Role of B cells as antigen presenting cells after passive uptake of plasmid DNA

By

Ichwaku Rastogi

A dissertation submitted in partial fulfilment of

the requirements for the degree of

Doctor of Philosophy

(Cancer Biology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2022

Date of final oral examination: 06/22/2022

The dissertation is approved by the following members of the Final Oral Committee:

Douglas McNeel, Professor, Cancer Biology
Anna Huttenlocher, Professor, Cancer Biology
Jacques Galipeau, Professor, Oncology
Paul Lambert, Professor, Cancer Biology
Paul Sondel, Professor, Human Oncology

Abstract

Role of B cells as antigen-presenting cells after passive uptake of plasmid DNA

Ichwaku Rastogi

Under the supervision of Professor, Douglas G. McNeel

at the University of Wisconsin-Madison

Immunotherapy is the most recent advancement for the treatment of cancer patients. It is a clever approach as most immunotherapy-based treatments function by educating the immune system to identify tumor cells and target them specifically for cell death. Use of DNA vaccines is one such therapeutic approach, where a tumor antigen is encoded in plasmid DNA and delivered to cancer patients via injections. Upon vaccination, the DNA vaccine gets processed and presented by antigen-presenting cells to generate tumor-antigen-specific CD8 T cells. Recent pre-clinical and murine model studies have demonstrated promising results from the use of DNA vaccines. However, most clinical trials that evaluated anti-tumor efficacy of DNA vaccines have reported a limited response in cancer patients. One of the reasons for this subpar immunogenicity could be that upon delivery, plasmid DNA is processed by the bystander cells such as dermal cells and myocytes instead of professional antigen presenting cells such as B cells, dendritic cells (DCs) and macrophages. Antigen processing through non-professional antigen-presenting cells can result in lower production of the antigen, ultimately leading to poor cross-priming of CD8 T cells. To address this, we wished to evaluate if direct processing of DNA vaccines by professional APCs

could augment the immunogenicity of DNA vaccines. We had previously reported that upon passive uptake of plasmid DNA B cells, but not DCs or macrophages could transcribe the encoded antigen. We further reported that B cells captured DNA by micropinocytosis and translocated it to the nucleus, whereas DCs and macrophages captured DNA by phagocytosis, after which it underwent endosomal degradation. In this report, we evaluated the mechanism of antigen presentation of plasmid DNA by B cells and evaluated the phenotypes of CD8 T cells that could result upon antigen-priming through B cells and compared to that of DCs. We utilized plasmid DNA encoding ovalbumin as a model for antigen presentation studies. Utilizing B cells and DCs isolated from C57Bl/6 mouse splenocytes, and CD8 T cells from OT1 (ovalbumin specific) mouse splenocytes, we demonstrated that a co-culture of B cells and DCs was required for activation of antigen-specific CD8 T cells. Neither B cells, nor DCs alone were capable of processing plasmid DNA and generating an antigen-specific CD8 T cell activation. Furthermore, by using B cells and DCs from MHC I knockout mouse spleen, and by using re-purified B cells and DCs following the co-culture, we demonstrated that B cells were the primary antigen presenting cells for plasmid DNA upon passive uptake. However, B cells required licensing through DCs, which most likely occurred through cell-cell interaction(s) between B cells and DCs during the co-culture, as demonstrated by using trans-well plates. Lastly, from RNA-seq analysis of DNA-loaded B cells that had either been cultured alone or in presence of DCs we demonstrated that after interaction with DCs, B cells had dramatic changes in their gene expression patterns. DNA-loaded B cells that had been co-cultured with DCs represented an activated phenotype which is supportive of antigen presentation. GSEA analysis indicated that these B cells had similar gene expression signatures to TLR7/8 activated and/or BCR stimulated B cells. The exact interactions between B cells and DCs

