which is the process of coating the pathogens with antibodies to cause them being targeted by phagocytic cells of innate immunity [3]. By opsonization, complement system also helps to induce adaptive immune responses by antigen presentation via APCs. Innate immune system also recognizes pathogens by its specialized cells, but this recognition involves recognition common molecular patterns rather than antigen-specific recognition. These patterns are called pathogen associated molecular patterns (PAMPs). By recognizing these molecular patterns, innate immune cells act in their own ways. Macrophages, neutrophils and dendritic cells are phagocytic cells which phagocytes the pathogens [5]. Granulocytes show their effect via secreting enzymes and molecules such as heparin. Innate immune cells also secrete cytokines and chemokines to create inflammation and to orchestrate their trafficking to the site of infection. Through these mechanisms of actions innate immune system rapidly restrict the infected are and clear the pathogens to defend the host organism.

1.1.1.1 Pathogen Recognition Receptors

Pattern recognition receptors (PRRs) are the main receptors on the innate immune cells to detect PAMPs. Among PRRs there are many different types of receptors, which detect different types of PAMPs such as Toll like receptors (TLRs), Nucleotide oligomerization receptors (NLRs), C-type lectin receptors (CLRs), RIG-1 like receptors (RLRs), AIM2 like receptors and cytosolic DNA sensors[4]. When these PRRs get activated phagocytic capacity of innate immune cells increase and pro-inflammatory cytokines and chemokines are secreted as part of the innate immune defense.

1.1.1.1.1 Toll Like Receptors

Toll like receptors are one of the most well-known PRRs. They are capable of distinguishing broad range of pathogenic patterns such as fungal, bacterial and viral subcellular structures. Despite that they recognize many different PAMPs, they all share similar structures. They are membrane bound glycoproteins composed of 3 main compartments which are Leucine rich repeats in extracellular domain, transmembrane domain and Toll-interleukin-1 receptor domain (TIR) in the intracellular part [6]. So far, 13 different TLR proteins have been found and humans have 10 of them. These proteins are localized in either on the cell surface (TLR 1,2,4,5,6,10) or in the endosomal compartments (3,7,8,9,) [7]. Cell surface TLRs recognize mainly bacterial products while endosomal TLRs recognize nucleic acids. Activation of NF-$_{K}$B is the major pathway mediating TLR-induced immune cell activation, while some TLRs such as TLR4 and TLR3 can also activate IRF3 transcription factors to induce type-I interferon release. Activation of various MAP kinases also plays important roles for TLR mediated release of cytokines and co-stimulatory molecules used for effective antigen presentation by some innate immune cells. [8, 9]

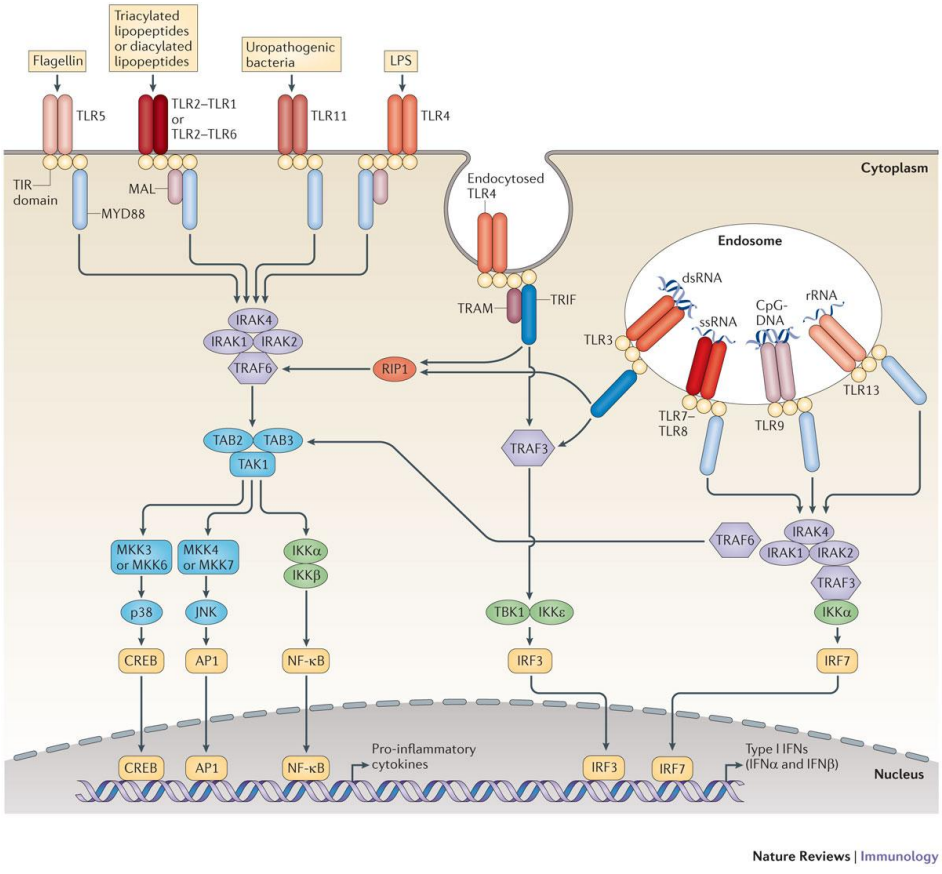


Figure 1.1: Toll like receptor localization and signaling pathways [10].

As can be seen in the figure 1, TLRs utilize two different adaptor proteins, MyD88 and TRIF, to mediate their effects. MyD88 plays a major role for inflammatory cytokine production such as TNF-α, IL12, while the adaptor TRIF is responsible from Type I Interferon production downstream of TLR3 and TLR4. Endosomal TLRs such as TLR7 and TLR9 interacts with MyD88. However, their molecular assembly with TRAF3 can lead to activation of IRF7 transcription factors and release of Type I interferons [11, 12]. By these different recognition patterns and usage of different pathways, TLRs can induce or regulate immune response in a well-organized fashion.

1.1.2 Adaptive Immunity

Adaptive immunity represents the second phase of immune response. It is highly specific to antigens and type of infection. Adaptive immunity requires innate immune cells to get activated, process the antigens and present them on their cell surface. Therefore, it takes longer periods of time to elicit adaptive immune responses. Adaptive immune system is composed of T and B lymphocytes, which rely on antigen specific receptors expressed on their surfaces [2]. These antigen specificity is the key characteristics of adaptive immunity. Gene rearrangements and following somatic hyper mutations plays parts to create this repertoire of antigen specific receptors [3]. The response of innate immune system with its cytokines and receptors leads to proliferation and activation of T cells and B cells. Afterwards, T and B lymphocytes constitute much more sophisticated and complicated cascade of events with their subtypes. Adaptive immunity is also the key in the formation of memory with its memory cells. Adaptive immune system shows its effects mainly by cytotoxic enzymes, cytokines and antibodies to clear out the infection.

1.1.2.1 T Lymphocytes

The cellular immunity of the adaptive immune system is carried out by T lymphocytes. T cells originated from lymphoid progenitors in the bone marrow and then they go into thymus. Here, maturation of T lymphocytes occurs with differentiation, positive and negative selection based on TCR affinity to self-antigens, which is also called thymic selection. After primary maturation steps, T lymphocytes contact with antigens and they become fully functional effector cells. There are two different types of T cells which are CD8+ or CD4+ T cells [13]

[14]. They can be also divided into 3 different functional groups: cytotoxic, helper or regulatory T cells. T cell polarization into different functional subsets is controlled by environmental factors related both to pathogens and host organisms. A small fraction of activated T lymphocytes can potentially differentiate into memory cells with self-renewal capacity. Memory cells are maintained for long periods time and in low numbers until they recognize their cognate antigen and expand [15].

CD4+ and CD8+ T cells create two main groups. CD8+ T lymphocytes also known as cytotoxic T lymphocytes (CTLs), mainly acts on altered self-antigens and pathogens. They show their effects via inducing apoptosis in those cells or organisms. Apoptosis induced by CTLs is done by variety of mechanisms. The first mechanism is the ligand-receptor interaction. CTLs have Fas ligands on their membranes. These Fas ligands, when encounter with their receptors on pathogens, activates the apoptotic cascades [16]. The other and more common mechanism is by use of enzymes. Perforin and Granzyme B are enzymes that are secreted by CTLs. Perforin can create pores on the target cell membranes and helps Granzyme B to enter the cell. Inside the cells, by affecting many different pathways, Granzyme B can induce apoptotic cascades [17, 18]. By these ways, CTLs are important components of immune system to clear the infection. Other than cytotoxic abilities, CTLs can also secrete cytokines such as IFNγ to activate other immune cells and inhibit replication of pathogens, especially viruses in normal cells [19].

CD4+ T lymphocytes are the other main group of T lymphocytes. Helper and regulatory T lymphocytes are among this subtype. Unlike CD8+ T lymphocytes, CD4+ cells have more regulatory roles in immune system [21]. They can regulate the activation or suppression of both innate and adaptive immune responses such as influencing B cells to produce different isotypes of antibodies, an event called “class switch”, enhancing neutrophil recruitment and their bactericidal activity and improving or suppressing CD8 T cell cytotoxicity [22]. Considering these roles, CD4+ T lymphocytes controls the fine-tuning of the immune response in an antigen specific manner. Therefore, depending on how they are educated they can suppress the immune response for a given antigen and cause peripheral tolerance, they can promote chronic inflammation or they can further enhance antigen-specific killing of pathogens or infected cells. Depending on how they shape the immune response, CD4+ T cells can polarize into many different functional subsets. Th1, Th2, Th17, Tfh and Tregs are

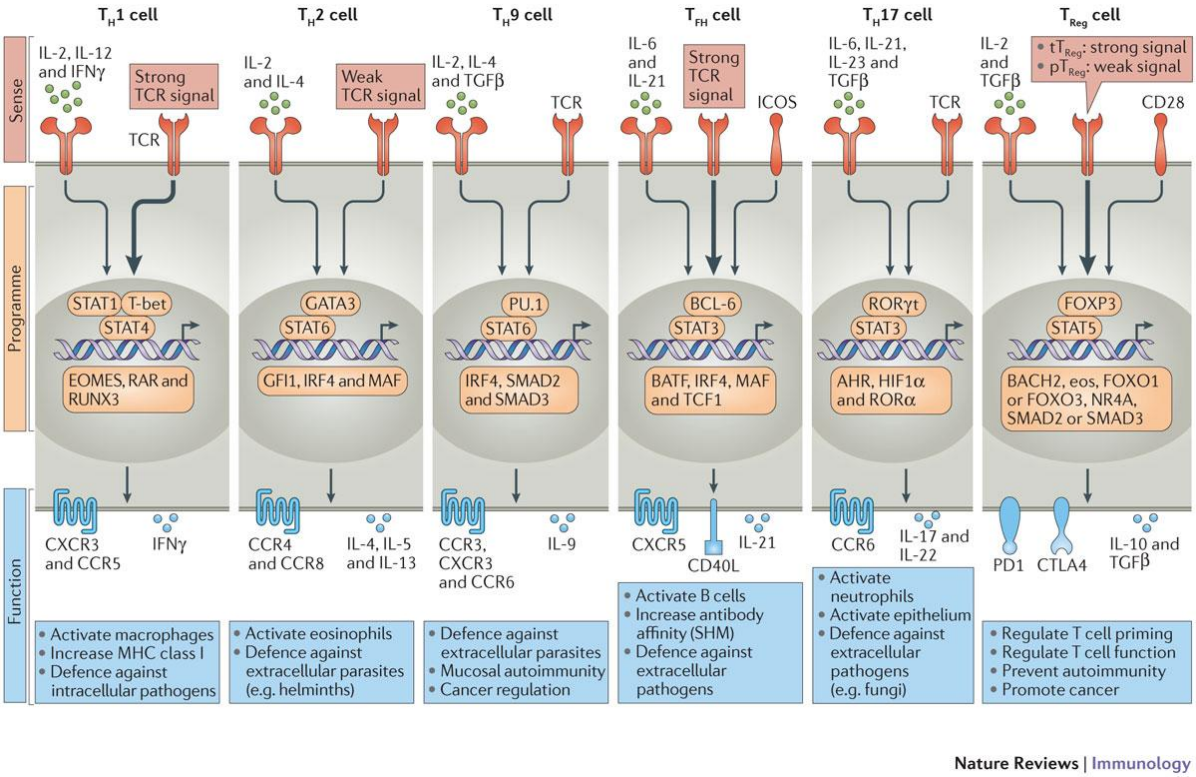


Figure 1.2: Subsets of CD4+ T Lymphocytes and their functions [20]

some of the well-known functional subsets of CD4+ T lymphocytes [23]. Th1 cells take part in intracellular pathogen clearance and autoimmunity by secreting IFNγ, lymphotoxin α, and IL2. By these cytokines, they can increase enhance phagocytosis by innate immune cells and help activation of CTLs [24, 25]. On the other hand, Th2 cells activated when extracellular parasites and allergic reactions and produce cytokines such as IL4, IL5 and IL25. Th1 and Th2 subsets creates 2 major groups of the CD4+ T cells [24, 26]. Follicular helper T cells (Tfh) are another important subset and they mainly responsible from mediating humoral response by interacting with B lymphocytes [27]. Although all these subsets have somewhat immunostimulatory role, regulatory T cells (Treg) are another subset with a role of maintenance of immunological tolerance and limit excessive inflammation. They can produce anti-inflammatory cytokines such as IL10 secretion, they can scavenge immunostimulatory IL-2 or they can directly interact with the target cells to suppress the immune responses[28, 29].

With all these roles T lymphocytes, encompasses an important part of the immune system to fight infections and to control excessive inflammation in an antigen specific and microenvironment-dependent manner.

1.1.2.2 B Lymphocytes

The humoral immunity of the adaptive immune system is carried out by B lymphocytes. B lymphocytes are originated from a common lymphoid progenitor same as T lymphocytes. They undergo gene rearrangements, negative and positive selection processes in the bone marrow and then they leave bone marrow as immature B cells. After bone marrow, B cells go to spleen to continue their development [30, 31]. Phases of B cell development can be seen in the figure 3.

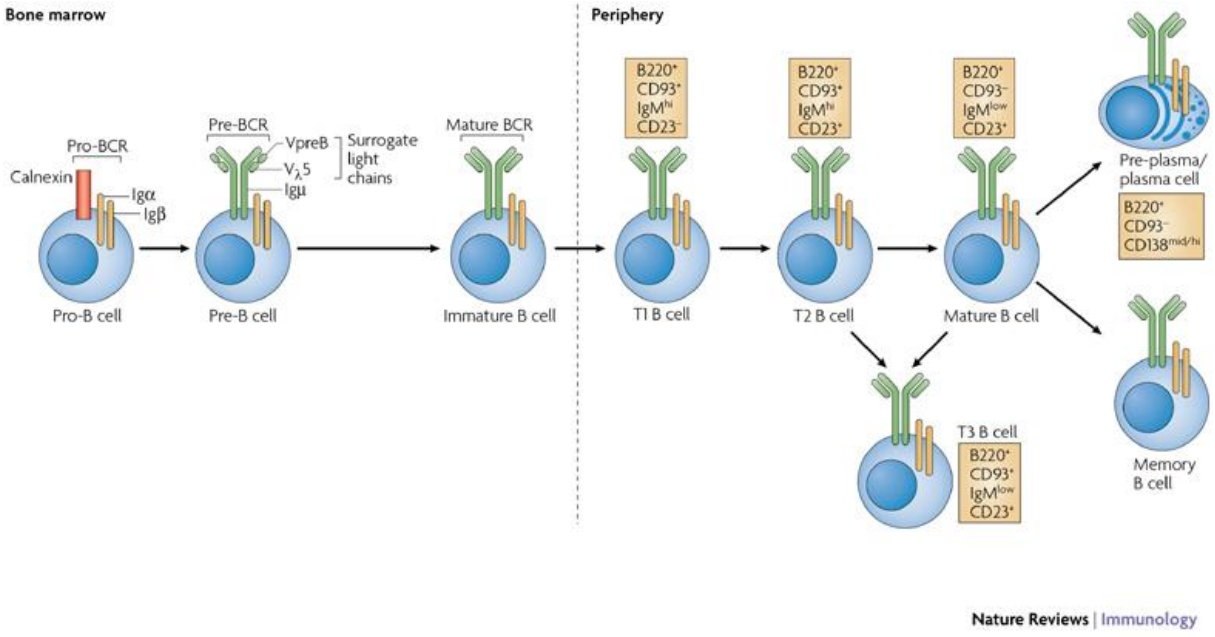


Figure 1.3: Developmental stages of B Lymphocytes [32].

After development, B cells get activated when there is an infection and their B cell receptors recognize their cognate antigen. Their activation occurs in secondary lymphoid tissues [33]. There are two different ways of activating B cells. These are i) T cell dependent or ii) T cell independent ways [34]. T cell dependent activation takes part when there are T cell dependent antigens such as ovalbumin. In this way of activation B cells needs costimulatory signals from T cells such as CD40L. In this way of activation, germinal center formation occurs and more long lived plasma cell and high affinity memory cells produced. In contrast, some antigens can activate B cells independent from T Lymphocytes such as foreign polysaccharides and unmethylated CpG DNA. This type of activation creates more rapid responses but and yield B cells producing or expressing lower affinity receptors or antibodies. In all these activation steps, B cells undergo steps such as affinity maturation and class switching [35-37]

B cells show their effects mainly by producing antibodies. Antibodies can be found on the cell surface as a B cell receptor (BCR) for B cells to mediate their activation and expansion and later to internalize the antigen for processing and presenting to the T cells [38]. After their activation and expansion B cells secretes antibodies into circulation, which can provide constant support for longer periods of time. When they find their antigens, they can prevent pathogens interacting with the host cells and mark them for more efficient phagocytosis [3].

Up on activation B cells can undergo rearrangements in their immunoglobulin locus to secrete different classes of antibodies. Mature B cells express IgM, IgD isotypes and these isotypes are switched to IgA, IgG and IgE variants. All these variants have different effects for different situations; for example, IgA is for protection of mucous membranes by activating complement system [39]. IgG classes is the most important class for the immunity. IgG type is generally secreted by plasma B cells. Complement activation and neutralization of toxins is some of the functions of IgGs. They also take part in antibody dependent cell mediated cytotoxicity. IgG1 and IgG2 are important subclasses of IgGs. Their ratio also gives insight about the type of inflammation. IgG1 dominant response is associated with allergic reactions or Th2 dominant immune reactions while IgG2 dominant response is associated with protective immunity against intracellular pathogens and more associated with Th1 type polarization of T cells [40].

1.1.3 Integrated Immune Response

Immune system is a very complicated and well-organized system with many differentiated cells in play. There is no immune cell type that works by itself to create sufficient immune response. They all work in harmony. Innate immune cells create the first line of defense by non-specific and rapid response to pathogens. When they first encounter with the pathogens they start secreting cytokines and chemokines to create an inflammatory environment and call for the other immune cells. Phagocytic cells and other players eliminate the pathogens. If they are not enough, antigen presenting cells (APC) come into play. APCs are the key players in between innate and adaptive immunity. Because T lymphocytes cannot recognize pathogens by their own. APCs are the cells to educate T cells for the specific antigens. They phagocyte the cells and lyse them inside, then they present specific antigens with major histocompatibility complex molecules. Macrophages, B cells and dendritic cells constitute the

APCs [41]. There are 2 different types of MHC class molecules. MHC-I can present antigen to CD8+ T lymphocytes while MHC-II presents to CD4+ Lymphocytes [42, 43]. They are not enough by their own as well. T cells needs costimulatory molecules to get activated and accumulate. One example for this co-stimulatory interaction is interaction between B7 proteins on the surface of APCs interacting with the CD28 on the surface of T cells to provide proliferative and survival signals. In the absence of co-stimulation, T cells get into anergic state. To prevent uncontrolled T cell activation molecules such as Cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed death 1 (PD-1) and killer inhibitory receptors (KIRs) are upregulated on the surface of T cells and negatively regulate T cell activation [44-46]. Some of these molecules compete for B7 proteins while the others interact with their own ligands on APCs such as PD-L1.

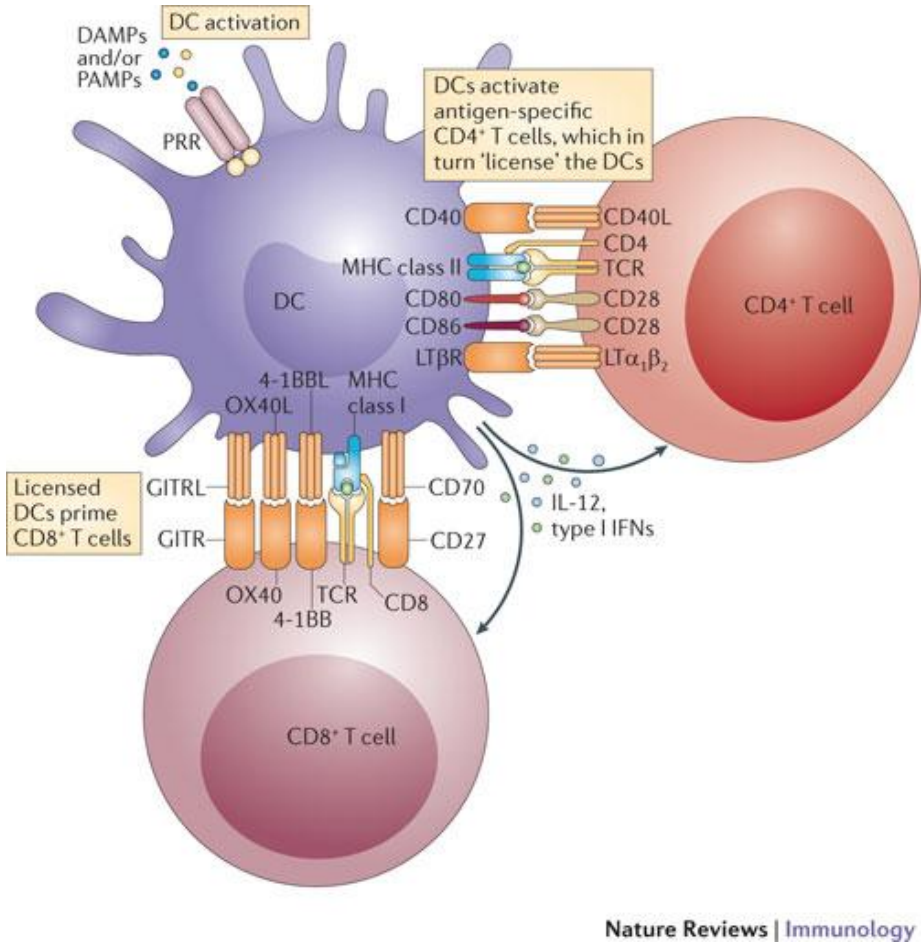


Figure 1.4: APC – T cell interaction in immune system [47].

Other than direct interaction between the cells, soluble factors such as cytokines can provide a third signal regulating immune cell polarization into different functional subsets. Because different immune cells can be unevenly distributed in the body and their initial response to infection or inflammatory stimuli may change, same pathogen or antigenic agent may trigger different modes of immune responses such as allergic response, tolerogenic response or strong protective immune reactions depending on the milieu.

1.2 Purinergic signaling

Purine is a heterocyclic aromatic organic compound which can be found in every cell and extracellular environment [48]. They take a key role in many cellular processes such as metabolic processes, tissue injury, circulatory system and nucleic acids. Purines can act inside the cell in many ways; however, they also have cell surface receptors to show their effects [49, 50]. Purinergic signaling is a type of extracellular signaling which is mediated by G-protein coupled receptors (GPCRs). There many different classes of purinergic receptors. P2Y receptors can recognize nucleotides such as ATP and UTP. P1 receptors, also named as adenosine receptors, are specialized to recognize extracellular adenosine. [49]

1.2.1 Adenosine Receptors

Adenosine can be found in extracellular environment. It can be released from apoptotic cells or concentrative or equilibrative nucleoside transporters (CNTs and ENTs, respectively). Adenosine can also be generated from ATP and ADP molecules via CD39 and CD73 ectonucleotidases [51]. Adenosine as an important signaling molecule first investigated in nervous system. Adenosine molecules can act as neurotransmitters. Other than nervous system, adenosine can play important roles in bone formation and lipid metabolism[52].