are currently unknown, but there are several possibilities, and their identification is one of our future directions. To directly evaluate the phenotype and efficacy of CD8 T cells that resulted from activation through B cells, in comparison to DCs, we utilized SIINFEKL peptide (ovalbumin specific) loaded B cells and DCs in their mature (by LPS) and immature states. We demonstrated that CD8 T cells that have been primed through peptide loaded immature B cells and immature DCs, resulted in similar activation, exhaustion, and cytotoxic profiles. Whereas LPS-matured B cells and LPS-matured DCs resulted in different phenotype of CD8 T cells. Mature DCs resulted in increased activation of CD8 T cells, whereas matured B cells resulted in activated yet non-responsive CD8 T cells (anergic). Additionally, in tumor bearing mice we demonstrated that adoptive transfer of CD8 T cells primed through either immature B cells, mature DCs or immature DCs resulted in similar anti-tumor efficacy. This suggested that B cells and DCs both were similarly capable of activating CD8 T cells. Taken together our data warrants additional investigations into understanding the function of B cells as APCs, specifically after passive uptake of plasmid DNA. A deeper analysis of DNA-loaded B cells that had been licensed through DCs is required and necessary for development of B cell-centered therapeutic strategies. We also explored options for delivery of DNA plasmid, which involved either targeting of plasmid DNA directly to B cells or required *ex vivo* generation of DNA-loaded autologous B cells for therapeutic use. Each of these methods demonstrated challenges and raised concerns that still remain unresolved. These strategies are still under development; however, we believe use of DNA-loaded B cells as immunotherapy would demonstrate to be the most efficient approach for eliciting DNA vaccine-mediated immune response in murine models and humans.

Dedication

This thesis is dedicated in memory of my paternal grandfather, Mr. Banshi Dhar Rastogi

I want to dedicate this thesis to my grandmother Mrs. Sarojini Rastogi and my parents, Mr. Rajiv Rastogi and Mrs. Jyoti Rastogi, who have always promoted in me curiosity and a desire to learn. Their guidance, care, love, and unconditional support is the primary reason for any and all of my accomplishments.

Then, to all my teachers who have nurtured me over several years and made me capable enough to achieve the highest level of academic qualification. Of special mention, Mr. A. N. Chidambaram, for instigating the love of biological research in me during my bachelor's degree and Dr. Neelu Puri, for enriching my technical skills and encouraging critical thinking during my master's degree.

Next, I dedicate this work, to all my family and friends, who are irreplaceable and without their support through good and tough times, this work would never have been possible. I want to give a special mention to my wife, Mrs. Aishwarya Raghuwanshi, who always inspired me towards progress with positivity and confidence. Her love, care and affection have always bolstered my self-believe and kept me going.

I also want to thank Dr. Douglas McNeel for his valuable guidance and innumerable efforts in shaping me as a scientist. Through my time during this thesis, he has been my friend and family away from home. Best boss ever!!

Acknowledgements

Graduate Research Advisor

Douglas G. McNeel, MD PhD

Thesis Committee Members

Jacques Galipeau, MD

Anna Huttenlocher, MD

Paul Lambert, PhD

Paul Sondel, MD PhD

Lab Members

Laura Johnson, PhD

Hemanth Potluri, PhD

Donghwan Jeon

Anusha Muralidhar

Jena Moseman

Ellen Wargowski

Melissa Gamat-Huber, PhD

Chris Zahm, PhD

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List of Abbreviations

APC – Antigen-presenting cells

ANOVA – Analysis of Variance

BAFF – B cell-activating factor

BCR – B cell receptor

Breg – B regulatory cells

Btk - Bruton's tyrosine kinase

CD40-B cells - CD40-activated B cells

CD40-KO – CD40 Knock-out

CFSE - Carboxy-Fluorescein Succinimidyl Ester

DC – Dendritic cells

GSEA – Gene Set Enrichment Analysis

HSC - Hematopoietic stem cells

ICOS – Inducible co-stimulator

ICOSL – Inducible co-stimulator ligand

iNKT – invariant Natural killer T cells

MHC – Major histocompatibility complex

MHCI-KO – MHC I Knock-out

ODN - Oligodeoxynucleotides

PAMP - Pathogen Associated Molecular Patterns

SCID - Severe Combined Immunodeficiency

TIL – Tumor-infiltrating lymphocytes

TIL-B – Tumor-infiltrating B cells

TLR – Toll-like receptors

TLS – Tertiary lymphoid structures

Treg – T regulatory cells

Chapter 1

Introduction on B cells, different types of B cells, antigen presentation and known mechanisms of antigen presentation by B cells

Most of the work in this chapter has been prepared as review article and has been submitted for publication in the journal "Frontiers in Immunology." Anusha Muralidhar, Jena Moseman, Donghwan Jeon, Hemanth Potluri and Dr. Douglas McNeel contributed towards writing and editing of this chapter.

Abstract

B cells have been long studied for their role and function in the humoral immune system. Apart from generating antibodies and an antibody-mediated memory response against pathogens, B cells are also capable of generating cell-mediated immunity. It has been demonstrated by several groups that B cells can activate antigen-specific CD4 and CD8 T cells and can have regulatory and cytotoxic effects. The function of B cells as professional antigen presenting cells (APCs) to activate T cells has been largely understudied. This, however, requires attention as several recent reports have demonstrated the importance of B cells within the tumor microenvironment, and B cells are increasingly being evaluated as cellular therapies. Antigen presentation through B cells can be through antigen-specific (B cell receptor (BCR) dependent) or antigen non-specific (BCR independent) mechanisms and can be modulated by a variety of intrinsic and external factors. In this chapter, we will discuss the pathways and mechanisms by which B cells present antigens, and how B cells differ from other professional APCs.

Introduction

B cells, commonly considered as antibody factories, are best known for their contribution to humoral immunity. They were first identified and described by Max Cooper in the 1960s, when he demonstrated that an irradiated chicken completely loses its ability to generate antibodies after removal of the Bursa of Fabricus, the primary site for B cell development in birds (1). Ever since, researchers have continued to explore the biology of B cells, with a focus on their role in antibody production. In humans, B cells originate and develop in the bone marrow and undergo selection and maturation in secondary lymphoid organs, predominantly in the spleen (2). B cells can be identified by the presence of a B cell receptor (BCR) on their surface, which also defines the antigen specificity of the cell. However, B cells also express both major histocompatibility complex (MHC) I and MHC II and are equipped with all the machinery required for antigen uptake, processing, and presentation. Hence, they are also classified as professional APCs, like dendritic cells (DC), monocytes and macrophages.

Recent studies have demonstrated that B cells can affect cancer progression. The role of B cells in cancer is complex, with some reports demonstrating pro-tumorigenic behavior for B cells and others showing enhancement of anti-tumor responses. Studies in murine models, in particular, have shown that B cell knockout mice exhibit better tumor control, that B cells can cause increased angiogenesis, and that B cells can cause circulating immune complex deposition which favors carcinogenesis (3-5). Furthermore, an IL-10-producing subset of B cells, known as B regulatory cells (Bregs), has been identified in both murine and human cancers. Bregs have been shown to be capable of converting conventional CD4+ T cells to T regulatory cells (Tregs) and have been associated with reduced survival in humans (6-8).