There are four different adenosine receptors expressed on cell surface. These are A1, A2A, A2B and A3 receptors. They are GPCRs and their signaling cascade mainly relies on cAMP signaling. A1 and A3 receptors are G_i coupled receptors and they decrease the cAMP amounts while A2A and A2B receptors are G_s coupled and they increase the cAMP [53] [54].

1.2.2 The Role of Adenosine Receptors in Immune System

Expression of all 4 types of adenosine receptors in immune cells is reported [55]. Adenosine by itself has a regulatory role in immune system. It generally suppresses the immune cells [56]. Adenosine's effects on immune cells become clear when there is inflammation and immune cell activation because these events can both rise extracellular adenosine concentrations and expression of mainly A2 adenosine receptors. Adenosine through its receptors, can regulate the cytokine secretion from macrophages. It can decrease pro-inflammatory cytokines and increase suppressive ones. It can also direct macrophage polarization to M2 phenotype [57-59]. In the dendritic cells, adenosine receptor signaling has a role in two ways. Through A1 and A3 receptors, it can lead to actin cytoskeletal reorganization and chemotaxis of immature dendritic cells. However, through A2A and A2B signaling, adenosine can lead to suppression of dendritic cells [60, 61]. Adenosine has also some effects on neutrophils. A2A receptor activation decreases the expression of adhesion molecules and by that, neutrophils can no longer attach endothelial surfaces as effective before [62, 63].

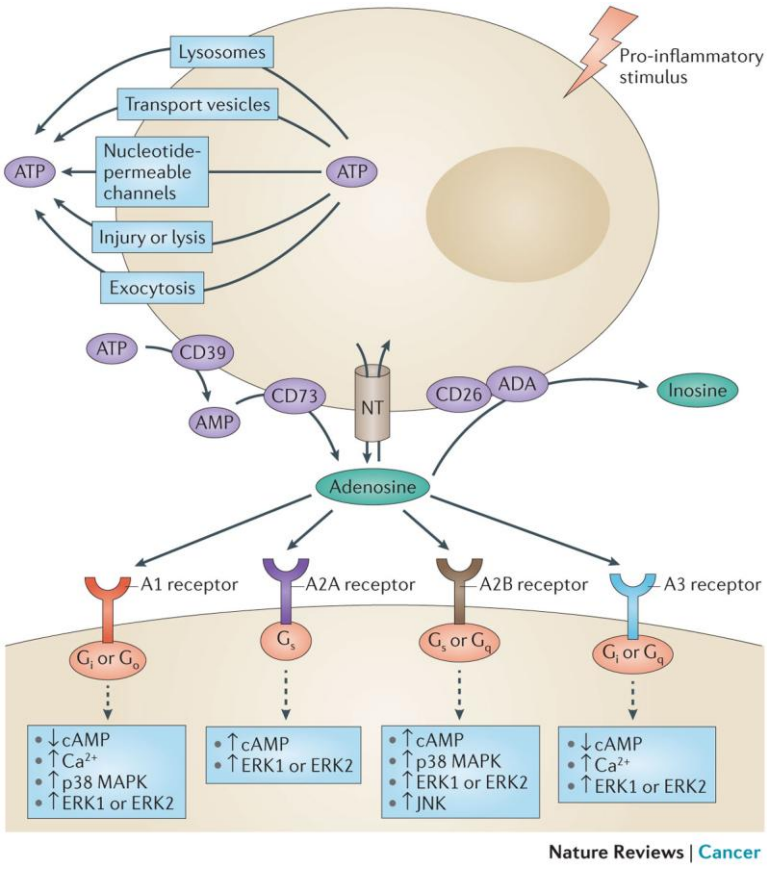


Figure 1.5: Adenosine metabolism and its signaling through its receptors [64]

Adenosine affects not only innate immune cells but also adaptive part of the immune system. Adenosine can inhibit the proliferation of T lymphocytes by decreasing the IL2 production [65]. It also decreases the production of IFNγ and IL-4, which changes the polarization status of lymphocytes [66]. Besides cytokine profiling, adenosine increases the expression of suppressive molecules such as CTLA-4, PDL1 and decreases the expression of immunostimulatory CD-40L [67]. There are also many evidence showing that adenosine can affect class switching mechanisms on B lymphocytes. It can activate class switch recombination during the maturation and activation of B lymphocytes [68].

1.2.2.1 A1 and A3

A1 and A3 adenosine receptors are G_i coupled GPCRs. They decrease cAMP levels and increase intracellular calcium levels. Adenosine can interact with A1 and A3 receptors with IC50 values between 0,1 and 1 μM. Intracellular adenosine levels (less than 1μM) are enough to activate these two receptors [69]. They have many role in nervous system and metabolism; however, they do not have strong anti-inflammatory effects on immune system. They are mainly involved in chemotaxis process with the help of rearrangement in the cytoskeleton. There is also evidence that adenosine can lead to neutrophil apoptosis via A1 and A3 receptors [70].

1.2.2.2 A2A and A2B

A2A and A2B adenosine receptors are G_s coupled GPCRs and their activation increases cAMP levels. These are the main 2 receptors of adenosine signaling which can lead to immune suppression. Adenosine can interact with A2A receptor with IC50 value between 0,1 and 1 μM. IC50 value for A2B receptors is 10 μM [69]. In a healthy tissue, extracellular adenosine level is enough to activate A2A receptors but not A2B. Therefore, A2B receptor activation is generally associated with pathological conditions such as hypoxia and inflammation, which can cause more than 10-fold increase in extracellular adenosine levels [71]. Pathological conditions such as hypoxia, infection and inflammation also increases the expression of both A2A and A2B receptors in immune cells. Therefore, adenosine-regulation of immune system predominantly occurs through these two adenosine receptor subtypes. In

general activation of these receptors play a tissue protective role by suppressing excessive immune reactions and favoring angiogenic inflammation to promote tissue oxygenation [49].

1.2.3 Targeting Adenosine Receptors as a Therapeutic Approaches

As described above adenosine signaling can regulate not only physiological but also pathological processes. Some of its physiological effects are regulation of sleep cycle (through A1), vasodilation and hypotension (through A2A), vascular integrity and cardiac preconditioning (through A2B) [72-75]. Among the pathological cases that adenosine receptors play a role, cardiac preconditioning (through A1), neurodegeneration (through A2A), fibrosis (through A2B and A2A), and inflammatory pain (through A3) can be shown [76-80]. Other pathological conditions that adenosine influences can be found in the figure 3.

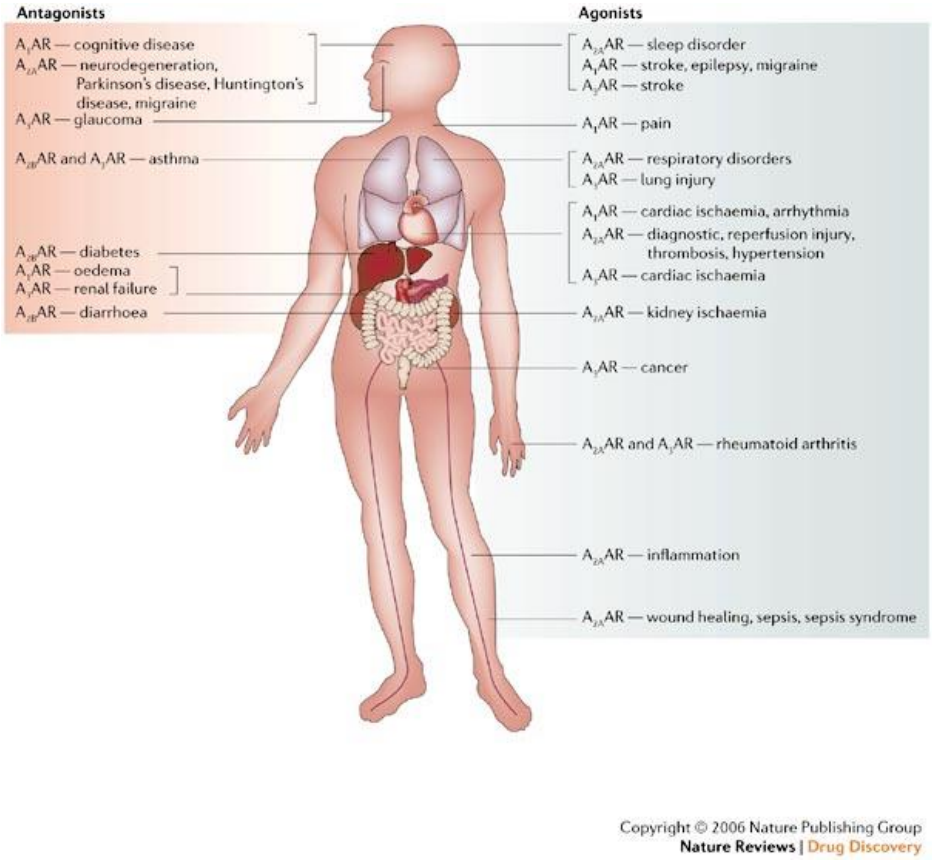


Figure 1.6: Relation with Adenosine Receptor Subtypes and Diseases [81].

Because of their role regulating many physiological and pathological processes adenosine receptors are considered as potential therapeutic targets. Therefore, different adenosine receptors agonists and antagonists are approved by FDA [82] or being tested in clinical trials (Examples are Regadenoson for sickle cell anemia and istradefylline for Parkinson's disease) [83-85].

Suppressive effects of adenosine receptor signaling in immune system makes antagonists suitable for immunotherapy, especially in the conditions that stimulate adenosine A2 receptors and that increases extracellular adenosine. This gets more apparent in the case of cancer. Due to hypoxic environment and increased level of cell death, extracellular adenosine levels increase significantly in tumor microenvironment, which leads to suppression of infiltrating immune cells [86, 87]. There are many evidence showing that adenosine receptors significantly influence cancer progression and they have a great value in cancer immunotherapy. Usage of adenosine antagonists especially A2A and A2B specific ones, can inhibit the tumor growth and metastasis and increase the immune cell infiltration in tumor microenvironment [88] . Adenosine receptor blockade can also change the cytokine profile in tumor microenvironment to a more inflammatory phenotype. It was also shown that usage of antagonists can increase the M1 type macrophages in tumor microenvironment [89, 90]. All these researches show that, adenosine receptors antagonists as well as agonists hold a great promise for use as therapeutics, particularly for cancer.

1.3 Vaccine Adjuvants

Vaccines are one of the most useful and common tools to gain protection against disease due to infections. Their main goal is to educate immune system for a possible future infection. In the case of infections, antigen specific and more effective immune response is relatively slow because it includes primary activation of innate immunity before antigen specific adaptive immune responses. However, with the help of vaccination, memory lymphocytes can induce adaptive immune responses faster and infection can be cleared before it causes irreversible damage or event death [91, 92].

Adjuvants are one of the main contents in vaccines that drastically increase the efficacy of vaccines while decreasing the need of using high dose of antigen to establish long-lasting immunity [93]. Some of these adjuvants work immuno-stimulatory roles while some works as

a delivery systems. There are many different adjuvants such as bacterial products, inorganic compounds such as alum, and cytokines used in clinical and preclinical setting. Immunological adjuvants are capable of inducing both cellular and humoral responses. However, side effects such as acute immunotoxicity, limit their use in clinic. Therefore, adjuvant and vaccine development is under strict control by governmental regulatory agencies. Despite these concerns, immunological adjuvants that are safer but more effective is still being developed by major pharmaceutical companies [94].

1.3.1 Mechanisms of Adjuvants

Antigens alone as vaccines induce modest antibody responses because they lack the intrinsic immunostimulatory structures of the infectious pathogens they represent. Their effects on T lymphocytes are also very little or none. Adjuvants come into stage at this point to cause a more portent immune response that leads to longer lasting immunological memory. Adjuvants can show their effects in many different ways. They are used as delivery systems to extend the presence of antigen as in the case of Alum and MF59. As stated earlier some others

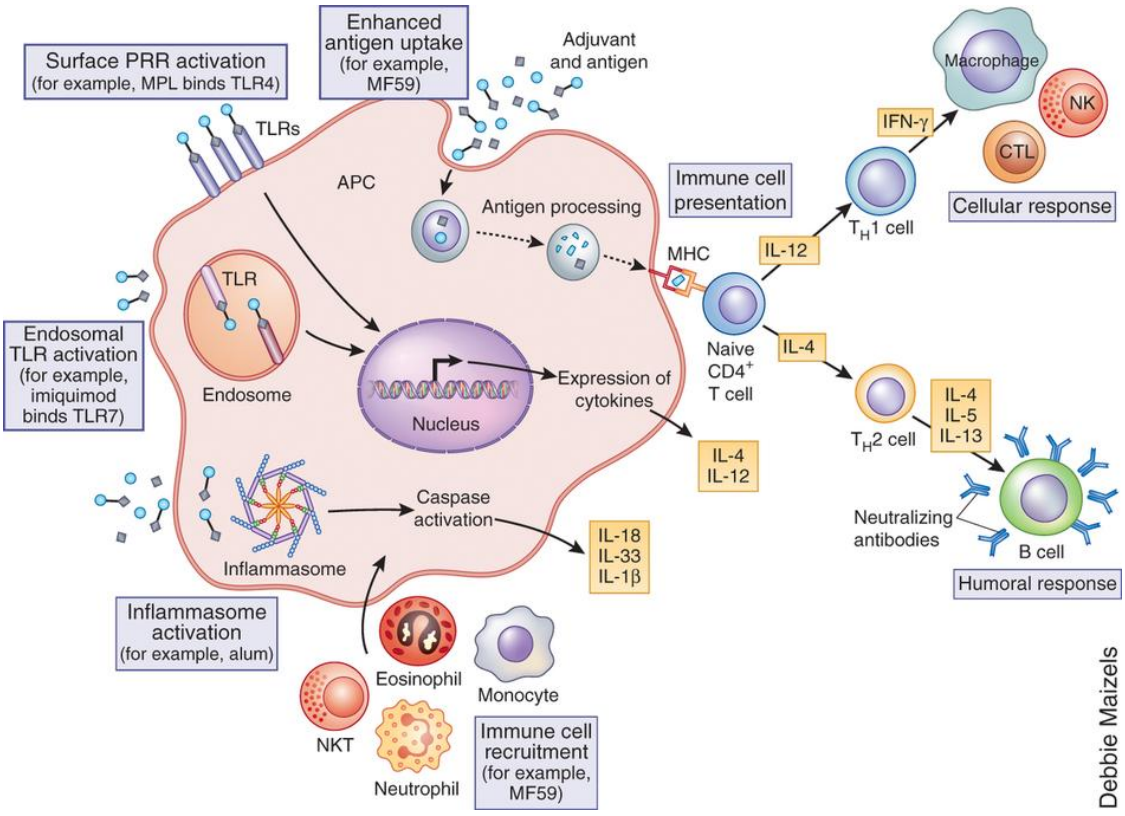


Figure 1.7: Mechanism of actions for different types of vaccine adjuvants [93].

are immunological adjuvants, which stimulate innate and adaptive immune cells and cause release of various cytokines. Immunological adjuvants stimulate polarization of T cells ad B cells into memory cells and substantially decrease the dose of antigen needed to induce long lasting immunity [95].

1.3.2 Delivery Systems

Rationale behind the use of delivery systems lies in the concept of antigen persistence. Antigens used vaccines, cannot be in high doses due to safety concerns, costs or manufacturability issues. When they were given directly, due to fast distribution to whole organism, their effective concentrations for APCs to pick up, process and present decrease rapidly. However, with the use of delivery systems, antigens can be trapped in a close vicinity longer periods of times [96]. Liposomes and some emulsions are among these delivery systems.

1.3.2.1 Oil in Water Emulsions

Oils in Water Emulsions are successfully used as vaccine adjuvants for many the years. Freund's adjuvant is a water-in-oil emulsion that is based on a type of mineral oil and it counted as the first successful oil in water emulsion as effective adjuvant formulation [97]. MF59 is another oil in water emulsion, which is used as an adjuvant in many different vaccine formulations. It is squalene bases rather than paraffin as in Freund's adjuvant and squalene metabolizes easily. Its safety was shown by many different studies [98, 99]. By itself MF59 can elicit both cellular and humoral responses [100]. The use of TLR stimulatory molecules with MF59 did not show any increase in the antibody titers; however, they cause a shift towards a Th1-assocaited phenotype [101]. Addavax is also a squalene based oil in water emulsion and it was shown that it can elicit both cellular and humoral responses while it has a competitive advantage as compared to Alum in inducing humoral response [102].

1.3.3 Immunostimulatory adjuvants

Using immunostimulatory molecules as adjuvants causes more potent immune responses towards the given antigens. They can effectively stimulate APCs and elicit both cellular and humoral responses [103] [104]. Detailed mechanism for how different adjuvant modalities works can be seen in figure 1.7. TLRs, NLRs, or cytosolic sensors are used in clinic or preclinical tested as immunostimulatory vaccine adjuvants.

1.3.3.1 Usage of Toll Like Receptors

TLR stimulation cause potent immune responses, which makes their agonists potential candidates for being used as immunostimulatory vaccine adjuvants. Then can both induce APCs, creates better antigen presentation and elicit adaptive immune responses. There are many researches and clinical trials on TLRs as vaccine adjuvants. Some of them are Imiquimod for the cancer therapy, CpGs against HPV, malaria and Flagellin for the influenza [105-107]. RC-529 is also licensed for HPV [108].

1.3.3.1.1 Monophosphoryl Lipid A as a Immunostimulatory Adjuvant

TLR agonists and their immunostimulatory effects are well-defined. However, their human use is limited due to potential immunotoxicity associated with them. Monophosphoryl Lipid A (MPLA) is among the licensed and most successful ones (MPL^{TM} adjuvant). It is an LPS derivative from *Salmonella Minnesota*. MPL is the lipid A portion of the LPS with reduced numbers of phosphate groups attached to its disaccharide group. MPL formulations also contain variable numbers of acyl chains attached to the hydrophilic disaccharide group as opposed to six acyl chain attached to a typical Lipid A molecule. MPLA stimulates TLR4 in a more TRIF biased way thereby causing only limited immunotoxicity while potently improving antigen-specific immune responses [109]. Being a low-toxicity derivative of LPS is an important for its human use in vaccine formulations. AS04 is one of the licensed adjuvant formulation and includes MPLA and Alum. It was used for the vaccines against HPV and can stimulate both humoral response with the memory B cell formation and cellular immune response effectively [110]. MPL-based vaccines are also being tested against the allergy due to its ability to induce strong Th1 response while inhibiting the Th2 responses [104].

1.4 Aim of the Study

Vaccines can be classified in two major groups, which are subunit vaccines and attenuated vaccines. Attenuated vaccines can cause strong immune responses towards their antigenic epitopes; however, they can also induce many side effects such as fewer, allergic reactions, and even contraction of the disease if the vaccine is a live attenuated vaccine or not heat-killed efficiently. On the other hand, with subunit vaccines, these side effects are decreased but the efficacy of the vaccines are also decreased and there is a need for repetitive vaccinations. Because they most often lack the intrinsic ability to stimulate immune responses by there is limited numbers of subunit vaccines for infectious diseases and repetitive immunizations with these vaccines is necessary to establish a long-lasting immunity. Therefore, vaccine and in particular adjuvant formulations should be developed or improved to decrease the need for repetitive injections and to develop effective vaccines towards a broader range of diseases. Previous studies including ours have clearly shown the immunosuppressive/immunomodulatory effects of adenosine and adenosine receptors in different immune cells and in different disease conditions [88, 90, 111, 112]. Adenosine receptors are targeted everyday by millions of people worldwide because they are targeted by caffeine, a common ingredient in coffee, tea and many other cold beverages. Also, adenosine A2A receptor blockade is currently being tested in humans as a cancer immunotherapy or to treat Parkinson's disease. Therefore, there is hundred years of human experience and recent clinical data showing that adenosine receptor blockade will potentially be safe for human use.

In this study, we aim to test the hypothesis that adenosine receptors can be targeted to improve the efficacy of subunit vaccines. To carry out this aim, we created our road map in two main steps. As the first step, we try to find the adenosine receptor subtype responsible from adenosine mediated immunosuppression using primary APC cultures. The second step is investigating whether adenosine receptor blockade will improve the antigen-specific immune responses *in vivo* using MPLA as an immunostimulatory adjuvant platform and a positive control and ovalbumin as model antigen.

Chapter 2

Materials, Solutions & Buffers

2.1 Materials

2.1.1 General Laboratory & Cell Culture Reagents and Materials

Cell culture medium RPMI-1640 was purchased from Gibco, USA. FBS (gamma irradiated) was from Biowest, USA. Pen-Strep, HBSS and AccuGENE molecular biology water and trypsin were purchased from Lonza, Switzerland and RPMI-1640 supplement sodium pyruvate was from Sigma, USA. StemPro® Accutase® as another chemical for cell detachment was purchased from Merck Millipore, Germany. Trypan Blue solution which used in the cell counting was purchased from Biological Industries, USA. DMSO (Sterile) used for dissolving lyophilized chemicals and reagents was purchased from Life Technologies, USA. Stericup-GP 500mL Express Plus, PES .22 μm for filtering solutions such as FBS, PBS and RPMI-1640 was from Millipore as well. For cell freezing, freezing container, Nalgene® Mr. Frosty was used and purchased from Nalgene, USA. Adenosine Deaminase (ADA) used for the degradation of endogenous adenosine from cells was purchased from Roche, USA. Plastics which were used in general cell culture processes such as cell culture plates and flasks and 2 ml cryogenic preservation vials for storage of frozen cells were from Greiner Bio-One GmbH, Austria. 40 μm cell strainers to disrupt the tissue integrity and preparing single cell suspensions were purchased from Corning Life Sciences Inc., USA.

2.1.4 Mice Experiments Reagents and Materials

Reagents for the mice experiment were all suitable for in vivo use and they can be seen in table 2.1. 26,5 G needles (Cat. No:300015) used for the intraperitoneal injection and 27G needles (Cat. No: 300635) for subcutaneous injections were purchased from BD Biosciences, USA.

Chemical	Catalog No	Brand
AddaVax™	vac-adx-10	Invivogen, USA
EndoFit Ovalbumin	vac-pova	Invivogen, USA
Thioglycollate Medium, Brewer Modified	211716	BD Biosciences, USA

Table 2.1: Information of chemicals used on mice.

2.1.5 Adenosine Receptor Agonists and Antagonists

Agonists antagonists used for stimulation of adenosine receptors for either in vitro and in vivo experiments can be seen in tables 2.2 and 2.3.

Agonists:

Chemical	Catalog No	Brand
2'-MeCCPA (A1 agonist)	2281	TOCRIS Bioscience, Bristol, UK
CGS 21680 hydrochloride (A2A agonist)	124431-80-7	TOCRIS Bioscience, Bristol, UK
BAY 60-6583 (A2B agonist)	4472	TOCRIS Bioscience, Bristol, UK
2-Cl-IB-MECA (A3 agonist)	1104	TOCRIS Bioscience, Bristol, UK
NECA	35920-39-9	TOCRIS Bioscience, Bristol, UK

Table 2.2: Information of adenosine receptor agonists.

Antagonists:

Chemical	Catalog No	Brand
PSB36 (A1 Antagonist)	2019	TOCRIS Bioscience, Bristol, UK
SCH 58261 (A2A Antagonist)	160098-96-4	TOCRIS Bioscience, Bristol, UK
PSB 603 (A2B Antagonist)	3198	TOCRIS Bioscience, Bristol, UK
THEOPHYLLINE	2795	TOCRIS Bioscience, Bristol, UK

Table 2.3: Information of adenosine receptor antagonists.

2.1.6 PRR Ligands

Chemicals used to stimulate TLR4 for either in vitro and in vivo can be seen in table 2.4

Chemical	Catalog No	Brand
Ultrapure LPS from Salmonella Minnesota R595	tlrl-smlps	Invivogen, USA
MPLA-SM VacciGrade™	vac-mpla	Invivogen, USA

Table 2.4: Information of TLR4 Ligands.

2.1.7 Recombinants and Other Agents

Recombinants GM-CSF (Cat. No: 576306) used in differentiation of bone marrows to dendritic cells was purchased from Biolegend, USA.

2.1.8 ELISA

Nunc-Immuno™ MicroWell™ 96 well solid plates (MaxiSorp™) for the ELISA experiments were purchased from Sigma-Aldrich, USA. TMB solution used for the develop the signals was purchased from Biolegend, USA.

2.1.8.1 Cytokine ELISA

ELISA experiments for the detection of TNFα, IL12 and IL10 was purchased as a kit and their information can be found on table 2.5

Chemical	Catalog No	Brand
Mouse IL-12 (p40) ELISA MAX™ Standard	431602	Biolegend, USA
Mouse TNF-α ELISA MAX™ Standard	430902	Biolegend, USA
LEGEND MAX™ Mouse IL-10 ELISA Kit with Pre-Coated Plates	431418	Biolegend, USA

Table 2.5: Information of ELISA Kits.

2.1.8.2 Antibody ELISA

Antibodies and reagents used in antibody ELISA for the determination of serum antibody levels can be found in table 2.6.

Material	Catalog No	Brand
HRP Goat anti-mouse IgG (minimal x-reactivity) Antibody	405306	Biolegend, USA
Goat anti-Mouse IgG2c Secondary Antibody, HRP	430902	Biolegend, USA
Ovalbumin	vac-stova	Invivogen, USA

Table 2.6: Information on chemicals and antibodies used in antibody ELISA

2.1.9 RNA Isolation, cDNA Synthesis and Q-RT-PCR.

For the determination of gene expression, the first step, RNA isolation was done with the NucleoSpin® RNA (Cat. No: 740955.50) kit from Macherey-Nagel. For the cDNA conversion from RNA, High-Capacity cDNA Reverse Transcription Kit (Cat. No: 4368814) was used from Applied Biosystems, USA. As a RNase inhibitor SUPERase In™ RNase Inhibitor (Cat. No: AM 2694) was used from Life Technologies, USA. Qualities of both RNAs and cDNAs were confirmed with the Thermo Scientific™ NanoDrop™ found in the Bilkent University. Quantification of cDNAs by Quantitative-Real Time PCR technique was done by using Taqman Gene Expression Assay. For that TaqMan® Universal Master Mix II, no UNG (Cat. No: 4440040) was purchased from Thermo Scientific, USA. Information about the probes used in these experiments can be found at table 2.7

Chemical	Catalog No	Brand
Mm00802075_m1 (Adora2a taqman probe/ primer)	4331182	Life Technologies, USA
Mm00839292_m1 (Adora2b taqman probe/ primer)	4331182	Life Technologies, USA
Mm00607939_s1 (beta actin probe/primer)	4331182	Life Technologies, USA

Table 2.7: Information on Taqman probes.

2.1.10 Flow Cytometry

For the flow cytometry experiments, antibodies and streptavidin conjugated dyes can be seen in tables 2.8 and 2.9. For the Ovalbumin specific CD8 T cell staining, tetramer from SIINFEKL peptide were prepared as explained in the methods.

For all of the flow cytometry experiments, Nunc™ 96-Well Polypropylene MicroWell™ Plates (Cat. No: 249944) which are purchased from Thermo Scientific, USA, were used. For the live cell determination, LIVE/DEAD® Fixable Green Dead Cell Stain Kit, for 488 nm excitation (Cat. No: L34970) was purchased from Life Technologies, USA.

Antibody Name	**Species**	**Catalog No**	**Brand**
Anti-CD11b FITC	Mouse	11-0112-41	Ebiosciences, USA
Anti-CD11b PerCP/Cy5.5	Mouse	65-0112-U100	Tonbo, USA
Anti-CD11c (N418) PE-Cyanine7	Mouse	60-0114-U100	Tonbo, USA
Anti-CD138 (Syndecan-1) PE	Mouse	142504	Biolegend, USA
Anti-CD16/CD32	Mouse	14-061-85	Ebiosciences, USA
Anti-CD185 (CXCR5) Brilliant Violet 421™	Mouse	145512	Biolegend, USA
Anti-CD19 FITC	Mouse	553785	BD Biosciences, USA
Anti-CD19 PerCP-Cy5.5	Mouse	45-0193-82	Ebiosciences, USA
Anti-CD25 Brilliant Violet 421™	Mouse	102034	Biolegend, USA
Anti-CD25 PE	Mouse	553075	BD Biosciences, USA
Anti-CD279 (PD-1) PE/Cy7	Mouse	109110	Biolegend, USA
Anti-CD4 FITC	Mouse	553046	BD Biosciences, USA
Anti-CD4 PerCP/Cy5.5	Mouse	65-0041-U100	Tonbo, USA
Anti-CD44 biotin Rat monoclonal antibody	Mouse	10232	Stem Cell, CANADA

Anti-CD45 Alexa Fluor® 700	Mouse	103128	Biolegend, USA
Anti-CD62L PECy7	Mouse	560516	BD Biosciences, USA
Anti-CD86 PE	Mouse	12-0861-83	Ebiosciences, USA
Anti-CD8a (53-6.7) APC-Cyanine7	Mouse	25-0081-U100	Tonbo, USA
Anti-CD8a FITC	Mouse	553030	BD Biosciences, USA
Anti-FOXP3 AF647	Mouse	320014	Biolegend, USA
Anti-GL7 Antigen Pacific Blue™	Mouse	144614	Biolegend, USA
Anti-H-2Kb Pacific Blue™	Mouse	116514	Biolegend, USA
Anti-I-A/I-E Brilliant Violet 510™	Mouse	107635	Biolegend, USA
Anti-IgD APC/Cy7	Mouse	405716	Biolegend, USA
Anti-IgG1 APC	Mouse	560089	BD Biosciences, USA
Anti-IgG2a Biotin	Mouse	553504	BD Biosciences, USA
Anti-IgM PE/Cy7	Mouse	406514	Biolegend, USA
Anti-NK1.1 FITC	Mouse	553164	BD Biosciences, USA
Anti-SA-APC	Mouse	17-4317-82	Ebiosciences, USA
Anti-TCR β chain Brilliant Violet 510™	Mouse	109234	Biolegend, USA
BV510 Streptavidin		563261	BD Biosciences, USA

Table 2.8: Information on antibodies in flow cytometry experiments

Antibody Name	**Catalog No**	**Brand**
Streptavidin-APC	17-4317-82	Ebiosciences, USA
Streptavidin-PE	S866	Invitrogen, USA

Table 2.9: Information on streptavidin conjugated dyes used in flow cytometry experiments

2.2 Buffers and Solutions

2.2.1 Cell Culture Media

RPMI-1640 (with L-Glutamine) (Gibco)

- 10% FBS heat inactivated at 55 °C and filtered
- 50 g/ml Penicillin/Streptomycin
- 0,11 mg/ml Na Pyruvate
- Ingredients dissolved in 500 ml medium
- Storage temperature: +4 °C

BMDC Differentiation Buffer

- Complete RPMI
- 50 μM 2-mercaptoethanol
- 5 ng/ml GM-CSF

Freezing Medium

- 10 ml DMSO
- 90 ml FBS (Filtered)
- Storage temperature: +4 °C

2.2.2 Spleen and Lymph Nodes Collection Buffer

- 2 ml FBS
- 100 ml HBSS
- Store at $4\,^{0}C$

2.2.3 Flow Cytometry Buffers

Flow Cytometry Buffer

- 500 ml 1x HBSS
- 2% FBS (10 ml)

- 125 mg Sodium Azide (0.25%)
- Storage temperature: +4 °C

Fixation Buffer

- 4% Formaldehyde in HBSS
- Storage temperature: -20 ^{o}C

Permeabilization Buffer

- Dissolve following in 500 mL HBSS:
- - 5 mL FBS
- - 0.5 g Sodium azide
- - 0.5 g Saponin
- - Adjust pH to 7.4-7.6
- - Filter (0.2 um) before use.

2.2.4 ELISA Buffers

2.2.4.1 Cytokine ELISA Buffers

Coating Buffer:

- 8.4g $NaHCO_3$
- 3.56g Na_2CO_3
- 1L ddH_2O
- Adjust pH to 9.5
- Store at 4 ^{o}C.

Assay Diluent

- 10% FBS Heat inactivated at 55 ^{o}C (10ml)
- 100 ml PBS

- Store at 4^0C

Wash Buffer

- 100 ml 10x PBS
- 0,5 ml Tween20
- 0,9 lt ddH_2O
- Storage temperature: room temperature

10X PBS (Phosphate Buffered Saline)

- 80 g NaCl
- 2 g KCl
- 15,2 g Na_2HPO_4. H_2O
- 2,4 g KH_2PO_4
- 1 lt ddH_2O
- pH adjustment: 7.4
- Sterilize By filtering
- Storage temperature: Room temperature

Stop Solution

- 2N H_2SO_4 in dH_2O
- Filter before use

2.2.4.2 Antibody ELISA Buffers

Wash Buffer

- 100 ml 10x PBS
- 4 ml Tween20
- 0,9 l ddH_2O
- Storage temperature: room temperature

Blocking Buffer

- 4 gr NFDM
- 100 ml wash buffer
- Storage temperature: +4^{o}C

Dilution Buffer

- 1 gr NFDM
- 1 l wash buffer
- Storage temperature: +4^{o}C

Stop Solution

- 2N H_2SO_4 in dH_2O
- Filter before use

ACK Lysis Buffer

- 8.3g NH_4Cl
- 1g $KHCO_3$
- 1l double distilled water
- Adjust Ph to 7,4

Chapter 3

Methods

3.1 Protocols for Cell Culture Experiments

3.1.1 Cell Freezing, Thawing & Cryopreservation

Cell freezing: Cells to be frozen should be reached 70-80% confluency before freeze them. After they reach the appropriate density, they were washed with HBSS and collected with appropriate collection method (Trypsin, Scraping etc.). Collected cells were centrifuged at 300g for 5 minutes and suspended in 1ml FBS. They were counted with hemocytometer and additional FBS were added so that final cell concentration would be $1x10^6$ cells in 500 µl FBS. Cells were mixed with same volume of FBS with 20% DMSO therefore final concentration of DMSO would be 10%. Suspension was aliquoted as 1ml into each cryovial immediately and put into -80°C freezer in Mr. Frosty container. They were stored in -80°C if they were used in a month, however for a longer-term storage they were placed into liquid nitrogen tanks.

Cell Thawing: Cryovials were taken and they were put into 37°C water bath for fast thawing. After partial melting, by using preheated medium they were collected and put into 7ml medium to reduce the DMSO concentration. Cell suspension centrifuged at 300 g for 5 minutes, then re-suspended in appropriate medium and seeded into appropriate cell container (T25 flask, flow tubes etc.)

3.1.2 Cell Counting

Hemocytometer is used to count cells. After cells were collected into fresh and appropriate medium, 10 µl of cell suspension was diluted with empty medium to make the counting easier and accurate. There was no standard dilution amount it was changed according to expected total cell number. 10 µl form cell suspension and 10 µl trypan blue mixed so that dead cells can be recognized and additional 2X dilution was performed. 10 µl form cell-trypan blue solution was taken into hemocytometer and counted for every 4 corner and average cell number was multiplied with appropriate dilution and 10^4 to find cell number in 1ml.

3.2 Isolating Cells from Bone Marrow

For bone marrow collection, desired number of C57/BL6 mice were sacrificed by cervical dislocation. They were sterilized with 70% ethanol and taken to dissection board. After a small incision, skin was peeled down through the leg and removed. With the help of tweezers, femur and tibia were collected. They were rinse with HBSS. To be able to collect cells inside, they were cut on one edge and placed into 0,6 ml tubes with the wholes at the bottom and these tubes placed into 2 ml tubes. They were centrifuge at 7000rpm for 15 seconds. Bone marrows can be seen at the bottom of the 2 ml tubes after the centrifugation. They were suspended in 0,5 ml HBSS and transferred into 50 ml falcon tubes. Then, for the get rid of red blood cells, 3 ml ACK lysis buffer were added on cells and incubated 3 min. After incubation time, rest of the tubes were filled with HBSS and centrifuged at 300g for 5 minutes. Pellet were re-suspended in 1ml in RPMI and counted with haemocytometer. They were used in BMDC or BMDM generation or they were frozen according to freezing protocol.

3.3 Bone Marrow Derived Dendritic Cell Generation

Collected bone marrows were centrifuged at 300 g for 5 minutes and then re-suspended in 1ml BMDC medium. After counting the cells, more BMDC differentiation medium were added so that final cell concentration would be $2x10^5$ cell/ml. 10 ml from this cell suspension would be taken and put into 10 cm^2 bacterial dishes and they were incubated at 37^oC. At day 3, 10 ml fresh BMDC medium were added onto the old medium. After that, at day 6, 10 ml of the media were taken and cells were centrifuged. Pellet were re-suspended in fresh 10 ml BMDC medium and added back to dish which contains 10 ml of old media and cell suspension. At day 8, same protocol is done with day 3 so that final volume would be 30 ml. 2 days after (day 10), half of the medium were collected and with the remaining half were pipetted therefore we can collect poorly adherent cells as well. Collected medium were centrifuged and can be re-suspended in desired medium or frozen according to cell freezing protocol. During this process, cells were controlled daily for their physiology and health. If there are any non-expected situation such as contamination or high levels of cell proliferation, these plates were disposed. At the end of 10 days, for the quality control, some of the cells were taken, stained with CD11b, CD11c, Gr1, Live-dead according to flow cytometry protocol and analyzed under flow cytometry.

3.4 Collection and Isolation of Murine Peritoneal Macrophages

3% Thioglycollate medium was taken into syringe under the hood and 3 ml per C57/BL6 was injected intraperitoneally. For both BMDC and peritoneal macrophage collections male mice were used. Mice were rested for 5 days to attract cells into peritoneal cavity. After 5 days of incubation, mice were sacrificed with cervical dislocation and wiped with 70% ethanol. With a small incision, skin of the mouse was peeled. Then, sterile 10 ml ice cold HBSS was injected into mice intraperitoneally. By keeping the needle inside the peritoneum and being careful not to puncture any organs, shake the belly to ensure homogeneous cell distribution. Then, HBSS with cells were collected back while avoiding the clogging from fat and organs. If at this point, the withdrawn HBSS and cell suspension has blood contamination, these cells were discarded and not used in any experiments. Cells were centrifuged at 300g for 5 min than re-suspended in regular RPMI 1640 with 10% FBS and they were seeded to 10 cm cell culture plates. After incubation for 4 hours, suspension cells were discarded and adherent

cells were collected by using Accutase. After this point, cells were ready to use and they can be counted and re-seeded for stimulations.

3.5 Mice Experiments

All mice used in experiments in this thesis were supplied by Bilkent University Animal Facility. For the animal experiment, related with adenosine receptor antagonist usage in adjuvant formulations, 72 female C57/BL6 mice were taken. They were 3-4 months old. They were grouped according their age differences. Younger ones were chosen for longer days of incubations while the old ones were chosen for the incubation with less time. Mice were shaved from their back at the right sides one day before injection. Injections done subcutaneous as 100 μl per mice. For the antigen re-challenge at day 28, injection made to same side of the mice as the first injection. They weight between 20 gr and 23 gr so they take injections according to that. After each incubation period, they were anesthetized by IP injection. Afterwards, their blood samples were collected into glass tubes from heart then spleen, draining and non-draining (according the side of injection) inguinal lymph nodes were collected.

3.6 Primary Murine Cell Suspension Preparation from Spleens and Lymph Nodes

Organs were sustained in Spleen and Lymph Nodes Collection Buffer while all the organs were collected and they were wait on the ice. After all organs were collected, organs were mashed by using 40 μm cell strainers. For the mashing plunger of the syringe were used. After mashing, strainer washed with more buffer so that we can collect remaining cells as well. Then suspension of cells was centrifuged at 300 g for 5 min. They were washed with HBSS at with the same centrifugation. After discarding the supernatant, cells were re-suspended in Flow cytometry buffer. Spleen were suspended in 2,5 ml while lymph nodes were suspended in 0,6 ml. They were keep at +4 ^{o}C until they were stained for flow cytometry.

3.7 Serum Collection from Blood Samples

Blood samples inside the glass tubes were put into 37^{o}C incubator for 2 hours to let the plasma clot. After clotting, remaining supernatant includes serum of the blood. Supernatant was collected into new tube. To get rid of the remaining plasma contamination, samples were centrifuged at 8000 rpm for 5 min at room temperature. after this step serum as a supernatant can be seen easily with its colourless nature. Collected serum samples were put into 96-well plates and stored in -20 ^{o}C until they were used in experiments.

3.8 ELISA

For the detection of TNFα and IL10 cytokine ELISA kits were used. For the detection of antigen specific antibody amounts, no kits were used. Before ELISA experiments, stimulated cell medium or the serum from mice were collected and stored at -20^{o}C until they are needed.

3.8.1 Cytokine ELISA

TNFα Detection: Mouse TNF-α ELISA MAX™ Standard (Biolegend) were used for the detection of TNFα. Nunc-Immuno™ MicroWell™ 96 well solid plates (MaxiSorp™) plates were coated with 50 µl anti-TNFα coating antibody and incubated overnight at 4^{o}C. Next morning, plates were washed 4 times with the ELISA wash buffer (0,05% Tween 20 in PBS) and 1 time with dH_2O. Any remaining buffer was blotted by tapping the plate upside down to absorbent paper. For blocking the wells, 200 µl assay diluent was used per well and incubated 1hr at room temperature. Same washing steps were done after blocking. After blocking, 50 µl standards and samples were put the wells and incubated 2 hour at room temperature. Samples were diluted 10 times to get the proper range. Highest standard concentration is 1000 pg/ml and goes by 2 times serially (1000, 500, 250 …). Assay diluent were used for diluting the samples and standards. It is also used as blank. Biotin conjugated detection antibody were diluted 1:200 in assay diluent and added to wells as 50 µl after washing the plates as previously indicated. After 1 hour incubation with the detection antibody, plates were washed again and incubated in 50 µl 1:1000 diluted avidin-HRP for 30 minutes. After final washing, 50 µl TMB solution were added per well and waited for enough time to get a good color change in the standard (usually 10-15 minutes). After getting the suitable color difference, 50

μl stop solution were added to each well to stop the reaction and wells were read with the endpoint protocol of ELISA reader (Biotek) at absorbance of 450 nm.

IL10 Detection: Detection of IL10 were done with the Biolegend IL10 ELISA kit with the pre-coated plate. Protocol is the same with the TNF- α with a few minor changes. Due to the usage of pre-coated plates, overnight coating step was discarded. Wash buffer is provided as 20X in the kit and it was diluted with distilled water to 1X working concentration. Assay buffer A supplied with the kit was also used in place of assay diluent.

3.8.2 Antibody ELISA

96-well Nunc maxisorp plates were coated overnight with 20 μg/ml Ovalbumin as 100 μl per well. They were put at +4^{o}C. Next day, plates were washed 4 times with 0,4% Tween 20 in PBS and one time with distilled water. Then plates tapped to absorbent paper. Blocking of the wells were done with antibody ELISA blocking buffer 200 μl per well. They were incubated 2 hr at room temperature. In these 2 hr, serum samples were taken from -20 ^{o}C and thawed. They were serially diluted (as the first sample is 1:5 than 5 times each as 1:25, 1:625 and so on) in dilution buffer. After blocking same washing step were done. 100 μl of diluted samples was put into each well and incubated for another 2 hr at room temperature for the Ovalbumin specific antibodies to bind the Ovalbumin. Dilution buffer was used as a blank. After diluted serum incubation, same washing step was performed again. For the detection of total IgG, HRP conjugated antibody was diluted as 1:4000 in wash buffer while IgG2c was diluted as 1:2000. Wells were incubated with 100 μl of diluted detection antibodies and incubated for 1 hr at room temperature. After this incubation, final washing was done 5 times with wash buffer and 2 times with distilled water. They were dried well. To develop, 100 μl of TMB solution were added each well and waited. After getting the suitable color difference, 100 μl stop solution were added to each well to stop the reaction and wells were read with the endpoint protocol of ELISA reader (Biotek) at absorbance of 450 nm.

3.9 RNA Isolation

RNA isolation was done with the NucleoSpin® RNA kit was used. This kit includes columns for the effective RNA binding. Isolation of RNAs done according to protocol of the kit with one change. In the final elution step, instead of eluting in 60 µl at once, elution performed as 2 step with 40 µl and 20 µl of elution buffers to get more RNA. Kit is suitable for working in room temperature however it was performed on ice. Cell lysates can also be stored for couple days however RNAs were isolated right after stimulation with fresh cell lysates. Quality of RNAs were assessed and they were put into -80 ^{o}C until they were needed or directly used for the cDNA synthesis.

3.10 cDNA Synthesis

cDNA synthesis was done with the High-Capacity cDNA Reverse Transcription Kit. Master mix were prepared according to table 3.1.

2X Master mix (for 1 reaction)	
Components	**Volume (µl) per Reaction**
10X RT Buffer	2
25X dNTP Mix (100mM)	0,8
10X RT Random Primers	2
Reverse Transcriptase	1
Rnase Inhibitor	1
Nuclease-free Water	3,2
Total per Reaction	10

Table 3.1: Ingredients of 2X master mix for cDNA synthesis for 1 reaction.

After enough master mix were prepared. 10 µl of master mix and 10 µl of each RNA sample was mixed and sealed. Then they were put into PCR reaction as indicated in table 3.2. After

the PCR reaction were completed, their quality was assessed and they were diluted 1:5 with RNase and DNase free water. They can be stored at -20ºC for. long term.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25	37	85	4
Time	10 min	120 min	5 min	∞

Table 3.2: PCR program for cDNA synthesis

3.11 Quantitative-Real Time-PCR

For the quantification of gene expressions, Q-RT-PCR technique was used. For this purpose, Taqman Probes were used rather than cyber green. cDNAs were taken and PCR reaction mixes were prepared for each gene except cDNA sample. cDNA sample were added at last after the 8 µl of master mix was added to each well. Preparation of master mix can be seen in table 3.3.

Components	**Volume per 10 µl Reaction (µl)**
20X Taqman Gene Expression Assay	0.5
2X Taqman Gene expression Master Mix	5
cDNA Template	2
RNase free water	2.5

Table 3.3: Preparation of master mix for Q-RT-PCR

Step	**Temperature (°C)**	**Duration**	**Cycle**
Initial Denaturation	95	5 min	1x
Denaturation	95	10 secs	50x
Annealing	60	10 secs	
Elongation	72	15 secs	

Final Extension	72	5 min	1x
Hold	4	∞	

Table 3.4: Protocol for Q-RT-PCR

After plates were ready. They were sealed and centrifuged. Then PCR protocol was started according to protocol in table 4.4. After the protocol was ended, CT values were calculated by the machine and analysis done based on these values. β-actin were used as housekeeping gene for the analysis.

3.12 Flow cytometry

3.12.1 Tetramer-PE preparation

After calculating the total moles of biotinylated monomer and the concentration of biotin binding sites in the streptavidin-PE conjugate stock. 25 μl biotinylated SIINFEKL monomers were mixed with 79,5 μl of Streptavidin conjugate PE molecules. However, for efficient tetramerization, they were not mixed directly. They were mixed in a controlled manner. First 25 μl of monomer was taken into Eppendorf then total Streptavidin were added slowly as 1/10 aliquots as 7,95 μl and waited 10 minutes between each addition. Tetramerization process was done in at room temperature and in dark. After protocol was done, Tetramer-PE conjugate were stored at +4^{o}C.

3.12.2 Surface Staining

Samples were taken into Nunc™ 96-Well Polypropylene MicroWell™ Plates for the flow staining. They were washed 2 twice with flow cytometry buffer with centrifugation at 300 g for 5 min. After final washing, samples were suspended in 50 μl of flow cytometry buffer with (1:50 diluted) Anti-CD16/CD32 antibody (Fc receptor blocker). After 5 min of incubation, another 50 μl of flow cytometry buffer containing 1:200 diluted antibody cocktail for surface staining were added. Inside the surface staining cocktail 1:400 diluted live/dead green and lineage negative antibodies were put (they all FITC conjugated). After 30 min of

incubation, they were washed with flow cytometry buffer again and they can be read at flow cytometry or they can be fixed. All of the surface staining protocol were made in 4 ^{o}C.
Staining including the biotinylated antibodies is also same with biotinylated antibodies in the antibody cocktail. The only difference here is that, after antibody incubation, samples were washed and incubated with 1:400 diluted streptavidin for another 30 min.