Contrarily, recent evidence supports the beneficial role of B cells in cancer immunotherapy. Studies have found that the presence of infiltrating B cells are associated with favorable outcomes across multiple human cancers (9, 10). Intratumoral B cells are frequently found within organized structures analogous to those found in lymphoid organs, which are called tertiary lymphoid structures (TLS). Greater number of TLS, or expression of TLS-related genes, has been additionally associated with increased survival in patients (11-13). Cabrita and colleagues showed that B cells within TLS have high expression of MHC I and MHC II, suggesting that these B cells are capable of presenting antigens (11). Several groups have also shown that the presence of TLS is associated with response to immune checkpoint blockade (11, 13, 14). Bruno et al., showed that infiltrating B cells from human non-small cell lung cancer (NSCLC) tumors were able to present to and activate CD4+ T cells in the presence of human tumor antigens. In addition, some patients had tumor infiltrating B cells (TIL-Bs) that could activate CD4+ tumor infiltrating lymphocytes (TILs) without exogenous antigen. When B cells from patients with NSCLC were co-cultured with CD4+ T cells, the CD4+ T cells took on a Th1 phenotype, whereas B cells with exhaustion markers yielded CD4+ T cells with a regulatory T cell phenotype (15). Taken together, these data suggest that activated B cells within TLS may act as APCs and elicit a recall response among T cells that were primed in lymph nodes, promoting their survival and proliferation, and allowing them to respond better to checkpoint blockade. This makes the function of B cells as APCs a potentially important and understudied process for cancer immunotherapy.

Antigen Presentation

Generally, antigen presentation is how the immune system generates a specific immunogenic response against a pathogen or cancer-associated tumor antigen. Typically, pathogenic/tumor specific proteins are digested by the immunoproteasomes and the resulting antigenic peptides are then loaded onto MHC molecules. They are transported to the APC cell surface for interaction with T cell receptors on T cells (16-18). This process varies widely depending upon the pathogen/tumor type and the cell type that is processing the antigenic protein. All nucleated cells are APCs, but only a few subsets are professional APCs, as they specialize in priming and expanding antigen-specific T cells (19). Another major difference between professional and non-professional APC is that MHC type II complexes are exclusively expressed on the surface of professional APCs along with MHC type I, whereas non-professional APCs only express MHC type I. Professional APCs include dendritic cells (DCs), monocytes, macrophages, and B cells, whereas all other cell types that express MHC I molecules are considered non-professional APCs (20-22). Generally, T cells are tolerant to interactions with self-antigen, as they undergo negative selection during their development in which most self-reactive high-affinity T cells undergo apoptosis. However, in the case of interaction with a foreign antigen, T cells get activated and can undergo clonal expansion to generate an adaptive immune response (23-25).

Antigens can either be directly presented or cross-presented depending upon the cell types that are involved in the process. Direct presentation is when a professional APC encounters and processes a pathogen to generate antigens and interact with T lymphocytes (26). On the other hand, if the antigen being presented is acquired, but not generated by a professional APC itself, then this is called cross-presentation (27). There are several ways of cross presentation of an antigen, one of

the most common mechanisms being internalization of free-floating antigen from the extracellular matrix, immunoproteasome processing, and then loading onto MHC I molecules for presentation (28). Other mechanisms include acquisition of pre-processed antigen from non-professional APC, which can happen either by cell-cell interaction or by cytosolic secretion of peptides. Another mechanism is by internalization of peptide-loaded MHC molecules from infected or apoptotic cells in the vicinity of a professional APC, a process also known as cross-dressing (29). Most of the cross presentation in humans is accredited to DCs (28), which survey their surroundings and capture any foreign protein/antigen from the peripheral tissue to cross present. Cross presentation is crucial in certain cases, such as viral infections that do not infect professional APCs, or tumors of non-hematopoietic origin. DCs are best suited for this task as they have been shown to have less lysosomal protease activity compared to other professional APCs (30). This results in slower degradation of the antigen and provide a safe storage space (endosomal compartment) that provides increased stability and half-life for the antigens (31).

Like DCs, B cells also participate in antigen presentation, and this can occur through direct or cross presentation. Antigenic peptide-loaded B cells interact with CD4+ as well as CD8+ T cells, leading to their activation and resulting in Th1 and Th2 type immune responses (32-34). B cells have also been shown to cross present while residing in the secondary lymphoid organs. They can encounter small soluble antigen in the lymphatic fluid that passes through the subcapsular sinus to the follicles (35). Follicular B cells can also interact with large antigens and immune complexes in the macrophage-rich subcapsular sinus, which are presented on the surface of follicular DCs (35). Some B cells that migrate through the lymph nodes can encounter antigen presented on the surface of resident DCs or newly migrated DCs located around high endothelial venules in the

paracortex (36). Although it is believed that B cells are relatively weaker when compared to dendritic cells in context of antigen presentation, some studies have shown that when B cells are exposed to certain stimuli their antigen presentation capacity can be significantly enhanced to match that of dendritic cells (32, 34, 37-39). These agents and their effects on B cells are discussed in more detail in Section 6 below.

B cell populations and development

B cell populations can be generally divided in to three groups, B1 cells, B2 cells and B regulatory cells (Bregs). B cells are generated throughout the human lifespan (40), and most of those B cells in adult humans are conventional B cells, also referred to as B2 B cells. B1 cells and Bregs constitute minor populations. In this section, we discuss the development and maturation stages of each of these B cell types, and what is known about the APC function of these different populations.

Conventional B cells (B2)

In the case of B2 B cells, a signal from stromal B cells to progenitor lymphoid cells initiates B cell development in humans. The stromal cells engage the progenitor cell by providing cytokine signals including CXCL12 and IL-7, along with SCF/c-Kit ligand (stem cell factor), that promote B cell development (41). The stages of development include pro-B cells (early and late), pre-B cells (large and small), immature B cells and mature B cells (Figure 1). These stages are defined by the expression of diverse antigen receptors (B cell receptor - BCR) on the surface of B cells, along with other cell surface and intracellular markers (Table 1). In the early stages of development,

components of the BCR start to assemble and undergo rearrangement to promote diversity, similar to T cell development, and as described below. Complete assembly and expression of BCR is observed in immature B cells as they depart from the bone marrow and migrate to secondary lymphoid organs. This developmental process also includes selection pathways that eliminate self-reactive B cells, favoring B cells that are reactive to foreign antigen.

In the first phase of B cell development that takes place in the bone-marrow, expression of RAG1, RAG2 and TdT is induced in CD34+ stem cells. Their expression is accompanied by joining of D (diversity) and J (joint) region on the H (heavy) chain of the immunoglobulin (42, 43). This phase is called early Pro-B cell. Following this, B cells enter the late Pro-B cell stage where V (variable) region binds to DJ region on the heavy chain (44). After VDJ joining, the B cell enters the pre-B cell stage, while still in the bone marrow. VDJ rearrangement begins and expression of membrane μ chain is induced. Along with the μ chain, surrogate L (light) chains are also expressed, which pair with the H (heavy) chains. Igα and Igβ accompany the H and L chains to form a functional pre-B cell receptor (pre-BCR) in the endosomal compartment of the cell (45). If any of the above-mentioned processes fail, then the developing cell will undergo apoptosis and will not proceed to the next stage (46). Once the pre-B cell receptor receives a signal from Igα and Igβ, the heavy chain recombination halts and the pre-B cell starts proliferating rapidly (47).

During proliferation, B cells transit from large pre-B cell stage to a small pre-B cell stage in the development process. This generates a huge amount of variability within the B cell population as different L chains are paired with the rearranged H chains. The daughter cells (small pre-B cell) start synthesis of L chains (κ or λ) and initiate V-J joining (48). Now the previously synthesized heavy chain combines with the newly synthesized L chain to form a functional antigen receptor

IgM, which migrates to the membrane. Once either IgMκ or IgMλ is expressed on the cell surface the cell is now designated an immature B cell (49).