3.12.3 Fixation and Permeabilization

Fixation and permeabilization were always performed before intracellular and after surface staining. For the fixation, samples were washed once with HBSS with the same centrifugation as in the surface staining. Afterwards they were suspended in 50 μl of HBSS and pipetted well to get rid of all clumps. Then they were mixed with 50 μl of fixation buffer. For efficient fixation, samples were wait on ice for 30 min. After fixation, samples were washed twice for permeabilization buffer. If there would be no intracellular staining, there is no need for washing with permeabilization buffer instead, they can be washed with flow cytometry buffer and analyzed.

3.12.4 Intracellular Staining

After samples were fixed and permeabilized, they were re-suspended in permeabilization buffer containing (1:50 diluted) Anti-CD16/CD32 antibody (Fc receptor blocker) and incubated for 5 min. Then another 50 μl of flow cytometry buffer containing 1:50 diluted antibody cocktail for intracellular staining were added and incubated for 30 min more. After incubation, they were washed with flow cytometry buffer and analyzed.

3.12.5 FOXP3 Staining Protocol

For the FOXP3 staining, Foxp3 / Transcription Factor Staining Buffer Set were. General Flow cytometry protocol is the same with this kit as well. Only difference is the fixation with its own fixation buffers according its own protocol and extracellular staining at room temperature. The purpose of using this kit is to get more effective fixation because, FOXP3 is a transcription factor and can localize in nucleus.

3.12.6 Subtypes of B Cell Receptor Staining

B cell staining was also same in many steps for staining. The difference is that there are 2 times of surface staining with different antibodies. Our antibodies for surface staining such as anti-IgD and CD138 has IgG2a or IgG1 isotypes that is why anti-IgG1 and anti-IgG2a was stained first and washed. Afterwards, other surface molecule specific antibodies were given.

3.13 Statistical Analysis

For the statistical testing and preparation of figures GraphPad Prism 6.0 software were used. Student t test were used to compare groups. $p<0,05$ were used as statistically significance value.

Chapter 4

Results

4.1 Adenosine Receptors and Their Effects on TLR4 Agonists, LPS and MPL-A

4.1.1 NECA Suppresses Inflammatory Phenotype Acquired by LPS and MPLA in Peritoneal Macrophages and Bone Marrow Derived Dendritic Cells

Lipopolysaccharide, also known as LPS, is a bacterial product that can induce strong immune responses. It is recognized as pathogen associated molecular pattern by TLR4 on many cells, most importantly, macrophages and DCs [113]. It is also widely used in immune system researches as a common proinflammatory stimuli. One reason for that is the strong immune response elicited by LPS due to the activation of both MyD88 and TRIF dependent signaling pathways [113]. LPS can be a good candidate as an immunological adjuvant in vaccines. However, its use is limited to in vitro studies due to its toxicity arise from Lipid A domain of this molecule. On the other hand, Monophosphoryl Lipid A (MPLA) is a low-toxicity derivative of LPS. It is also recognized by TLR4. Due to its reduced toxicity about a hundred-fold as compared to its parent compound LPS MPLA can be used as an immunological vaccine adjuvant in humans ([110] [109]).

In order to investigate the effects of adenosine receptors to LPS and MPLA on peritoneal macrophages and BMDCs, 5' -*N*-Ethylcarboxamidoadenosine (NECA) was chosen. NECA is a high affinity cell impermeable adenosine receptor agonist. It is an adenosine analog with non-selectivity against any adenosine receptor subtypes. Because NECA is cell impermeable its effects on other cells is mediated by adenosine receptors, making it an ideal adenosine analog to study adenosine receptor biology [114]. BMDCs and Peritoneal Macrophages were

collected as indicated in methods and quality control for BÖDCs can be seen in appendix figure 1. Before their stimulation, they were treated with adenosine deaminase (ADA) which converts adenosine to inosine so that endogenous adenosine levels remain in steady state levels and does not affect the results. LPS and MPL-A elicit the secretion of inflammatory cytokines TNFα and IL-12 from BMDCs with weaker response to MPLA as expected. NECA significantly suppresses the secretion of these cytokines after LPS or MPLA-stimulation. NECA also increases the secretion of anti-inflammatory cytokine IL-10 from BMDCs (Figure 4.1 A). This non-selective adenosine analog, also shows its effects on peritoneal macrophages as well. TNFα secretion from these cells also decreases in the presence of NECA (Figure 4.1 B). Consistent with the previous studies [115], these data show that NECA can change the cytokine profile of innate immune cells such as DCs and macrophages and cause the inhibition of pro-inflammatory phenotype and polarization of these cells into anti-inflammatory phenotype.

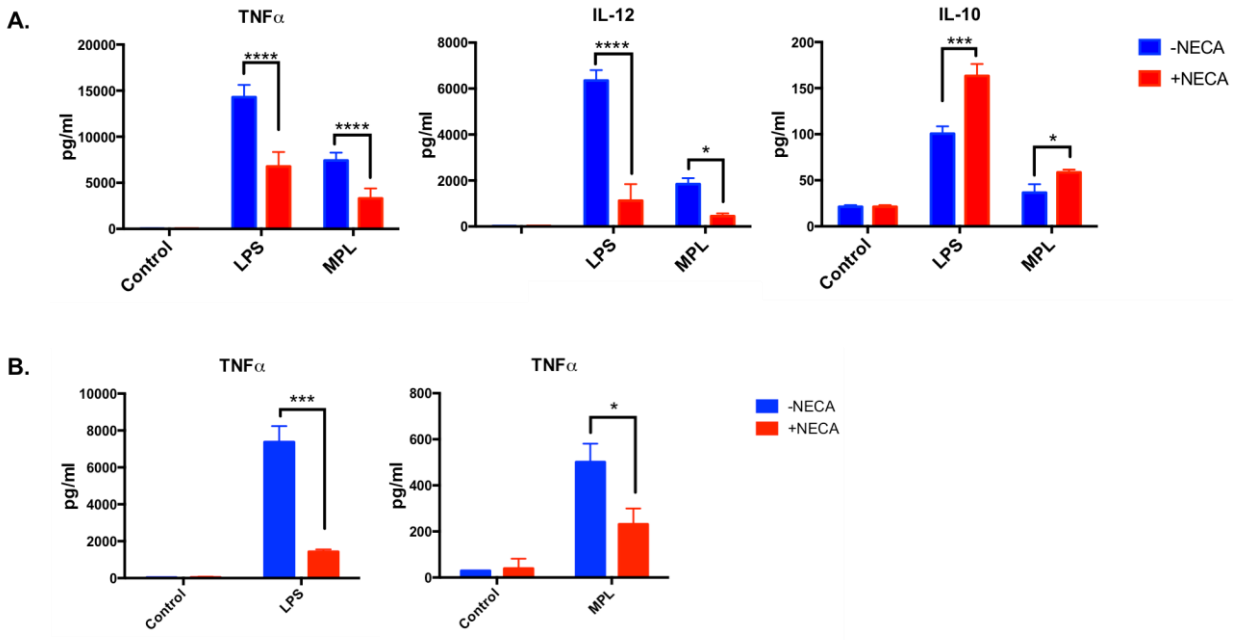


Figure 4.1: Adenosine receptor stimulation promotes an anti-inflammatory phenotype after LPS or MPLA stimulation in Dendritic cells and Macrophages. *ADA pretreated BMDCs* ***(A)*** *and peritoneal macrophages* ***(B)*** *were stimulated with indicated agonists (MPL-A: 1μg/ml, LPS:1μg/ml) for overnight. TNFα, IL10 and IL12 secretion were measured from the media with cytokine ELISA. For statistical analysis, student t test was used. * p<0,05, **p<0,01, ***p<0,001, ****p<0,0001. Results were representation of 3 independent experiments with 3 replicates each.*

After confirming the effects of NECA on some key cytokine profiles, we next examined its effects on expression of molecules important in antigen presentation to show that TLR4-mediated adjuvanticity can be directly influenced by adenosine receptor signaling. CD86 is expressed on the surface of APCs and provides a co-stimulatory signal for proper T cell activation. Upon activation CD86 is upregulated on the surface of antigen-presenting cells [116]. MHC I and MHC II are also expressed on cell surface. APCs present antigens through MHC I and MHC II and prime CD8 and CD4 T cells, respectively [3]. To determine the abundance of these proteins, one of the most important APC, BMDCs were used. Stimulations were done same with previous experiment. With the help of Accutase, cells were collected and they were analyzed by flow cytometry. As can be seen in Figure 3.2 addition of NECA significantly reduces LPS- or MPLA-induced CD86 (Figure 4.2A), MHCI (Figure 4.2B) and MHCII (Figure 4.2C) expression on DCs.

As a result, NECA suppresses APC responses after not only LPS stimulation but also TRIF-biased TLR4 agonist and vaccine adjuvant MPLA stimulation. Adenosine can provide an important negative feedback signal for priming T cells by limiting the expression of CD86, MHC I and MHC II molecules in APCs. Therefore, because APCs are important bridges between innate and adaptive immune responses we have investigated the effects of targeting of adenosine receptors to design novel vaccine formulations.

4.1.2 Adenosine-mediated suppression of APCs is mediated by adenosine A2A and A2B receptor subtypes.

There are four adenosine receptor subtypes: A1, A2A, A2B and A3. As explained in the Chapter 1, they can show their effects through different mechanisms and these effects may vary depending on the tissues and conditions. Because NECA can potentially target all the adenosine receptors we next investigated the specific adenosine receptor(s) playing the main role I adenosine-mediated suppression of immunostimulatory responses. We particularly used peritoneal macrophages because bone marrow-derived macrophages and dendritic cells predominantly responsive to A2B receptor stimulation and may not represent their primary counterpart directly isolated from mouse tissue.

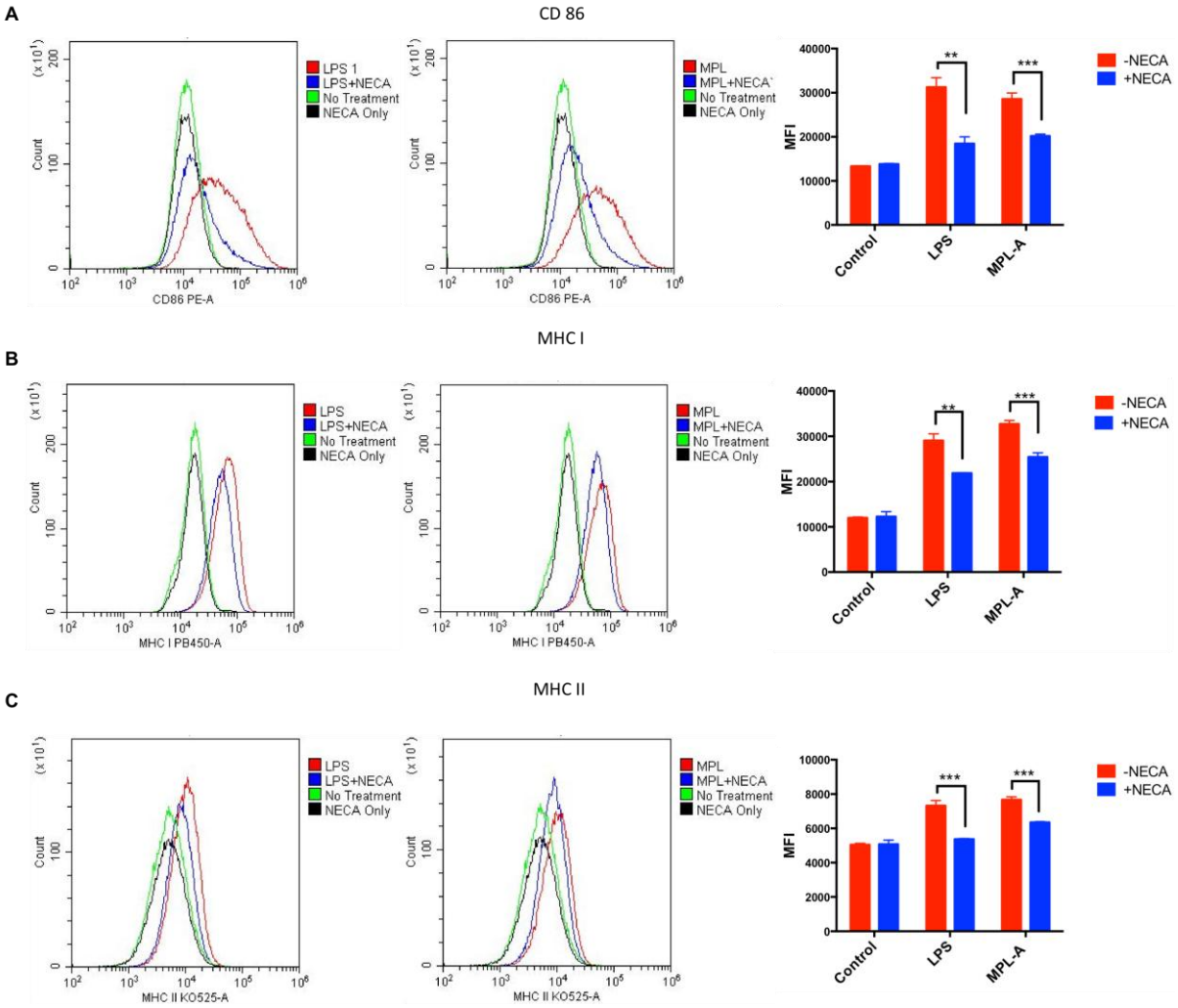


Figure 4.2: Effects of NECA on the Surface Markers of BMDCs activated with LPS (1µg/ml) or MPL-A (1µg/ml). *ADA pretreated BMDCs were stimulated with or without NECA in the presence of MPL-A or LPS for overnight. They were analyzed with for the MFI rates in flow cytometry. First 2 Graph is showing the histogram profiles and the 3 graph shows the quantitative results for CD86* ***(A),*** *MHC I* ***(B)*** *AND MHC II* ***(C).*** *For statistical analysis, student t test was used. * p<0,05, **p<0,01, ***p<0,001. Results were representation of 2 independent experiment with 3 replicates each.*

In fact, we replicated A2BR-dependence of adenosine regulation of BMDCs in Çekiç lab (Kayhan et al, Imran et al., unpublished results.) These specific agonists are 2' MeCCPA for A1, CGS 21680 for A2A, BAY 60-6583 for A2B and 2-CI-IB-MECA for A3. Peritoneal macrophages were stimulated with LPS or MPLA with or without one of these specific receptor agonists after ADA pretreatment. One of the most used activation marker TNFα was used as a read out and every sample were compared with the LPS and MPLA stimulation by itself.

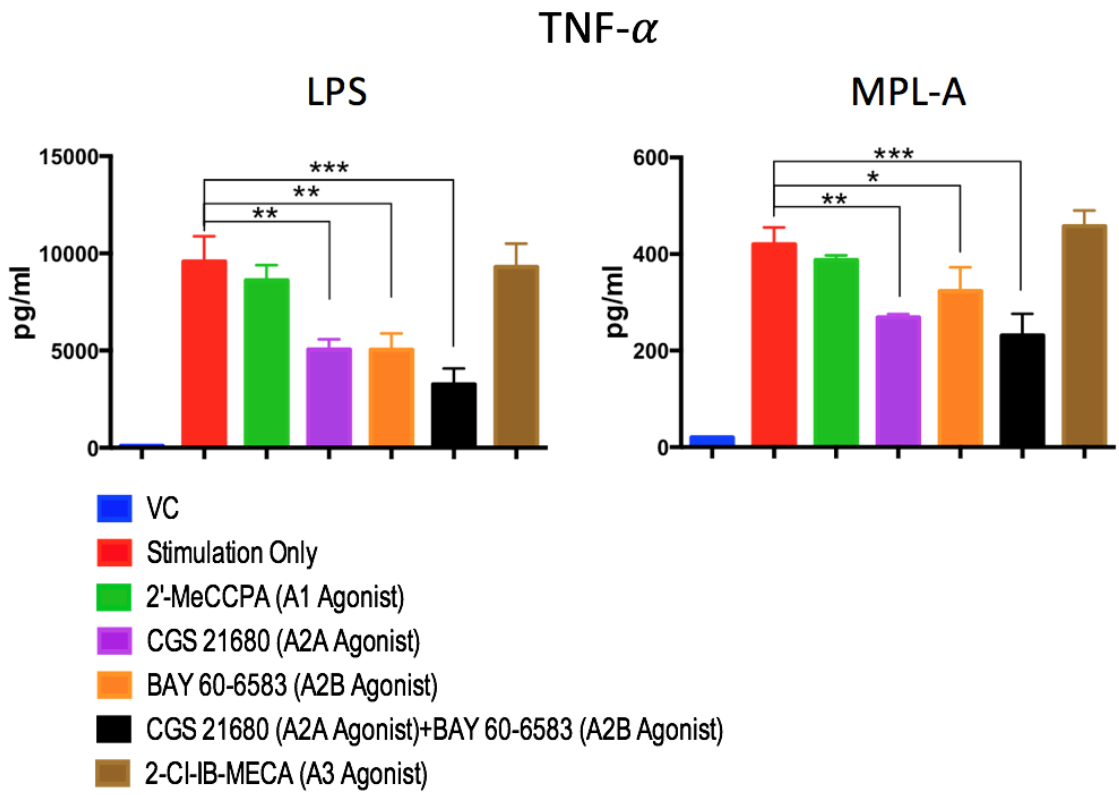


Figure 4.3: Effects of specific adenosine receptor agonists on peritoneal macrophages stimulated with LPS or MPL-A. *Peritoneal macrophages were stimulated overnight with LPS (0,3 μg/ml) or MPL-A (1μg/ml) and one of the specific receptor agonists, A2A and A2B together or DMSO control after ADA treatment. TNFα levels were measured by ELISA form the overnight culture media. For statistical analysis, student t test was used. * p<0,05, **p<0,01, ***p<0,001. Results were representation of 2 independent experiment with 3 replicates each.*

A1 and A3 receptors are G_i coupled GPCRs and decrease the cAMP levels while A2A and A2B receptors are Gs coupled and increase the cAMP levels [49]. Increase in cAMP level was shown to have anti-inflammatory effects [117]. Therefore, we hypothesized that adenosine A2A and A2B subtypes will reduce LPS- or MPLA-induced proinflammatory responses. As can be seen in Figure 4.3, A1 and A3 receptor agonists 2' MeCCPA and 2-Cl-IB-MECA did not inhibit the LPS- or MPLA-induced TNFα levels stimulation However, A2A and A2B receptor agonists, CGS 21680 and BAY 60-6583, significantly decrease TNFα release after LPS (Figure 4.3, left) or MPLA stimulation (Figure 4.3, right). When A2AR and A2BR agonists were given at the same time there is only a slight additive effect reducing TNFα release, especially after LPS stimulation.

Our proof-of-concept vaccine design targeting adenosine receptors will rely on the fact that there is adenosine present in tissues and adenosine receptors signaling due to endogenous adenosine present or generated after immune reaction to the vaccine will limit the effectiveness of the vaccines. Therefore, we next examined whether adenosine receptor antagonists can reverse adenosine-mediated immunosuppression. We also aimed to validate our results indicating A2AR and A2BR subtypes play the predominant role in adenosine suppression of macrophages using adenosine receptor specific antagonists. Peritoneal macrophages were stimulated by LPS or MPLA in the presence of NECA and in the presence of PSB 36 for A1 or SCH 58261 for A2A or PSB 603 for A2B or Theophylline as a non-selective antagonist and tested for TNFα secretion.

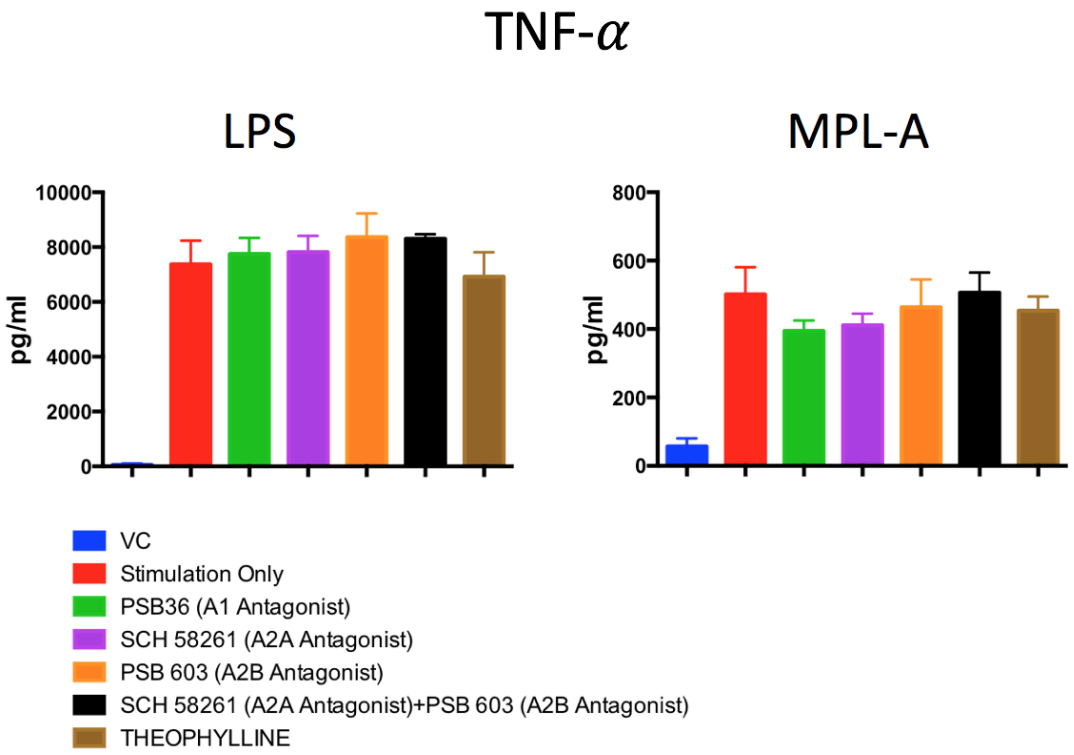


Figure 4.4: Effects of adenosine receptor antagonists on LPS or MPL-A stimulated peritoneal macrophages. *ADA pretreated peritoneal macrophages were stimulated overnight with LPS or MPL-A and one of the specific receptor antagonists, A2A and A2B together or DMSO control. TNF α levels were measured by ELISA form the overnight culture media. For statistical analysis, student t test was used. Results were representation of 2 independent experiment with 3 replicates each.*

We also tested whether addition of both A2AR and A2BR antagonists together will have any effect on proinflammatory response after MPLA or LPS stimulation in the absence or presence of NECA treatment because as can be seen in Figure 4.3. both of these receptors can potentially mediate adenosine-induced suppression and blockade of both of them may be necessary to reverse the effect of adenosine-mediated suppression of TNFα release. Addition of antagonists did not significantly change TNFα release due to LPS or MPLA stimulation (Figure 4.4).

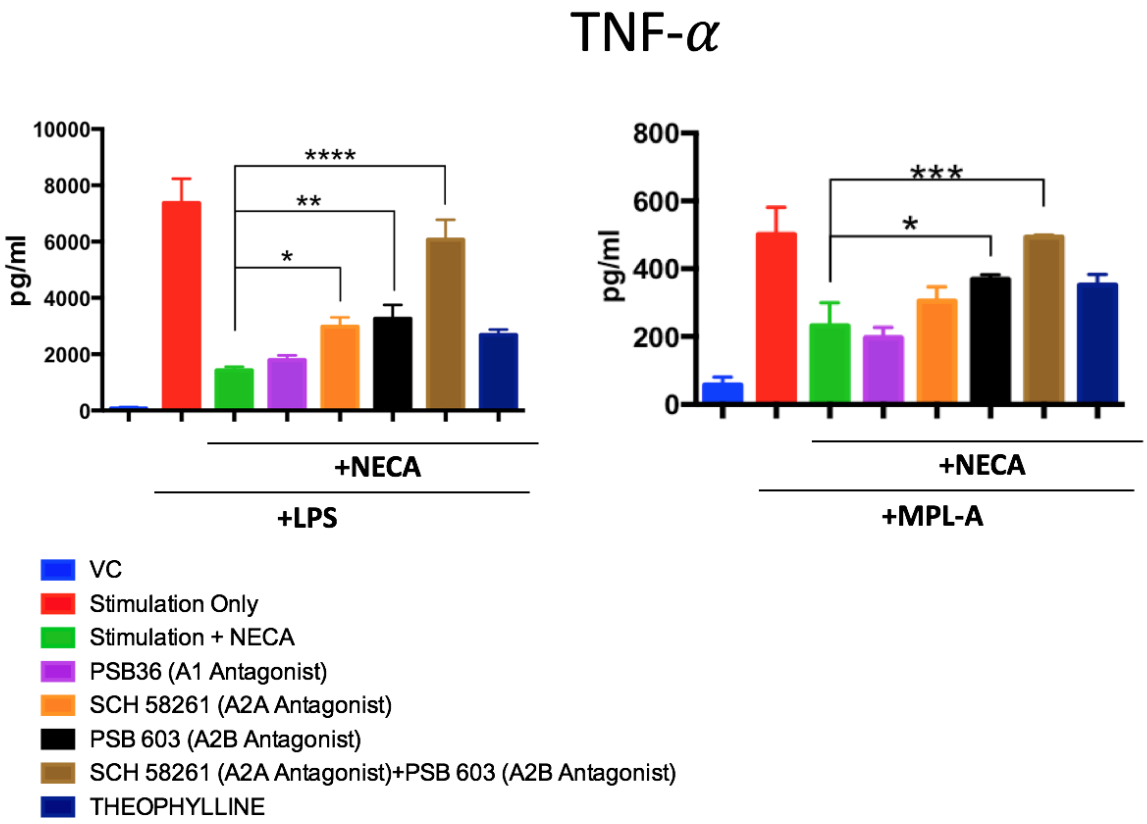


Figure 4.5: Effects of adenosine receptor antagonists on LPS or MPL-A stimulated peritoneal macrophages in the presence of adenosine analog. *ADA pretreated peritoneal macrophages were stimulated overnight with LPS or MPL-A and one of the specific receptor agonists, A2A and A2B together, Theophylline or DMSO control. All samples were also stimulated with NECA as well. TNFα levels were measured by ELISA form the overnight culture media. For statistical analysis, student t test was used. * p<0,05, **p<0,01, ***p<0,001. Results were representation of 2 independent experiment with 3 replicates each.*

Figure 4.5 shows that addition of A2BR or A2AR or non-selective antagonist theophylline can partially reverse the decreased TNFα release after NECA treatment. However, addition of both A2AR and A2BR antagonists together (Figure 4.5, brown bars) could completely reversed the suppressive effects of NECA on TNFα release by LPS or MPLA. Overall these results suggest that targeting of both A2A and A2B receptors is important to improve the vaccine efficacy

4.1.3 Usage of MPL-A and LPS increases the expression levels of A2A and A2B

As explained in the Chapter 1, A2B receptor is the low affinity receptor for the adenosine and responsiveness of A2BR profoundly related to i) Adenosine concentrations, ii) receptor expression level and iii) potentially their relative expression as compared to other ARs. To more clearly test the expression levels and its changes after proinflammatory stimuli or stimulation with MPLA we used BMDCs. Expression of both A2A and A2B receptors strongly increases after LPS or MPLA stimulation in a time dependent manner after 1 and 3 hours (Figure 4.6). Therefore, TLR4 targeting and using MPLA as a potential immunostimulatory adjuvant may increase the responsiveness of innate immune cells to adenosine by directly changing adenosine receptor expression.

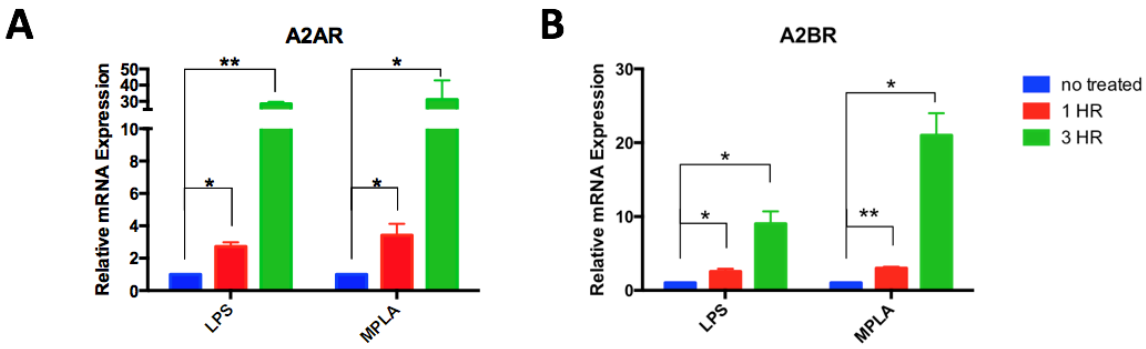


Figure 4.6: Effects of LPS and MPLA stimulations on adenosine receptor expression in BM-DCs. *ADA pretreated DCs were treated with indicated ligands for indicated time points and Q-RT-PCR protocol were performed as indicated. Effects of these ligands on A2A receptor* ***(A)*** *and A2B receptor* ***(B)*** *were shown. For statistical analysis, student t test was used. * p<0,05, **p<0,01. Results were representation of 2 independent experiment with 3 replicates each.*

4.2 Using A2AR and/or A2BR antagonists to improve subunit vaccine efficacy.

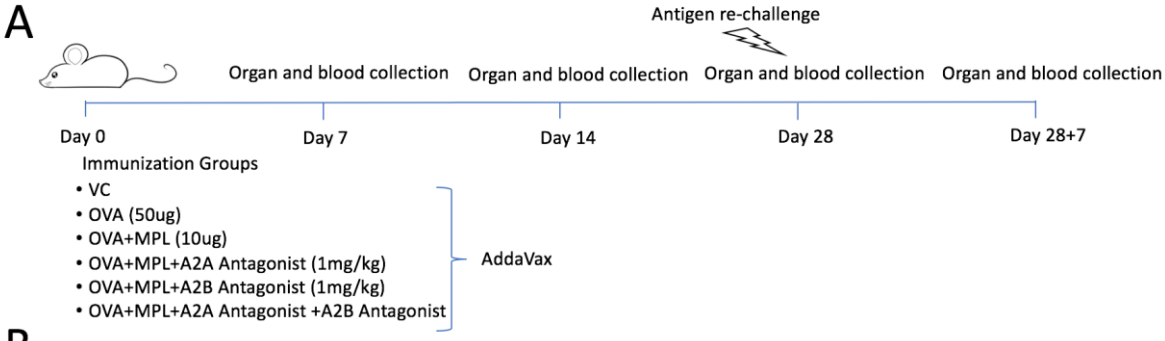
Previous studies and our results on adenosine receptor signaling and their effects on immune system, clearly show their effects on suppression in many immunological processes [49, 66, 118]. Because it is present in many tissues at concentrations high enough to activate its cognate receptors, adenosine and due to tissue-specific expression of its target receptors adenosine can regulate many physiological processes. Our preliminary studies so far clearly indicated that A2AR and A2BR expression is influenced by vaccine adjuvant MPLA and adenosine, by acting through these receptors, could limit the immunostimulatory effects of MPLA on antigen-presenting cells. Therefore, we hypothesized that adenosine A2A and/or A2B receptor blockade will improve the efficacy of MPLA in a subunit vaccine model. To test this hypothesis, we used SCH 58261 (A2A receptor antagonist) and PSB 603 (A2B receptor antagonist). To be able to show its effects, we also choose TLR4 ligand MPLA because it is readily used in licensed adjuvant formulations and it can be a suitable positive control as an immunological adjuvant. MPLA formulation in subunit vaccines also contains oil in water emulsion to improve the delivery of the antigen. Therefore, to model the clinical situation better we also add another licensed vaccine adjuvant, Addavax^{TM} into our formulation. Addavax is a squalene oil in water emulsion and it is similar to MF59 used in vaccine formulations for influenza [102]. Addavax can trap the immunostimulatory adjuvants and the antigens in a limited environment so that they can be found in higher concentrations for prolonged times.

4.2.1 Design and the Read Outs of the Mice Experiment for Determining the Effects of Adenosine Antagonist as Agents in Adjuvant Formulations.

Different combinations of MPL-A (10μg/mice), Addavax (1:1 ratio), SCH 58261 (1mg/kg) and PSB 603 (1mg/kg) were used as adjuvants and Ovalbumin (50μg/mice) as a model antigen. All groups including vehicle control, take 1:1 ratio of Addavax while all groups except VC takes OVA as an antigen. As a positive control, other than OVA and Addavax, MPL-A was used as adjuvant formulation while experimental groups take either A2A or A2B antagonists addition to MPL-A. Female C57BL/6 mice were grouped into 6 each immunized with a different combination subcutaneously. Each group contained 12 animals, three of which were sacrificed at indicated time points below. (Figure 4.7). Their spleens, lymph

nodes and blood samples were collected after indicated periods of time and single cell suspensions were prepared for FACS staining. One set of animals were re-challenged with 50μg of Ovalbumin/mice on day 28 and sacrificed 7 days after the re-challenge for sample preparation. Serum samples from all these animals were also collected for antibody ELISA. Details about the experimental design and the read outs can be seen figure 4.7 and the process regarding to gating strategy of flow cytometry experiments can be seen in appendix.

A



B

Tetramer Panel	
Lin- and LDG	FITC/GFP/Green
CD45	AF700
CD8	APC CY7
CD62L	PECY7
CD44	APC
Tetramer	PE
TCRb	BV510
CD25	BV421
CD4	PERCP CY5.5

B cell Panel	
Lin- and LDG	FITC/GFP/Green
CD45	AF700
CD19	PerCP-Cy5.5
IgM	PE-Cy7
IgG1	APC
IgD	APC-Cy7
CD138	PE
IgG2a	BV510
GI7	BV421

TFh Panel	
Lin- and LDG	FITC/GFP/Green
CD45	AF700
CD4	PerCP-Cy5.5
PD-1	PE-Cy7
FoxP3	APC
CD8a	APC-Cy7
CD25	PE
TCRb	BV510
CXCR5	BV421

Figure 4.7 Design and the Read Outs of Mice Experiment. ***(A)*** *Representative figure for the mice experiment. At day 0 primary immunizations and at day 28 antigen re-challenge were performed as indicated.* ***(B)*** *3 Different panels of flow cytometry for different purposes and parameters used.*

4.2.2 Effects of Adjuvants on CD4+ and CD8+ T Cell Numbers

One of the main purposes of our study is to perform a proof of concept study to show adenosine receptor blockade in subunit vaccine formulations can enhance antigen-specific immune responses. To dissect the broad effects of adenosine receptor blockade on the numbers of total CD4 and CD8 T cell populations and on the numbers of antigen specific CTLs we measured the changes in the numbers of these populations by flow cytometry. We also compared these populations from draining vs. non-draining inguinal lymph nodes and from spleen. Figure 4.8 shows that almost all vaccinated groups have increased numbers of overall T cell populations as compared to vehicle control in draining lymph nodes for days 7,

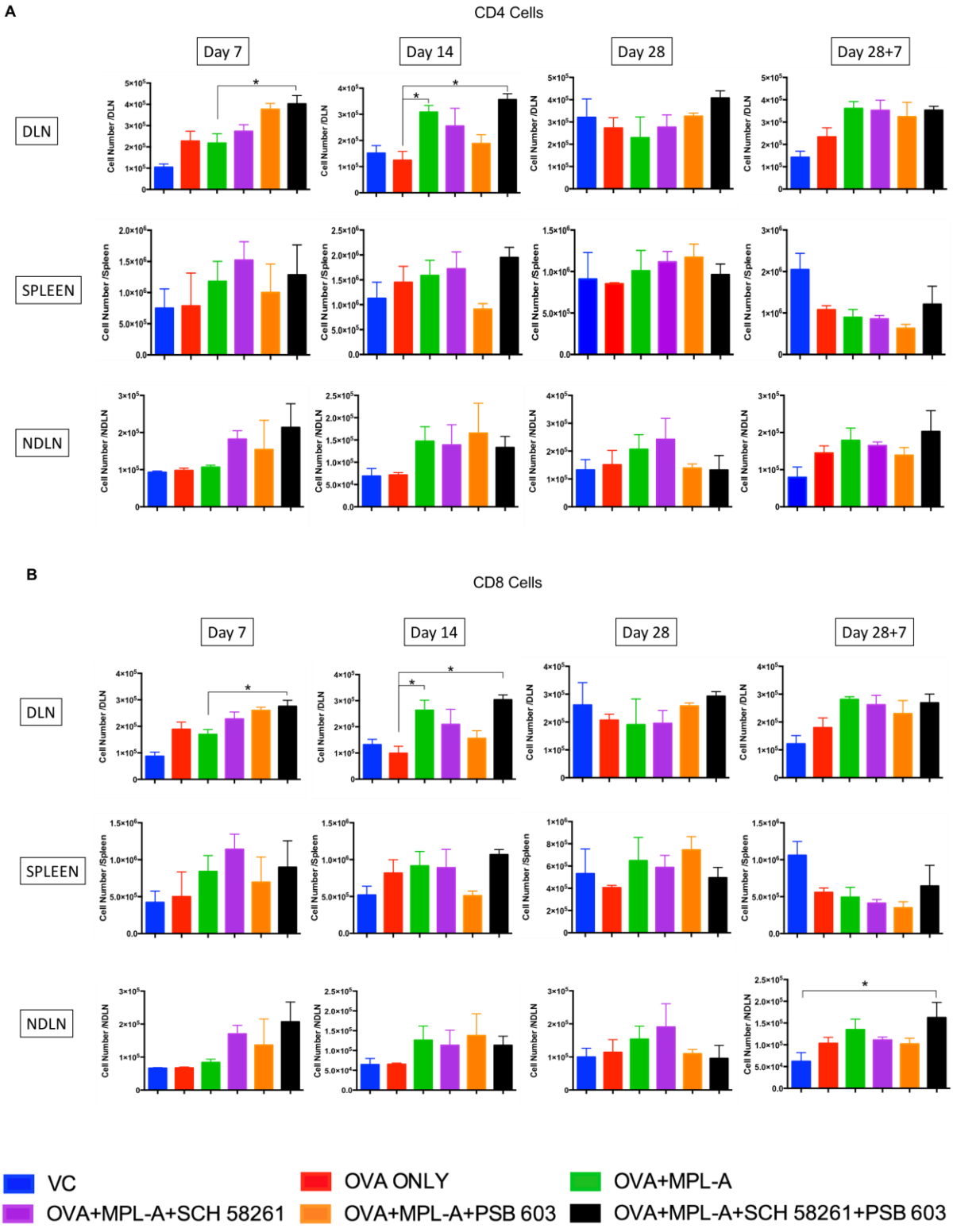


Figure 4.8: Effects of Adjuvants on CD4+ and CD8+ T Cell Number. ***(A)*** *CD4+ and* ***(B)*** *CD8+ T Cell Numbers. They were calculated according to read volumes for flow cytometry and total volume of tissue suspensions. For statistical analysis, student t test was used. * p<0,05, **p<0,01. 3 mice per group was used and AddaVax was used in all groups including VC.*

14 and 28+7. The difference is even more obvious when vaccine formulations contain A2B or A2A and A2B receptor antagonists. On day 28+7 total T cell numbers increased in lymph nodes while there is concomitant decrease in these populations in spleens of vaccinated.

Figure 4.9 and Figure 4.10 shows that despite the increases in these populations, proportions of naïve and effector populations in overall T cell populations did not significantly change after vaccination with the exception that on day 7 proportion of CD44+ (effector/memory) CD4 T cells increases by two-fold in the groups that receives MPLA. Overall these results suggest that although vaccination changes the numbers of overall CD4+ and CD8+ T cell numbers in draining lymph nodes this is not due to a particular increase in effector/memory populations especially for CD8+ T cells.

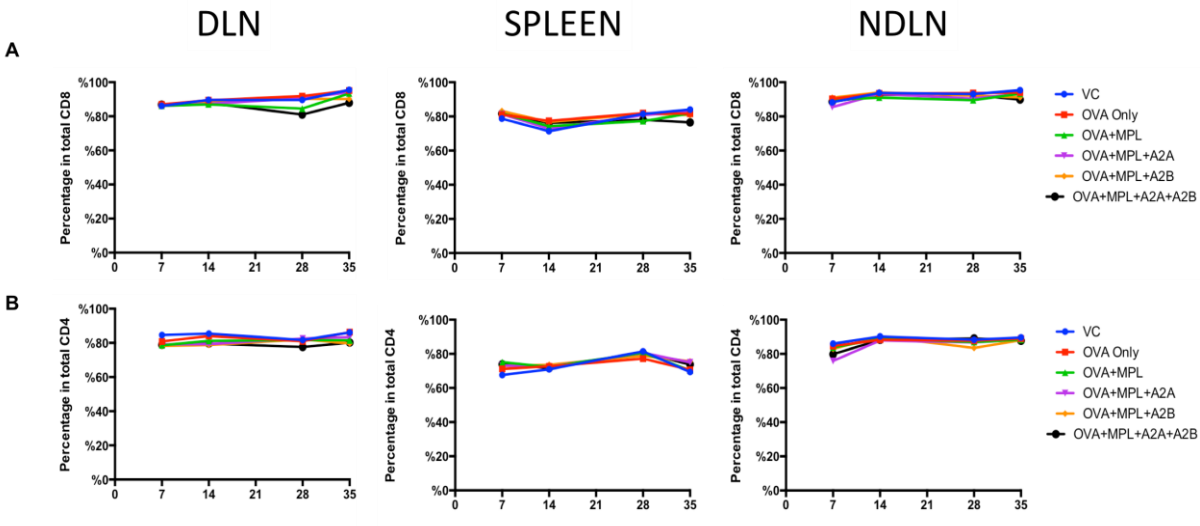


Figure 4.9 Naive CD4 and CD8 Percentages after Vaccination. ***(A)*** *CD44- CD8+ T cell percentages in total CD8+ T lymphocytes.* ***(B)*** *CD44- CD4+ cell percentage in total CD4+ T lymphocytes. 3 mice per group was used and AddaVax was used in all groups including VC.*

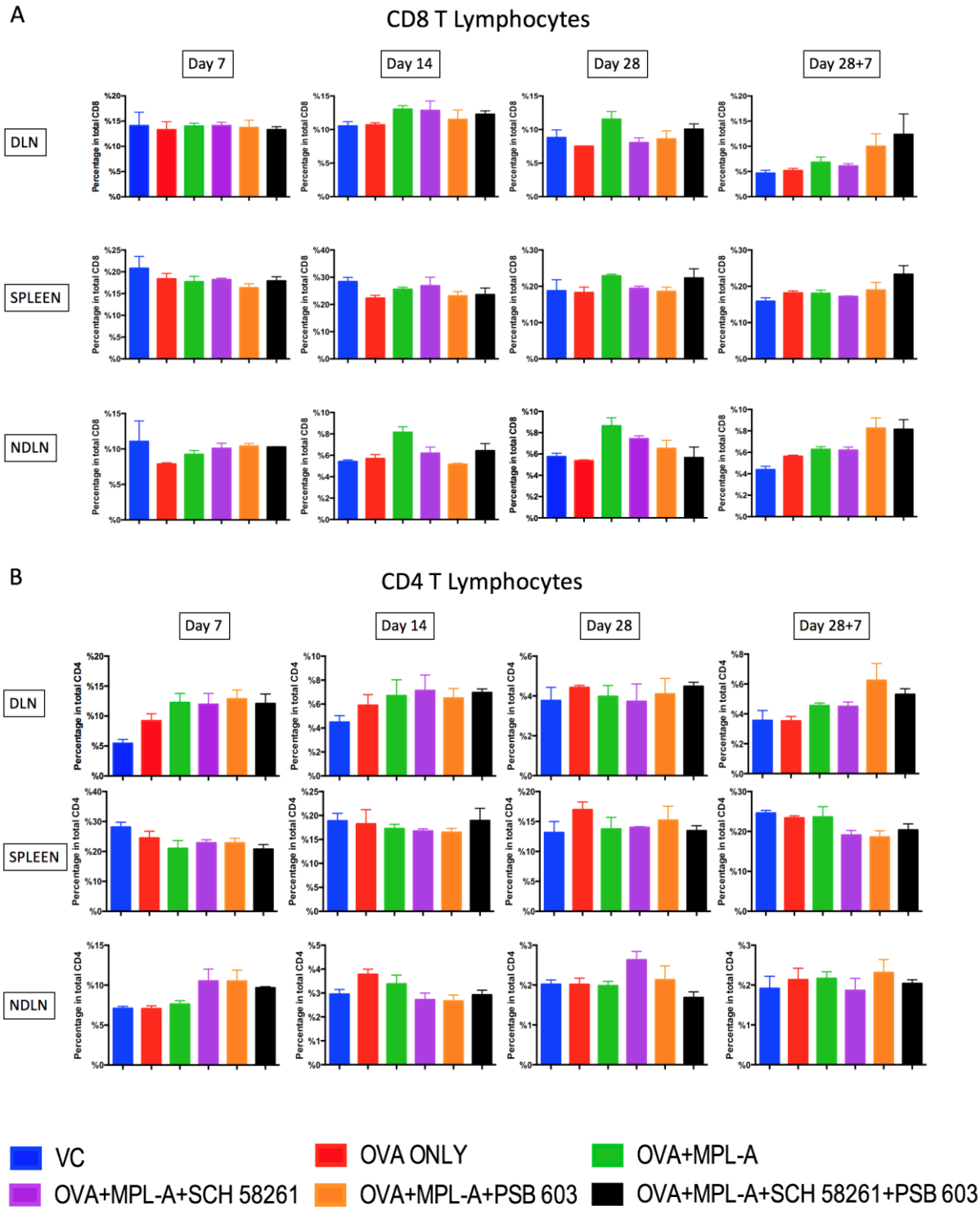


Figure 4.10: Activated CD4 and CD8 Percentages after Vaccination. ***(A)*** *CD44+ CD8+ T cell percentages in total CD8+ T lymphocytes.* ***(B)*** *CD44+ CD4+ cell percentage in total CD4+ T lymphocytes. 3 mice per group was used and AddaVax was used in all groups including VC.*

4.2.3 Changes in Ovalbumin Specific CTLs after Immunizations.

Activation status of overall T Lymphocytes can show us if there are general trends for immunostimulatory effects of the vaccines. However, the most important parameter to look for is the specificity this response. This is because among millions of T cells with unique TCRs only a few will carry the specific TCR for dominant epitopes of a given protein., To be able to determine antigen specific responses in T cells, we used biotinylated SIINFEKL loaded MHCI monomers tetramerized by fluorescently-labeled streptavidin molecules to detect T cells recognizing this dominant antigen epitope of ovalbumin for CD8 T cells.

DLNs are the first places to see the specific responses because antigens will be carried to nearest lymph nodes to be presented. First of all, all the vaccinated groups containing ovalbumin had increased numbers and percentages of CD8+CD44+Tetramer+ cells in lymph nodes and spleen as compared to that of animals treated with vehicle control, showing that vaccination triggered an antigen-specific CD8 T cell expansion. Also, we see an initial expansion in tetramer+ CD8 T cells on day 7 and contraction in their numbers on day 14 and day 28 (Figure 4.11). All groups receiving A2AR or A2BR or both antagonist had significantly increased numbers of tetramer positive CTLs on day 7 particularly in lymph nodes. A2AR blockade also significantly improved Tetramer+ CD8 numbers in spleens on this day. A2BR blockade alone increased both numbers and percentages of antigen specific CTLs in NDLNs on day 14. After 28 days, we can see decrease in the number of tetramer specific cells as expected however, the groups receiving both A2AR and A2BR antagonists had higher numbers of tetramer+ CTLs in their draining lymph nodes. On day 28+7, after the re-challenge with antigen only, we observed gradual increase with A2AR and then A2BR blockade. Including A2AR and A2BR antagonists in the formulations strongly and additively increased the numbers of tetramer positive CTLs in spleen. The mice immunized with A2BR antagonists also showed an increase in the numbers of tetramer+ CTLs in draining lymph nodes after the re-challenge. Overall, these results indicate that A2A and/or A2B receptor antagonists can increase antigen specific T cell responses when included in vaccine formulations.