In the final stages, immature B cells are positively selected for being able to bind an antigen and negatively selected for binding with a self-antigen. Once a functional BCR is confirmed, the cell starts expressing IgD molecules along with IgM on its surface and becomes a naïve mature B cell, which then leaves the bone marrow and migrates to secondary lymphoid organs such as spleen and lymph nodes (50). Immature B cells transit to spleen where they attain maturation and activation. This process can also be divided into several stages depending upon the location and activation status of the cell. B cells migrating from the bone marrow to the spleen are known as transitional B cell (51). In the spleen, B cells can be classified as marginal zone B cells, regulatory B cells, follicular B cells, activated B cells, germinal center B cells, plasma cells (short or long lived) and memory B cells (52). These B cell subsets can also be identified based on the differences in the expression of certain cell surface and intracellular protein markers, as listed in Table 1.

B2 cell development and maturation is fundamentally categorized by the generation of an antigen-specific and mature BCR. The BCR is the primary source of antigen presentation by B cells. There are two ways BCR can be involved in antigen presentation, either by interacting with a BCR-specific antigen that leads to B cell activation, or by internalization of BCR non-specific antigens for processing and presentation (53). The impact of BCR on the APC function of B cells is discussed in more detail in Sections 4 and 5.

Classically, upon encounter with cognate antigen in the spleen, B cells undergo activation, class switching, division and proliferation. Class switching, or isotype switching, occurs when naïve B

cells switch from expressing IgM and IgD to other isotypes such as IgA, IgG, IgE (54). This is determined by the type of cytokine signal B cells receive from T helper cells after presenting antigen. IL-4 (55), IL-5 (56), TGFβ (57) and IFNγ (58) are all known to induce isotype switching after activation in mice and humans. The two most prominent cell subtypes that occur after terminal differentiation of B cells are plasma cells, which produce antibodies against the antigen/pathogen, and memory B cells, which can respond quickly to subsequent exposure to the same antigen (59). Plasma cells are generated in large numbers and are short lived, whereas the memory cells are long lived and are generated in small numbers.

Most B2 B cells develop in the bone marrow, but a small population of B2 B cells have also been identified in fetal liver and fetal bone marrow (60). However, not much is known about these environments to study their development and capabilities. Most of the B cells that arise during fetal development are characterized as B1 B cells (described below), and these have been the most studied. It has, however, been shown that hematopoietic stem cells (HSCs) isolated from fetal liver can potentially differentiate into both B1 and B2 B cell types when exposed to adult microenvironment factors (61).

B1 cells

B-1 cells can be characterized as either B1a or B1b B cells based on expression of Ly-1 (CD5) on their surface (62). B1a B cells express CD5 and B1b B cells do not, while expression of other cell surface markers is common to both, as in Table 1. Both types predominantly develop in the fetal liver, and their functions are classified as innate immune type responses (63). B1 B cells can also develop during adulthood in the bone marrow, but their frequency is extremely low in comparison to B2 B cells (64). B1 cells primarily secrete IgM, but also IgA and IgG3, without any antigenic

stimulus. Hence these antibodies are called natural antibodies, as they have high reactivity but low affinity towards pathogens (65). The natural antibodies are secreted independent of T cell help and are the first barrier against a pathogenic infection, before the development of an adaptive response (66, 67). About 80% of all naturally occurring IgM is secreted by B1 cells, which is thought to maintain immune homeostasis, by regulating B-1 cell development as a feedback mechanism along with regulation of IgG2a production and promotion of B2 cell antibody responses (68, 69). Some studies have also correlated increased numbers of B1 cell populations with development of autoimmune diseases, such as in patients with Sjogren's syndrome (70) and rheumatoid arthritis (71).

Although the development and maturation mechanisms of B1 cells are well studied in murine models, their developmental pathway in humans has been controversial. As the progenitors to B1 cells in humans are yet to be characterized/identified, there are currently two theories about their lymphopoiesis. One proposes a lineage model, in which different progenitors are responsible for different subsets of B cells (72), and the other proposes that there could be interconversion between B1 and B2 cells based on a selection model (73). Others have also proposed a reconciled model that considers both lineage-based and activation-based B1 cell development models (73, 74). In adult humans, B1 cells primarily populate the peritoneal cavity, but are also present in pleural cavity, spleen, bone marrow, lymph nodes and blood in smaller numbers (75).