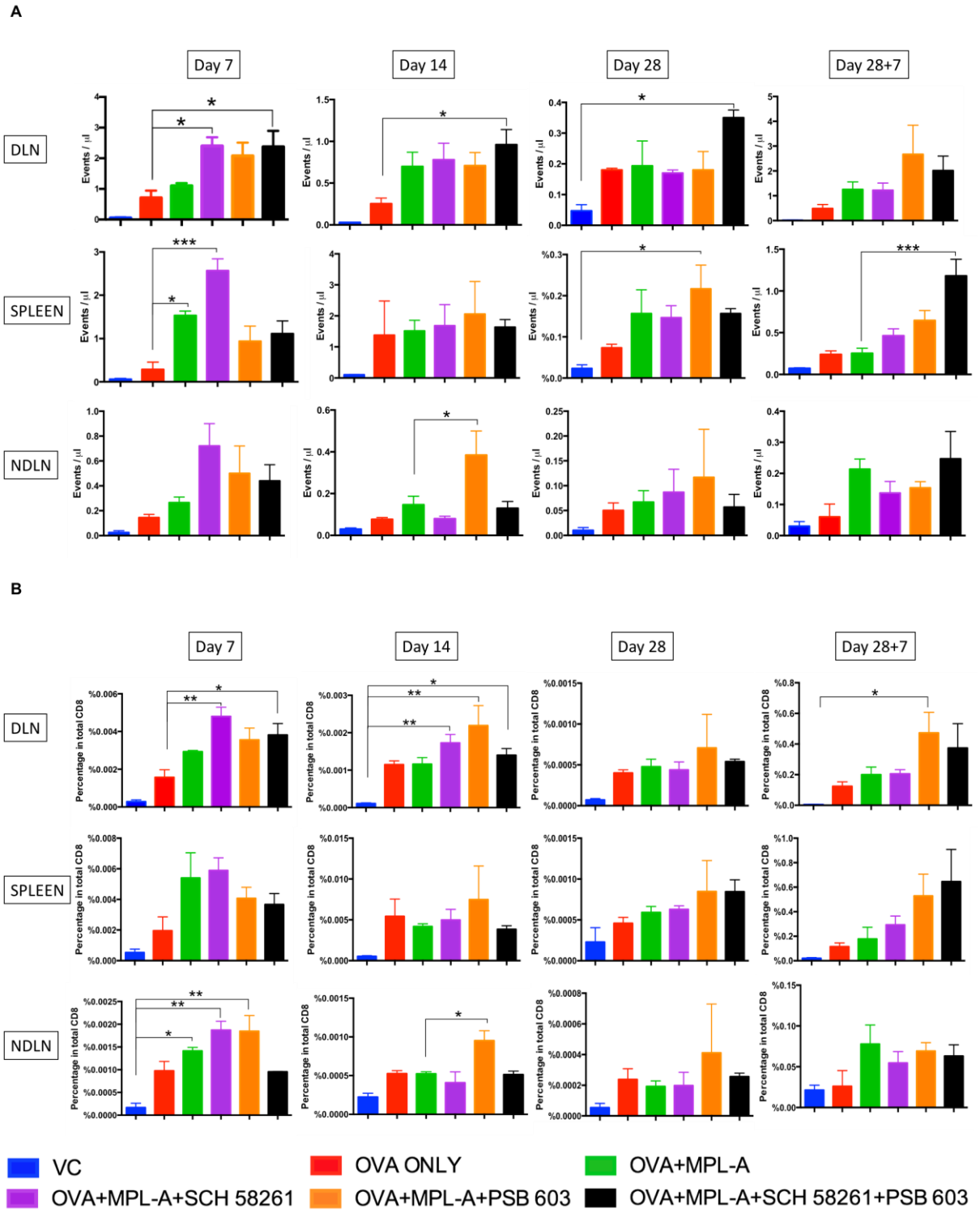


Figure 4.11: Antigen specificity of CD8 T Lymphocytes after Vaccination. ***(A)*** *CD44+ CD8+ Tetramer+ CD8+ T cell events.* ***(B)*** *CD44+ CD8+ Tetramer+ CD8+ T cell percentage in total CD8 T lymphocytes. For statistical analysis, student t test was used. * p<0,05, **p<0,01. 3 mice per group was used and AddaVax was used in all groups including VC.*

4.2.4 Regulatory and Follicular Helper T Lymphocyte Profiles after Immunizations

Other than cytotoxic components of the immune system, helper cells also show great importance. Regulatory T cells and follicular helper T cells are the 2 of them. Follicular helper T Lymphocytes are main cells in activation of humoral immune response and creating a memory against pathogens [27]. Follicular helper T cell differentiation was examined in figure 4.12. Proportions of Tfh cells (CXCR5+PD1+) increased particularly in DLNs. On day 7 all the groups that contain MPLA have even more pronounced increase in the proportions of Tfh population. The same population tends to increase on day 28+7 for the group of mice received A2BR antagonist. Overall these results suggest that adding immunological adjuvant MPLA increased the proportion of Tfh populations. A2A and or A2BR blockade did not further increased the proportion of these cells. Because these are overall Tfh populations, changes in the antigen specific Tfh cells will be monitored using ova peptide specific CD4+ T cells in the future as continuation of this project.

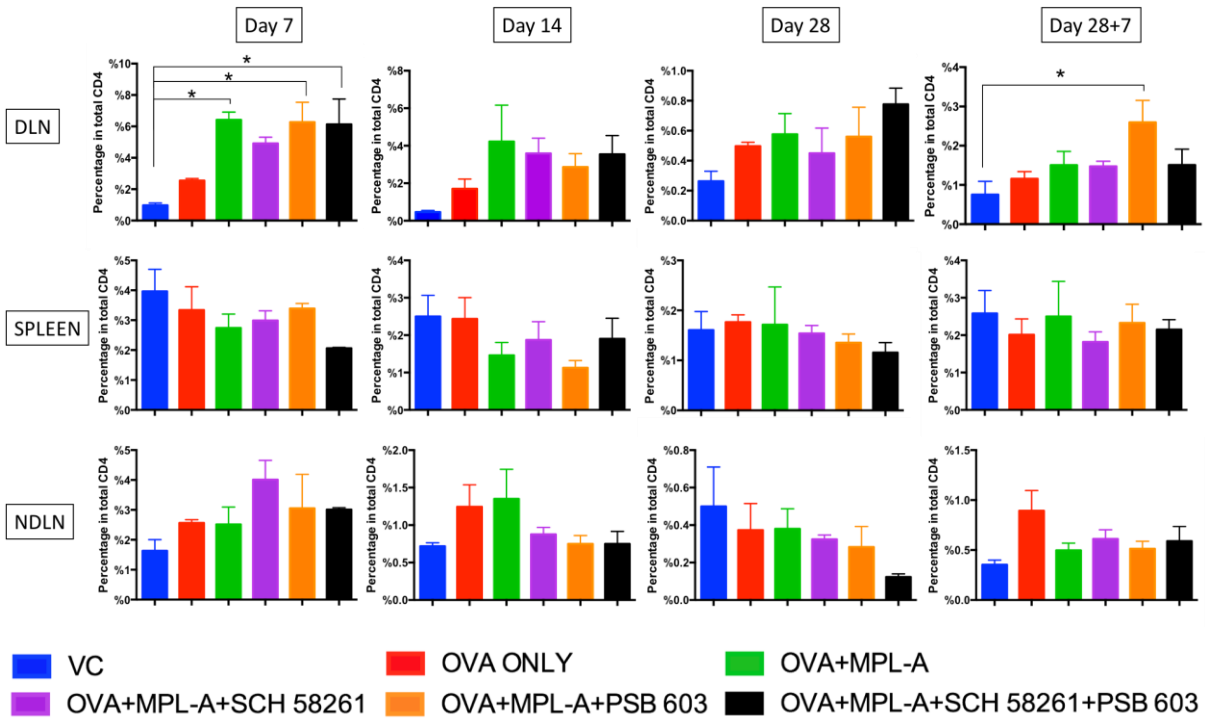


Figure 4.12: Effects of Adjuvants formulations on Tfh Profiles. *CXCR5 and PD1 double positive cell populations were chosen between CD4+ T lymphocytes. Graphs shows the percentage of these populations in total CD4 cells. For statistical analysis, student t test was used. * p<0,05, **p<0,01. 3 mice per group was used and AddaVax was used in all groups including VC.*

Regulatory T cells are another subsets of helper T lymphocytes, which are responsible from immunosuppression to prevent tissue damage due to overactive immune responses [29]. Adenosine receptor stimulation was reported to promote T cell differentiation into a regulatory phenotype [49]. Because regulatory T cells can suppress antigen-specific immune responses and causes immunological tolerance, it is important for vaccine adjuvants not to induce high levels of Treg responses. Therefore, we next evaluated the changes in proportions of T cells with regulatory phenotype. As it can be seen in figure 4.13, adenosine receptor blockade, interestingly, slightly increased the proportion of regulatory T cells on day 14. These results may indicate a potential feedback for increased immune cell activity due to adenosine receptor blockade. Similar to Tfh cells these cells we tested represent the overall regulatory T cell populations. It will be important to test whether antigen specific/induced regulatory T cell numbers will change after A2A and/or A2BR blockade in similar settings.

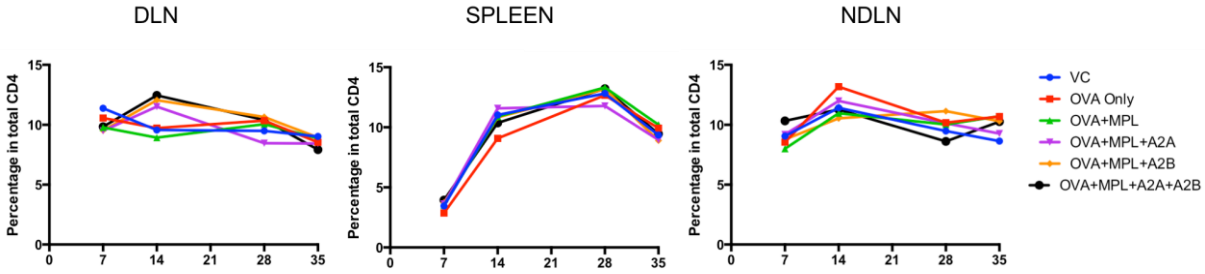


Figure 4.13: Treg Profile after Vaccinations. *FOXP3 and CD25 double positive cell populations were chosen among CD4+ T lymphocytes. Graphs shows the percentage of these populations in total CD4 cells. 3 mice per group was used and AddaVax was used in all groups including VC.*

4.2.5 Effects of Adjuvants on B Lymphocytes and Their Subtypes

T cells are important to create cellular immunity and clear infection. However, to be able to get proper immune response and long term immunity, humoral immune response is also important. B lymphocytes are the main cells to establish proper humoral immunity. The main mechanism B cells use to provide immune protection is secreting antibodies. Therefore, one of the main measures to assess the effectiveness of a vaccine is B cell responses. Using our model, we tested changes in B cell subpopulations as a general overview of the effects of our treatments. First we tested total B cell numbers. (Figure 4.14). In DLNs numbers of total B cells slightly increased after vaccination with ovalbumin on day 7. The groups immunized

with formulations containing A2AR and/or A2BR antagonists along with MPLA are the only groups causing significant increase in total B cell numbers as compared to vehicle group. On day 14 all the groups containing MPLA had significantly higher numbers of B cells. On day 28 only the group that received both A2AR and A2BR antagonists tend to have higher numbers of B cells. On day 28+7 all the groups containing ovalbumin had increased numbers of B cells. In spleen, B cell numbers did not significantly change between the groups after primary immunization. However, when mice were re-challenged by ovalbumin groups containing immunological adjuvants (MPLA alone or along with antagonists) had reduced numbers of B cells. Along with this reduction in B cell numbers similar and concomitant increases in B cell numbers are observed in both DLNs and NDLNs. For NDLNs all immunized groups tend to have more B cell numbers after antigenic re-challenge. There are also significant increases on day 7 especially in the group receiving A2AR antagonists. These results indicate that addition of MPLA strongly increase total B cell numbers in DLNs. This effect is more obvious in groups receiving A2 adenosine receptor antagonists for day 7 and addition of A2AR and A2BR antagonists together prolonged this effect on day 28.

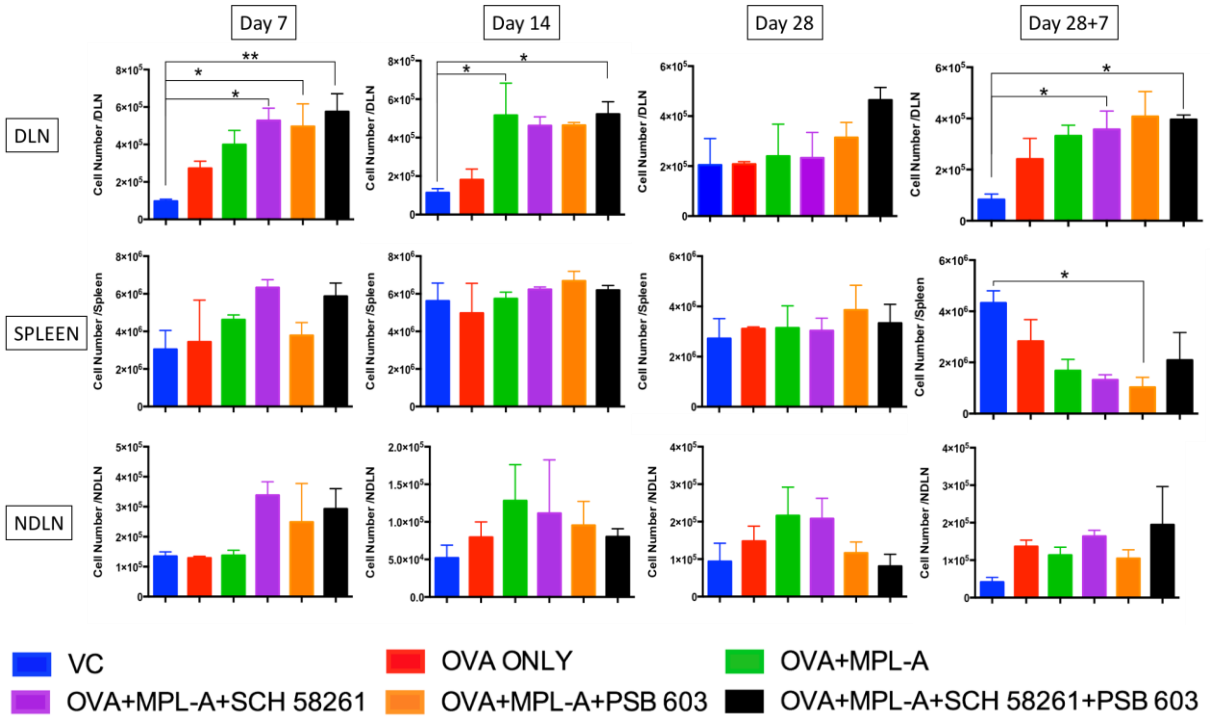
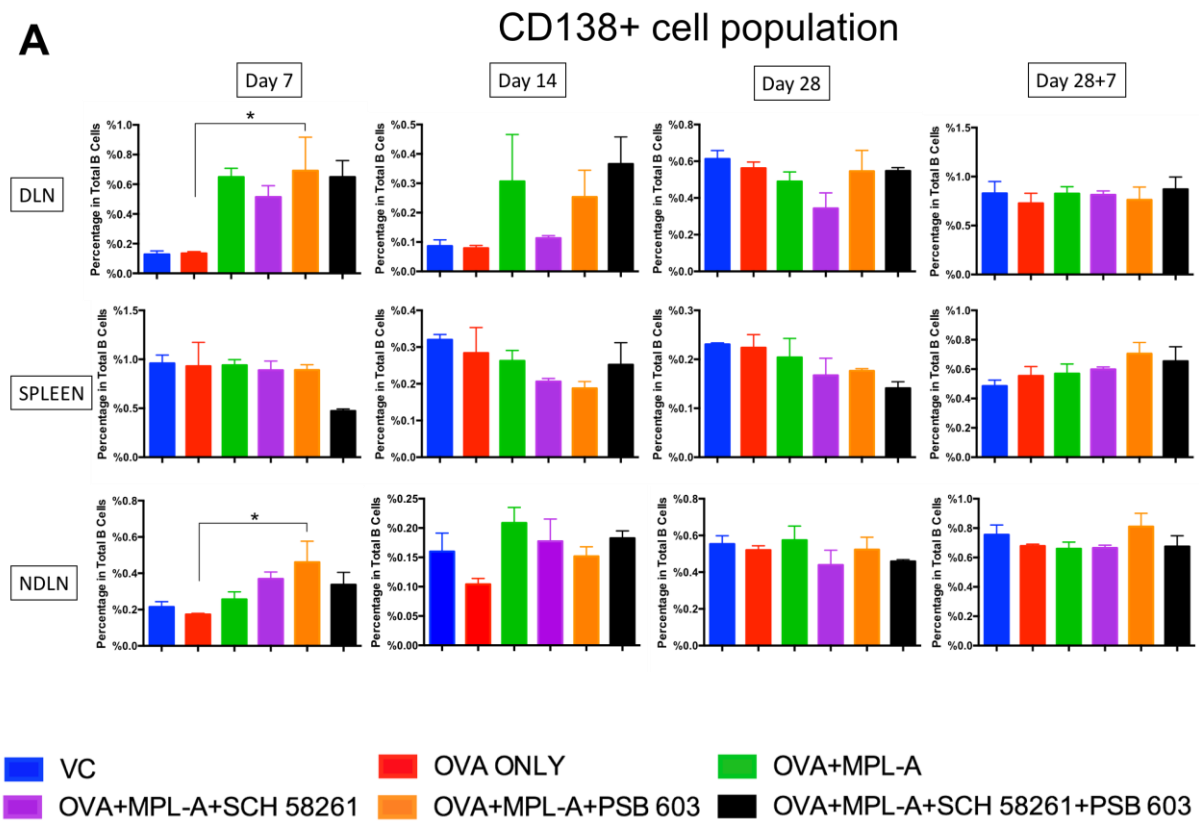


Figure 4.14: Effects of Adjuvants on B Cell Number. *B Cell Numbers from B cell panel. They were gated as CD19+ cells. They were calculated according to read volumes for flow cytometry and total volume of tissue suspensions. For statistical analysis, student t test was used. * p<0,05, **p<0,01. 3 mice per group was used and AddaVax was used in all groups including VC.*

CD138+ plasma cells and Gl7+ cells were important subtypes of B cells. CD138+ cells can be found in circulation and secrete antibodies to clear the infection while Gl7+ cells are related with germinal center formation and acquired memory in B lymphocytes [119, 120]. In DLNs all the groups containing MPLA had higher numbers of CD138+ and GL7+ populations on day 7. However, addition of adenosine receptor antagonists did not further increase their numbers. Same tendency continued on day 14 except for the group which was received A2AR antagonists. Plasma cell proportion was similar between the groups on day 28 and day 28+7. In Spleen and NDLNs plasma cell proportion among B cells did not change between all the treatment groups. Similar to plasma cells, in DLNs proportions of GL7+ cells in total B cell populations increases when MPLA is included in the formulations for day 7. Interestingly, addition of A2AR and A2BR antagonists particularly increased proportions of these cells for day 28 suggesting adenosine receptor blockade may prolong these populations in draining lymph nodes for better memory formation (Figure 4.15).



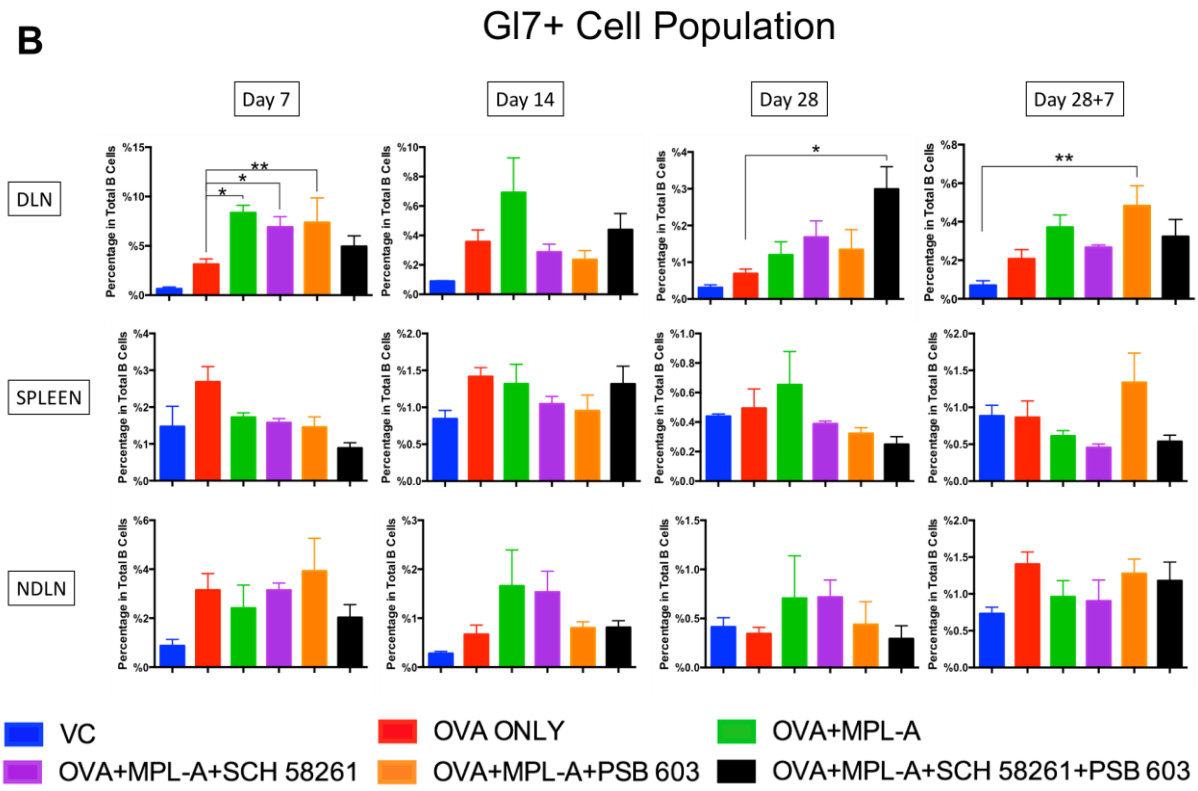


Figure 4.15: Differentiation into CD138+ and GL7+ cells upon vaccination. ***(A)*** *CD138+ cell and* ***(B)*** *Gl7+ cell percentages in total B cell populations. For statistical analysis, student t test was used. * p<0,05, **p<0,01. 3 mice per group was used and AddaVax was used in all groups including VC.*

4.2.6 Class Switching of B cells after Activation with Adjuvants

After their activation, B cells go into class switching process. Every B lymphocyte is a IgM and IgD positive in early developmental progress. However, after their activation, they lose the expression of these types and start to express other subtypes such as IgG [31]. To be able to understand the class switching in B cells, we evaluated the IgM and IgD expression levels on CD19+ CD45+ B cells (Figure 4.16). However, neither A2A nor A2B antagonism or both caused significant changes in the proportions of CD138+ nor Gl7+ cells. On the other hand, A2A and A2B antagonists together, slightly decreased the IgM+ cell percentage among GL7+ cells in DLNs. Decrease in the IgM+ cells may mean increase in class switched, activated B cells. It also supports the idea of more effective humoral responses which also indicates the higher memory formation possibility. Although decrease in IgM percentage shows activated B cell responses, it is not conclusive and other parameters should be checked. There are many other subtypes of immunoglobulins and they all differ in mechanisms. While IgG1 may indicate the allergic responses, IgG2 is more related with acquired immunity.

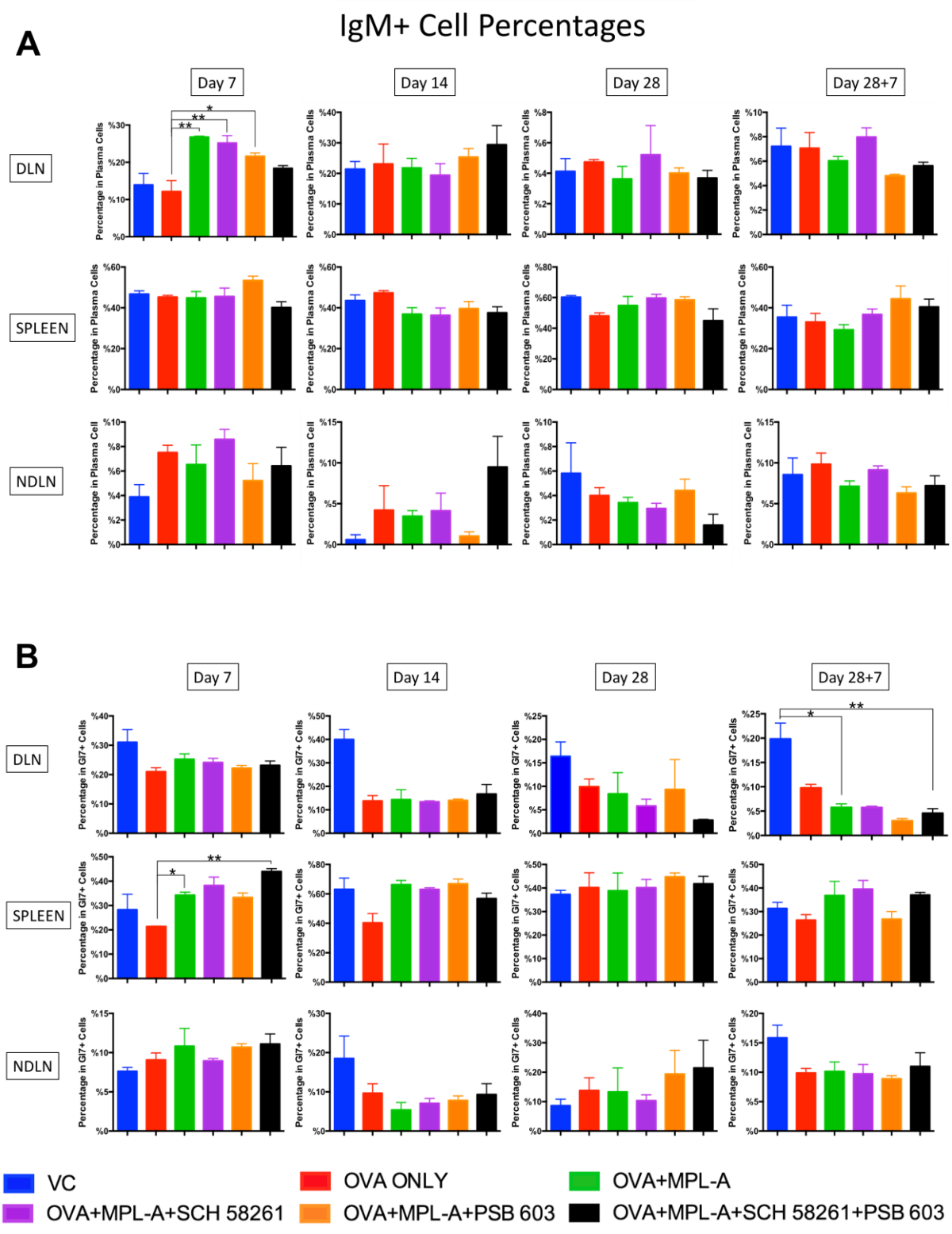


Figure 4.16: IgM+ B Lymphocyte Percentages. ***(A)*** *IgM+ B lymphocyte percentages in CD138+ cells and* ***(B)*** *IgM+ B lymphocyte percentages in Gl7+ cells from 3 different tissues and 4 different time points after vaccination with adjuvant formulations and proper controls. For statistical analysis, student t test was used. * p<0,05, **p<0,01. 3 mice per group was used and AddaVax was used in all groups including VC.*

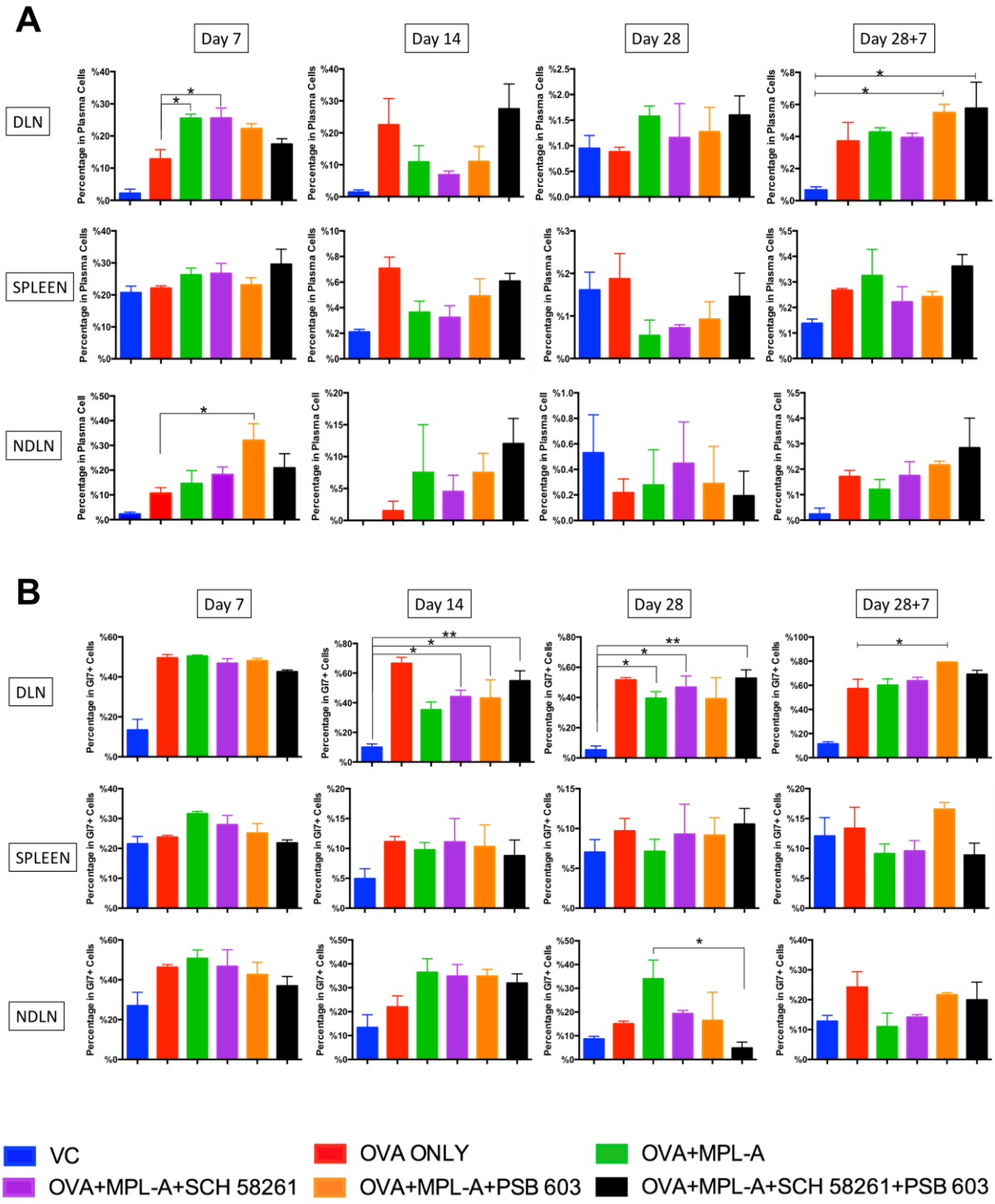


Figure 4.17: IgG1+ B Lymphocyte Percentages. ***(A)*** *IgG1+ B lymphocyte percentages in CD138+ cells and* ***(B)*** *IgG1+ B lymphocyte percentages in Gl7+ cells from 3 different tissues and 4 different time points after vaccination with adjuvant formulations and proper controls. For statistical analysis, student t test was used. * p<0,05, **p<0,01. 3 mice per group was used and AddaVax was used in all groups including VC.*

We next examined the proportions of IgG1+ cells in activated (IgD-) B lymphocytes to be able to gain more insight on their differentiation status. All adjuvant formulations show the increase in IgG1 responses compared to VC. However, there is not significant differences between adjuvant formulations neither in CD138+ cells nor in Gl7+ cells (Figure 4.17) Not having a significant increase in IgG1 responses may be important because it indicates that increased Th2 responses means that protective immunity against intracellular pathogens. As for the CD4+ T cells these tests evaluated overall B cell populations and do not indicate dynamic changes in OVA-specific B cell populations.

4.2.7 Antigen Specific Serum Antibody Responses to a Different Adjuvant Formulations

As in the case of CD4+ T cells, activation or class switching of B cells would not show any conclusive result by themselves. Antigen specificity of these responses should also be evaluated to better evaluate the efficacy of vaccines. Therefore, we analyzed the OVA specific total IgG, IgG1 and IgG2c responses by utilizing and ELISA-based assay to get the differences between specific antibody responses. 5-fold serial dilutions were done 8 times for the analysis of antibody levels.

After primary immunization antibody titers were increased in all groups. Addition of MPLA to the ovalbumin and Addavax drastically increased the titers. A2B antagonist containing shows slightly higher IgG titers on day 14 but other than that, there is no significant changes in total IgG measurements with adenosine receptor antagonist treatment after primary immunizations (Figure 4.18).

After analyzing the ovalbumin specific total IgG responses, we continue with checking the secretion of IgG1 and IgG2c subsets. As indicated before, B cells can secrete many different antibody types and IgG antibodies also can be grouped into mainly 2 which are IgG1 and IgG2c for CL57B/6 mice. Those IgG subsets are secreted for different conditions. IgG1s were secreted for extracellular pathogens whereas IgG2c causes protection against intracellular pathogens.

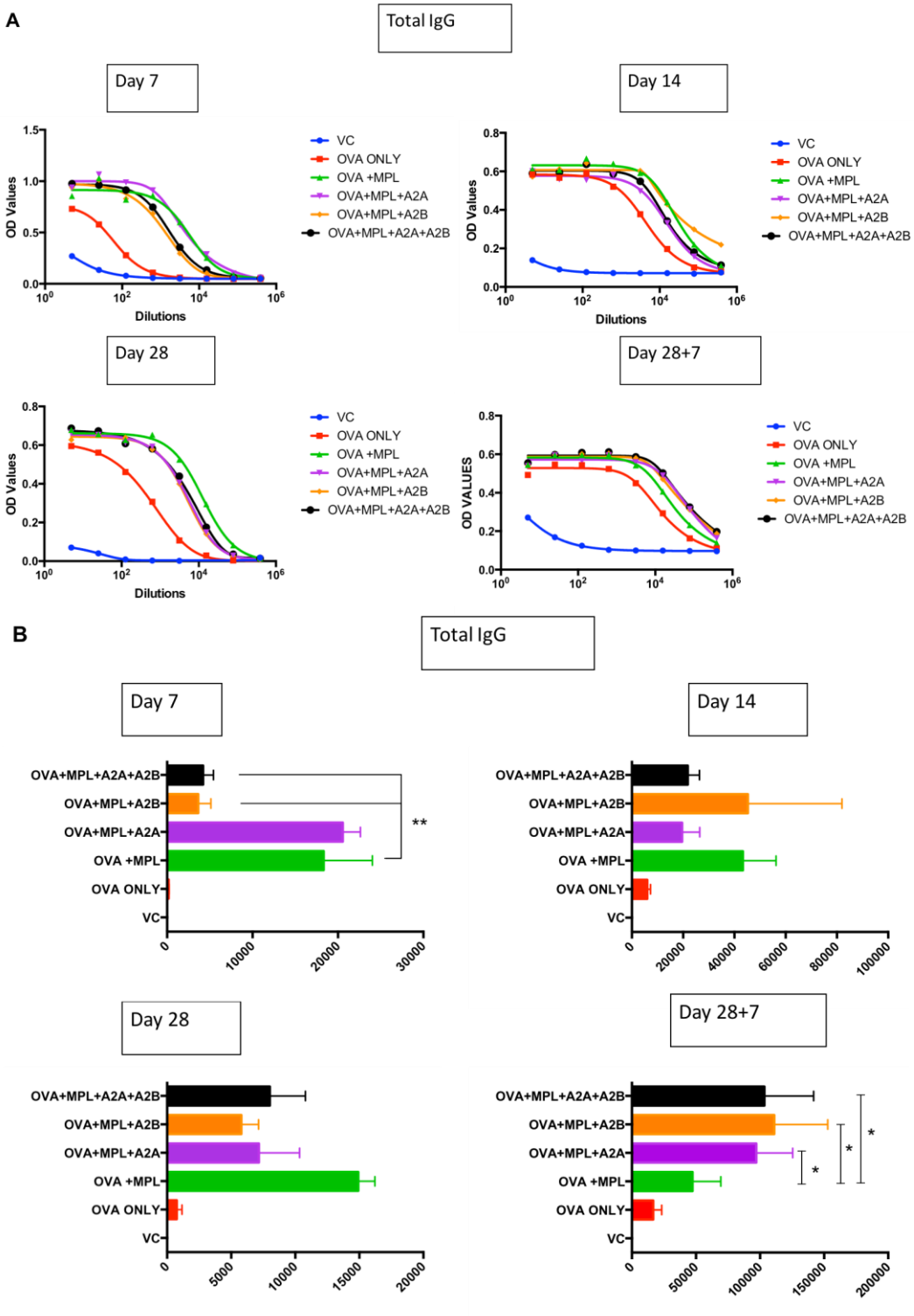


Figure 4.18: Ovalbumin Specific Total IgG Secretions. Total Mouse IgG measurements by antibody ELISA. *(A) OD values calculated from ELISA experiments with respected dilutions were drawn for 7, 14, 28 days after immunization and 7 days after antigen re-challenge (day 35). (B) Respected dilutions were drawn for 0,3 OD value for each sample at indicated time points. 3 mice per group was used and AddaVax was used in all groups including VC. For statistical analysis, student t test was used. * p<0,05, **p<0,01.*

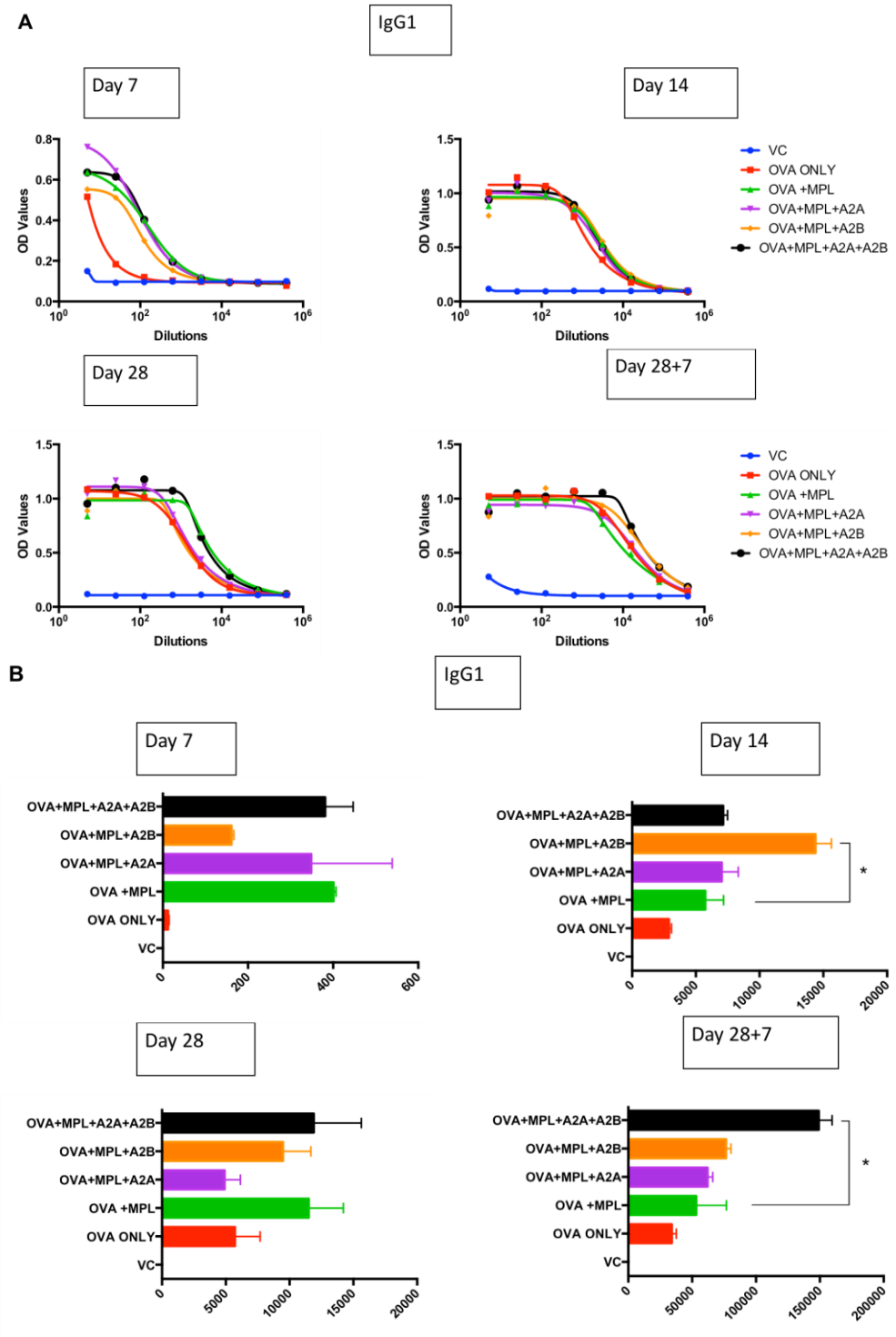


Figure 4.19: Ovalbumin Specific IgG1 Secretions. Mouse IgG1 measurements by antibody ELISA. ***(A)*** *OD values calculated from ELISA experiments with respected dilutions were drawn or 7, 14, 28 days after immunization and 7 days after antigen re-challenge (day 35).* ***(B)*** *Respected dilutions were drawn for 0,3 OD value for each sample at indicated time points. 3 mice per group was used and AddaVax was used in all groups including VC. For statistical analysis, student t test was used. * p<0,05, **p<0,01.*

When we consider IgG1 secretion, it can be seen that all groups taking adjuvant formulations were increases in IgG1 levels on day 7. Also, there is not significant and meaningful increase or decrease in levels after primary immunization. There is only a significant increase on day 14 after immunization with A2B antagonist containing adjuvants. However, after antigen re-challenge, A2A and A2B receptor co-blockade increases the IgG1 titers significantly comparing to only MPL-A and Addavax containing group (Figure 4.19).

We observed a similar trend with total IgG responses when comparing IgG2c titers after primary immunizations for the days 7, 14 and 28. However, groups which had received adenosine receptor antagonists produced more IgGs as compared to all other groups after antigen re-challenge (Figure 4.20).

Differences in IgG 1 and IgG2c titers are important for showing the type of immune responses as well. As indicated in introduction, Th1 and Th2 responses were important in creating potent immune responses. Th1 response causes protection against intracellular pathogens while Th2 responses were against extracellular pathogens. It was also considered that Th1 dominant responses were related with more protective immunity while Th2 dominant responses were related with autoimmunity and allergy. Increase in IgG1 titers were associated with Th2 response that is why ratio of IgG1 and IgG2 is important in evaluating vaccine efficacy. Figure 4.21 shows the ratio of IgG2c over IgG1. Except for day 14, there is an increased IgG2c to IgG1 ratio in all groups containing A2A receptor antagonist. Moreover, after antigen re-challenge, IgG2c/IgG1 ratio in adenosine A2A receptor antagonist containing groups increases. Overall our data suggest that Addition of A2AR antagonists will lead to a Th1 bias in antibody responses while adding A2BR antagonists will increase both IgG1 and IgG2c titers.

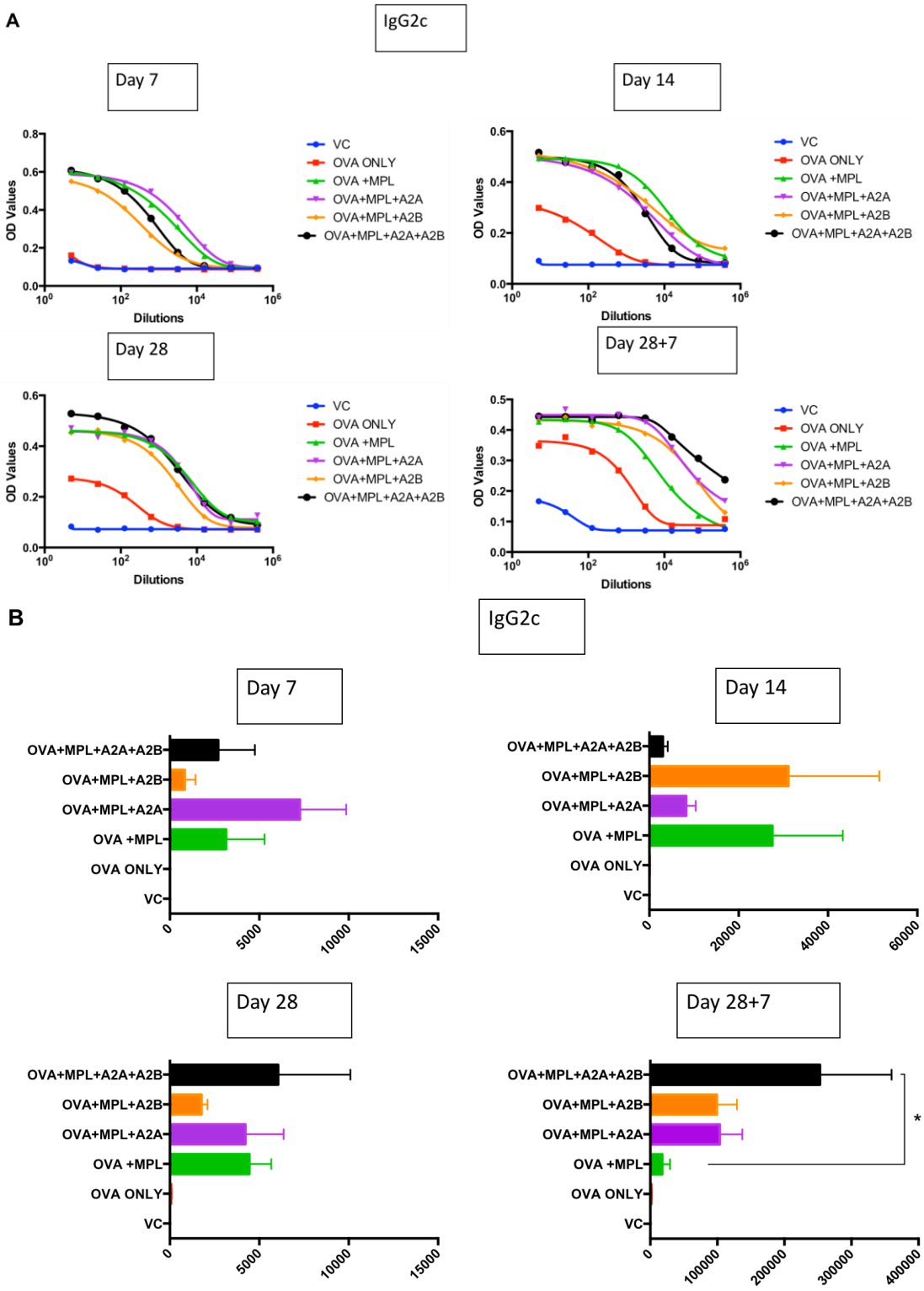


Figure 4.20: Ovalbumin Specific IgG2c Secretions. Mouse IgG2c measurements by antibody ELISA. ***(A)*** *OD values calculated from ELISA experiments with respected dilutions were drawn or 7, 14, 28 days after immunization and 7 days after antigen re-challenge (day 35).* ***(B)*** *Respected dilutions were drawn for 0,3 OD value for each sample at indicated time points. 3 mice per group was used and AddaVax was used in all groups including VC. For statistical analysis, student t test was used. * p<0,05, **p<0,01.*