In murine fetal liver, B1 B cells originate from fetal liver HSCs, differentiating into Pro-B cells, Pre-B cells, and then to immature B cells, after which they differentiate into either B1a cells or B1b cells (Figure 1) (64). In fetal liver, B1 cell development is dependent on BCR assembly; a weak/poor pre-BCR is allowed for further proliferation whereas cells with effective pre-BCR lead

to apoptosis. This is in contrast to the development of conventional B2 cells (74). In fact, there are three major differences between in the development process of B1 B cells when compared to that of B2 B cells: (i) B1 B cell-specific progenitor cells are responsive to thymic stromal lymphopoietin (TSLP) and proliferate upon exposure, but B2 B cell progenitors do not (76); (ii) Pro-B cells that result from B1-specific B cell progenitors can differentiate independent of IL-7, but B2 lineage-specific pro-B cells fail to differentiate further in the absence of IL-7 (77); and lastly (iii) differentiation of B1 lineage-specific B transitional cells into B1a and B1b B cells is not dependent upon B cell-activating factor (BAFF) (78) or NF-κB2 signaling, unlike their B2 lineage-specific counterparts (79).

The function of B2 B cells as APCs has been studied in more detail compared to B1 B cells. As previously discussed, B1 B cells are known for their ability to generate an innate type immune response following activation by T-independent antigens (63)(65). Some groups have also demonstrated their ability to generate T-dependent immune responses (80-85). More details about the findings of these studies are discussed in section 4. B1 B cells mostly remain anergic to BCR-antigen interaction. Several mechanisms are involved in suppressing the activation of B1 cells after BCR engagement. Recent studies have shown that BCR and TLRs play critical roles in regulating antigen uptake by B1 cells (86-88). However, some reports demonstrate macrophage-like phagocytic function of B1 cells, which enables these cells to function as APCs (89). Their phagocytic potential can be further augmented by use of adjuvants such as Propionibacterium acnes (90) and LPS (91, 92). Whether these stimuli also promote their antigen presentation activity, is not clear and is currently under investigation.

As characterized in mice, B1 B cell development occurs in three waves. The first wave takes place in the yolk sac and aorta-gonad-mesonephros (93), followed by the second wave primarily in the fetal liver, but also in the fetal bone marrow. The final wave occurs after birth in the bone marrow exclusively (61, 64). Most of the B1 B cell population that makes up the peripheral B1 B cell pool is the result of the second wave of development in the fetal liver (Fig 2). Similar observations have been made in humans as well, where B1 B cells in cord blood, fetal liver and fetal spleen have been reported (94). However, human B1 B cells cannot be classified by the expression of CD5 alone, as a large population of B cells in humans express CD5, not all of which demonstrate characteristics of B1 B cells (95). Classification of human B1 B cells at various stages of the development/maturation process based on surface markers has been controversial and is being actively researched. A recent study proposed CD20+ CD27+ CD43+ CD70- cells that spontaneously secrete IgM as human B1 B cells (96), but this has faced criticism (97, 98).

Regulatory B cells (Breg)

Regulatory B cells or Bregs are a minor B cell population, typically comprising of less than 1% of human peripheral blood mononuclear cells (PBMC) and are characterized by their immunomodulatory function to suppress inflammation (99). Most B cell subsets are capable of differentiating into Breg cells to regulate inflammation, and usually do so through IL-10 secretion (100), or IL-35 secretion (101). CD138+ plasma cells have also been shown to produce IL-10 and IL-35 and can differentiate into Bregs (101). TGF-β has also been shown to be produced by Breg cells to mediate induction of Tregs during tolerance induction after transplantation (102).