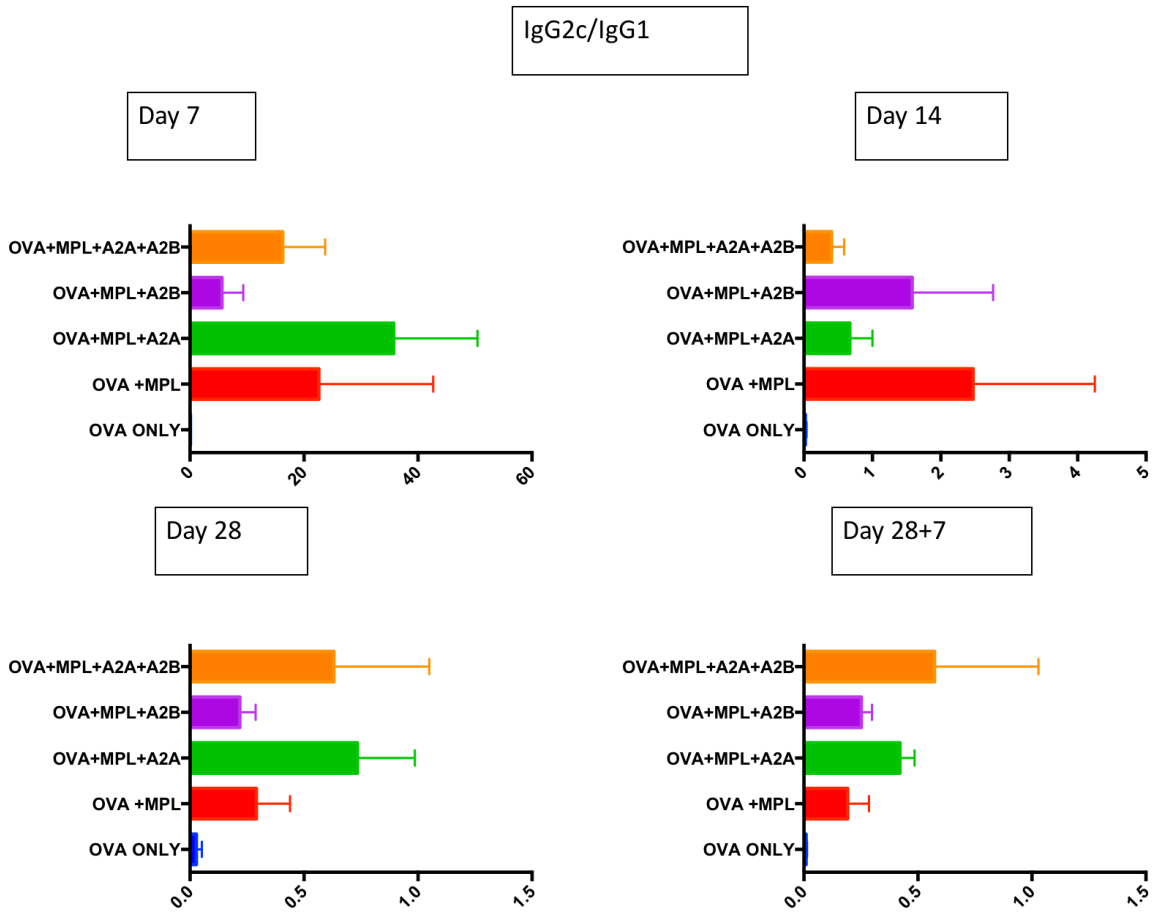


Figure 4.21: Ratio of Ovalbumin Specific IgG2c over IgG1. *3 mice per group was used and AddaVax was used in all groups including VC. For statistical analysis, student t test was used. * p<0,05, **p<0,01.*

Chapter 5

Discussion

Our body uses immune system as a defense against any pathogens or altered-self molecules. Immune defense starts with the physical and chemical barriers and continues with more sophisticated innate and adaptive arms [2]. Understanding the immune system and its regulation also makes this system a useful tool for many processes. We can now use many immune regulatory agents to create immunity in different disease states or in some cases we can use immunosuppressive agents. Vaccine technology is the main area that we can use our knowledge on immune system to extrinsically develop immunity against many different diseases. Although there are many vaccines on the market for different pathogens such as HPV, there is still an ongoing effort to develop vaccines to newly emerging pathogens such as zika virus or to develop vaccines for pathogens attacking directly to immune system such as HIV and tuberculosis [93]. There is also an ongoing effort to make the already existing vaccines more effective with fewer side effects. Therefore, we are in need of more effective vaccine formulations. Adjuvants are the functional units of vaccines, which helps them to create effective immunity [94]. By developing new adjuvant formulations, advancements in vaccines can be done. In this study, our aim was to increase the efficiencies of adjuvants to yield stronger antigen specific immune responses by using different supplementary agents.

Purinergic signaling mechanisms have regulatory roles in many different conditions. As part of purinergic receptors, adenosine receptors and their roles are appreciated more through the years. With advanced research, we gain more insight on their working principles and their effects on the organisms. These receptors affect immune system. They regulate immune responses by inducing a suppressive phenotype [55]. Usage of antagonist molecules shows that response caused by these receptors can be reversed. With this knowledge, we hypothesized that adenosine receptor antagonist usage in adjuvant formulations can enhance their capacity. Many different researches on adenosine receptor antagonist usage against immune response especially in the field of tumor immunotherapy also strengthened this hypothesis [75, 80, 81].

To study our hypothesis, we first demonstrated the suppressive effects of adenosine receptors by in vitro studies. To perform these experiments, we used cell impermeable, stable adenosine analog NECA. We also chose BMDCs and peritoneal macrophages as primary targets since our studies have identified adenosine receptor targeting particularly for these cell types yields enhanced antigen-specific immune responses against syngeneic tumors ([88, 90, 111]). Another reason for choosing these cells comes with their nature. They are primary cell cultures and they give the most similar response to what is observed in vivo. Other reason is their antigen presenting and phagocytic capacities. BMDCs as most important APCs, have a role in stimulating adaptive immune responses while peritoneal macrophages have central role in pathogen clearance by phagocytosis and producing effector cytokines to shape immune responses[2]. Adenosine receptor stimulation with NECA decreased the pro-inflammatory cytokine secretion while increase the anti-inflammatory IL10. NECA also decreased the surface expression of CD86, MHCI and MHCII, which are important in antigen presentation. By this result, we also showed that targeting adenosine receptors may influence antigen presentation. Later, we investigated the effects of signaling through different adenosine receptor subtypes in these cells. Our purpose was to find the most effective adenosine receptor subtype causing the anti-inflammatory phenotype. Different adenosine analogs act via different mechanism and they show different effects in different conditions. A1 and A3 acts via decreasing the cAMP levels however, A2A and A2B acts via increasing these levels. A3 receptor can have a role in neutrophil adhesion but A1 can act as a chemotactic agent [60, 62]. A2A and A2B was shown to create the immunosuppression [49]. Consistent with previous studies, we indeed see that A2A and A2B are the main receptors responsible from anti-inflammatory phenotype because specific A2AR and A2BR agonists could phenocopied

the effects of NECA and because A2AR and A2BR blockade successfully reversed the anti-inflammatory effects of NECA. Reversal of the effect of NECA was more clear when antagonists for both A2AR and A2BR were used together. Normally A2B is the low affinity receptor so it is somewhat surprising for it to have such a central role, however we also showed that TLR stimulatory agents, LPS and MPL-A, significantly increased the expression of A2A and A2B receptors. Because we defined both A2AR and A2BR receptors are involved in adenosine mediated immune suppression in APCs, we decided to use SCH58261 and PSB603 (A2A and A2B antagonists respectively) in our adjuvant formulations. Theophylline was also used in antagonists experiments due to its role as nonselective antagonism and its usage as a FDA approved asthma drug. Theophylline showed similar effects to A2A and A2B antagonist alone but we still choose to continue with the A2A and A2B rather than Theophylline because of their potent effects.

After confirming previous studies showing the central role for both A2AR and A2BR in regulating immune responses in APCs we tested whether targeting them alone or in combination in subunit vaccines will improve the antigen-specific immune responses. In our adjuvant formulations, we choose to use MPL-A and Addavax other than adenosine receptor antagonist. MPL-A is a non-toxic TLR4 agonist and licensed as an adjuvant supplement. Addavax on the other hand is a squalene based oil in water emulsion which can be used as a delivery method with its ability to trap antigens in the site of injection. It can also elicit both cellular and humoral responses. As in vivo experimental design, we choose to immunize mice once and study its effects for a month at 7, 14 and 28 days after the vaccination. After a month, we re-challenged mice with antigen to test the differences between immunological memory formed after immunization. Ovalbumin was chosen as model antigen because it is a well-established model and cell specific response can be analyzed easily with current tools.

In the first month after the immunization there was not significant changes in activation or number of the general CD4+ or CD8+ T lymphocyte responses compared to positive control mice on the contrary to our hypothesis however we see a slight increase in activation status of T Lymphocytes consistently in A2A and A2B combined formulation. They also show similar effects in regulatory T cell responses which is a positive result showing that immune systems of these mice do not try to suppress response from these antagonists. We also examined the Tfh cell percentages. Our results indicated that A2B alone or combinations of A2A and A2B showed slightly higher Tfh percentages in 7 days and 28 days after immunizations which

indicates their roles in memory formation. When we consider the B lymphocyte responses in the first month after immunization, there is also not significant changes in the endogenous total B cell population. However, there are increases in the Gl7+ cell to CD138+ cell ratio in A2A and A2B antagonist combined group. GL7+ shows the germinal center cell formation while CD138+ cells show the plasma cells. We can understand that combined A2A and A2B antagonist usage can increase the Germinal Center formation relative to plasma cells. This result also indicates the memory formation against antigen by humoral immune system cells.

When we studied the antigen specific responses after immunization, we observed more clear differences in groups immunized wit formulations containing A2AR and/or A2BR antagonists. There were more OVA specific cell formation after antagonist addition to adjuvants. This effect can be seen even after 7 days after immunization, especially in DLNs. Although we do not see significant increase in the active cell populations, there is increase in the antigen specific cell percentage in the active CD8+ T lymphocytes, which can be considered more important in measurement of antigen specific immunity.

Even though there were not significant and conclusive changes in the first month after immunization, after antigen re-challenge we can see some important changes. Tetramer specific CTL numbers and percentage reached its peak in all the tissue samples in the group received antagonists for both A2AR and A2BR. Spleen here is an important parameter because it shows the systemic protection against antigen. We also observed an profound increase in antigen specific serum IgG2c levels in mice received antagonists for both A2 receptor subtypes as compared to a conservative increase in total IgG levels. However, only addition of A2AR antagonist could lead to increased IgG2c to IgG1 ratio suggesting A2AR antagonists in vaccine formulations can be used to improve Th1 bias and more protective immune responses for intracellular pathogens. Although A2BR is considered low-affinity adenosine receptor our results indicated A2BR blockade increased overall antibody responses. This may indicate that A2-AR blockade can be a novel strategy to design new vaccine formulation which are effective but potentially safe due to the fact that caffeine, non-specific blockers of adenosine receptors have been used by humans for centuries.

Because we observed a more clear and significant effects for A2-AR blockade after re-challenge, blocking these receptors may particularly increases the memory formation. All the effects were even more visible when both A2A and A2B antagonists were used in adjuvants.

Primary immune responses were moderately improved after A2-AR blockade. This might be due to a couple reasons with the most important one being the half-life of these molecules. Adenosine antagonists have short half-lives. Many studies showing the effects of adenosine receptor blockade after repetitive injections[73, 75, 80, 81]. One time injection may not be enough to cause more significant results. More repetitive injections can also be tested or antagonists with longer half-life can be chosen to further improve the effect of these antagonists. Also these compounds can be incorporated in controlled delivery systems such as nanoparticles to prevent the need for repetitive injections and also to better substantiate their effects as these systems may also create a more sustained and controlled release rather than fast clearance after every injection. To sum up, despite their short half-lives, immunizations with only one time with these antagonists showed that they are valuable candidates for increasing the antigen specific immune responses and memory cell formation.

There are many different vaccines, which are in need for repetitive vaccination such as Hepatitis B. One of the adjuvants used in Hepatitis B vaccines is AS04 which consists of MPL-A and MF59 similar to Addavax and MPL-A case as in this study [121]. It might be possible that adenosine antagonists can be used in this type of vaccines to decrease the repetitive vaccinations required to induce memory as we see after antigen re-challenge. However, more studies are needed to conclude the effects of adenosine receptor antagonists' effects on vaccine efficacy. First of all, with more mice, these results should be repeated to get more conclusive results. There is also need for a more sophisticated study with disease models to show that these formulations will provide a better protective immunity.

Most outstanding disease model for adenosine antagonist usage seem cancer because, in tumor microenvironment, adenosine concentrations increase drastically due to increased cell death and hypoxic environment and the suppressive effects can be clearly seen. Previous studies also show that, adenosine receptor antagonists can be used as a therapeutic agent in regular intervals such as daily or 3 times a week, inhibits the tumor growth and metastasis [88, 90]. Because we showed that adenosine receptor targeting can increase antigen-specific immunity in vaccine setting, these formulations can be tested against cancer in addition to infectious diseases.

As future perspective, we aim to study more antigen specific response in CD4+ T lymphocytes and test antigen specific B-lymphocytes and their polarization to better

understand overall effects of this approach with broader perspective. We also plan to investigate the toxicity of these antagonists as well to evaluate their potential in clinical use and finally, to evaluate in disease models such as cancer and infectious diseases.

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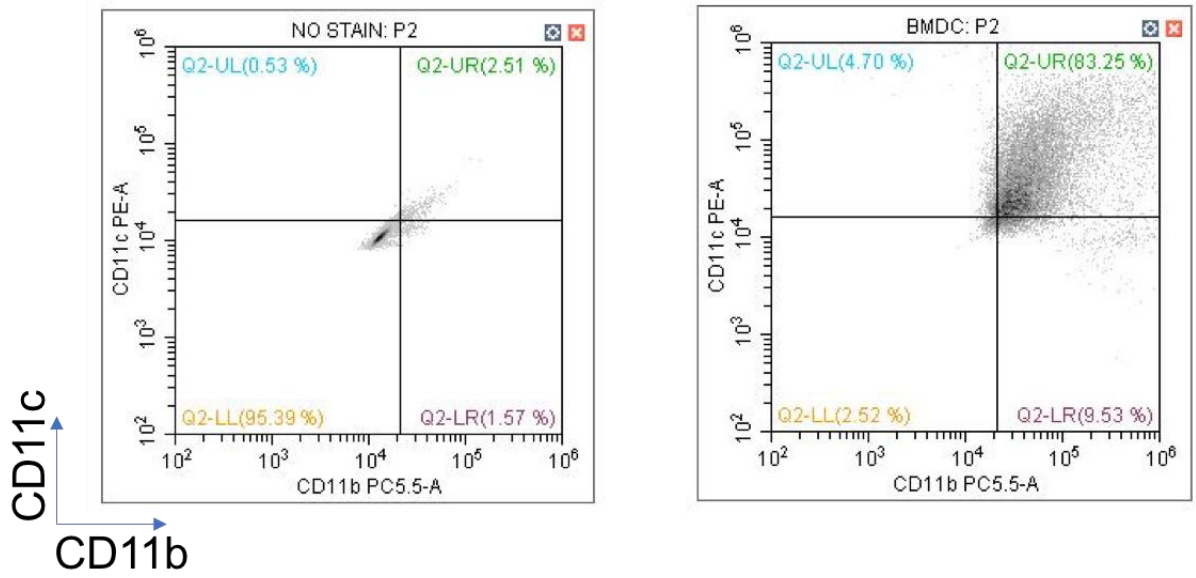
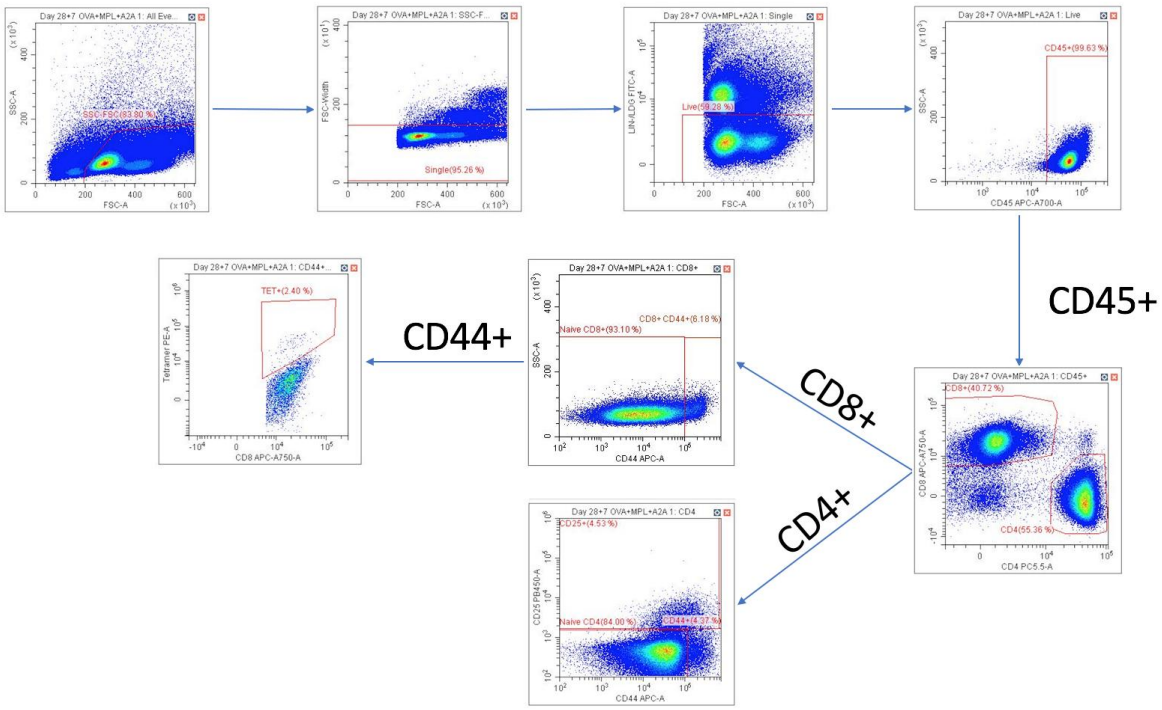
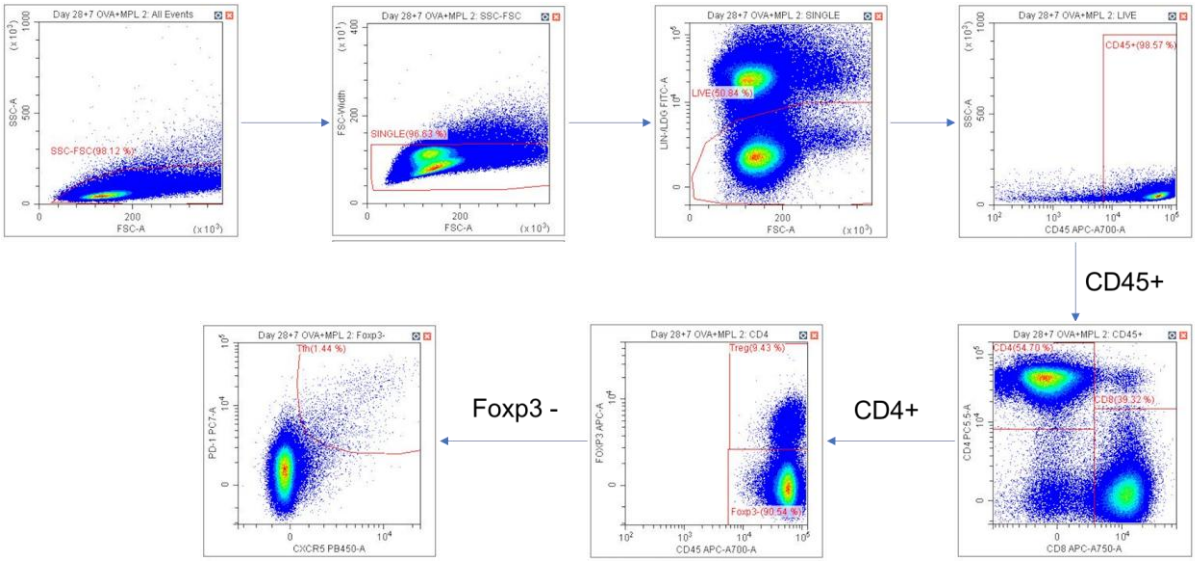
APPENDIX



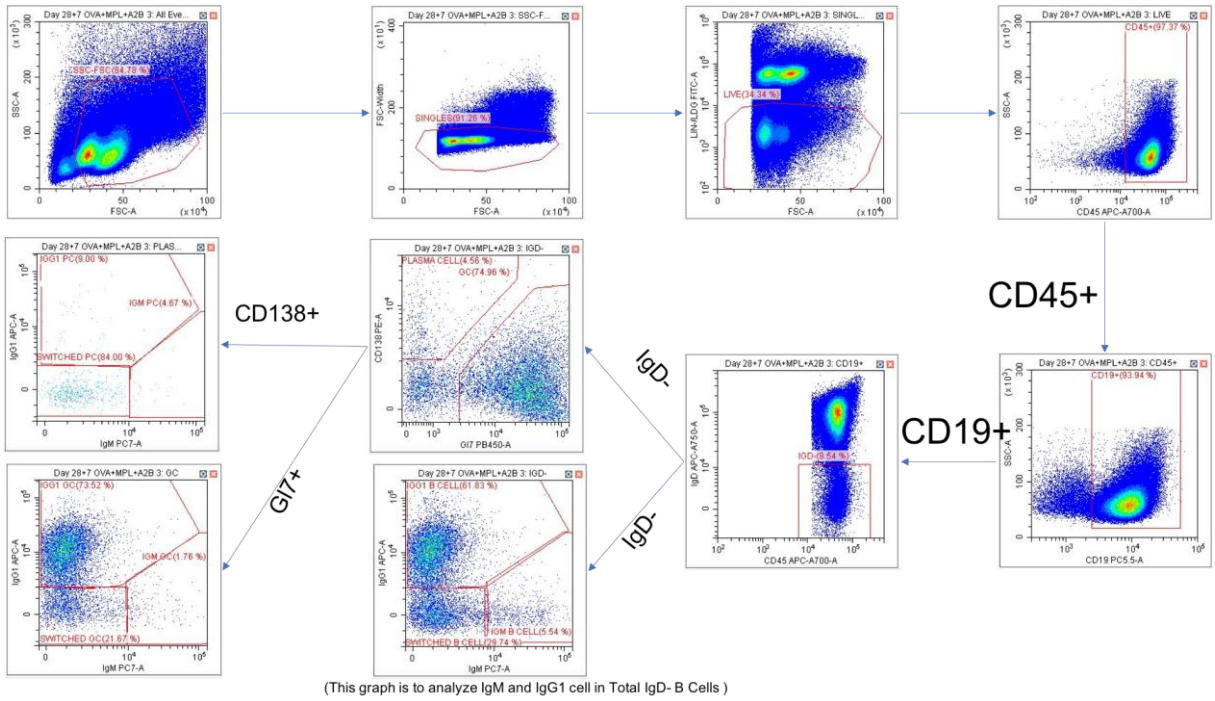
Appendix Figure 1: Quality Control for Bone Marrow Derived Dendritic Cells. *After collection of BMDCs, their quality was assessed by using CD11b and CD11c markers. With flow cytometry, these samples were stained as indicated in the methods chapter and their positivity were determined according to no stain control.*



Appendix Figure 2: Gating Strategy for Tetramer Panel. *First desired cell population were chosen from FSC vs SSC graph. Than cell duplets and triplets were eliminated with FSC-Width vs FSC-A Graph. Then Dead cells and Lineage negative cells were eliminated by FITC graph. As lineage negative markers, CD11b, NK1.1, and CD19 were used. After that, CD45+ events were gated then CD4 and CD8 cells were chosen. Both subsets were gated as CD44+ or CD44- and finally CD44+ cells were analyzed according their tetramer positivity.*



Appendix Figure 3: Gating Strategy for Tregs and Tfh. *First desired cell population were chosen from FSC vs SSC graph. Than cell duplets and triplets were eliminated with FSC-Width vs FSC-A Graph. Then Dead cells and Lineage negative cells were eliminated by FITC graph. As lineage negative markers, CD11b, NK1.1, and CD19 were used. After that, CD45+ events were gated then CD4 and CD8 cells were chosen. CD4+ events were gated according to FOXP3 marker. Finally, Foxp3- ones were separated to PD-1 and CXCR5 double positives.*



Appendix Figure 4: Gating Strategy for Tetramer Panel. *First desired cell population were chosen from FSC vs SSC graph. Than cell duplets and triplets were eliminated with FSC-Width vs FSC-A Graph. Then Dead cells and Lineage negative cells were eliminated by FITC graph. As lineage negative markers, CD11b, NK1.1, and CD4 and CD8 were used. After that, CD45+ events were gated then CD19+ cells were chosen as B cell population. Then IgD- CD19+ cells were separated. From These events, CD138+ and Gl7+ cells were separated and they each further separated into IgM+ and IgG1+ subsets.*

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