Within the last decade, several subsets of Bregs have been identified in mice and humans based upon differential expression of cell surface markers. A description of their expression profiles and associated phenotypes are listed in Table 2. Several B cell subsets, such as immature B cells, plasmablasts and mature B cells, have been shown to differentiate into IL-10-producing Bregs, upon stimulation with toll-like receptors (TLRs) and/or CD40 activation (103, 104). Differentiation of B cells into Bregs can also occur after stimulation of B cells through BCR signaling, following antigen encounter and presentation (105), and when this happens the resulting Bregs are antigen-specific (106). As mentioned previously, IL-10 is one of the major factors used to discriminate a Breg reliably from a conventional B cell, but their identification by cell surface markers has been controversial. As there is no known transcription factor or lineage-specific marker for Bregs (unlike FoxP3 as a marker for Tregs), it has been difficult to identify and define Breg populations. Current research has focused on understanding their development, and there are two proposed models. One suggests that Bregs are the result of a dedicated lineage, controlled by expression of specific genes. Another implies that B cells transition to a Breg cell type in response to certain stimuli at the site of inflammation. This latter model has gained credibility, due to the heterogeneous nature of Bregs (Figure 1) and the inability to identify a lineage-specific marker (107).

A direct relationship between the development and maintenance of Treg populations through Bregs has been proposed by many, in both mice and humans (108, 109). It has been demonstrated that the number of CD4 Tregs are similar or reduced in mice that are B cell deficient (110). Also, more specifically, if B cell-specific production of IL-10 is deleted, then the resulting mice are deficient of Tregs (111). In another study, IL-35-deficient mice (B cell-specific) have been shown

to have an increase in Th1 type response and aggravated experimental autoimmune encephalomyelitis (112). A greater protection was observed against Salmonella-induced sepsis and an increased number of macrophages in the spleen were also reported (112). Apart from supporting Treg function, Bregs also suppress other pro-inflammatory activities by inhibiting the differentiation of TNF-producing monocytes, IL-12-producing DCs (113), Th17 cells, Th1 cells and cytotoxic CD8 T cells (114, 115). These activities occur through secretion of IL-10, IL-35 and/or TGF-β (113). Interestingly, in humans it has been suggested that Bregs can also be involved in maintaining homeostasis of invariant natural killer T (iNKT) cells (116).

Although Bregs are known to impair antigen presentation, to our knowledge they have not been evaluated for their ability to present antigen. Given their intrinsic characteristics they would likely be poor candidates for priming and activating T cells.

B cell populations that serve as APC to T cells

B cells are protected from encountering antigens as they undergo development in the bone marrow. Consequently, their role as APCs becomes of importance once they become transitional B cells and transit to the spleen and other lymphoid organs. That is, through the developmental stages in the bone marrow, B cells interact with self-antigens during positive and negative selection. They are effectively shielded from interaction with foreign antigen(s) as they are not matured and can carry along an uncertainty towards generating an optimal and specific response. B cells express most of the machinery that is required for antigen processing and presentation, such as MHC II, invariant chain (li), calnexin and HLA-DM, during their early developmental stages (Pro-B, Pre-

B, and immature B cells). However, a mature BCR is absent, along with HLA-DO and CLIP (117-119). Once the immature B cells leave the bone marrow and are in the transitional stage, that is the first opportunity for them to naturally encounter and respond to a foreign antigen (120). Some reports suggest that when transitional B cells encounter an antigen they are marked for apoptosis, in contrast to mature B cells which get activated upon antigen encounter (121, 122). However, a recent *in vitro* study suggested that both T1 and T2 transitional B cells can process and present antigen just as well as mature B cells, but these cells need to be rescued from apoptosis by helper T cells (120, 123). Expression of HLA-DO and proteolytic cleavage of invariant chain (li) to CLIP peptide are initiated only in naïve follicular B cells and their subsequently differentiated B cell subtypes.

Expression of MHC II related antigen presentation components are lost in terminally differentiated antigen-specific plasma B cells, and these cells cannot present antigen or undergo class switching (124, 125). Immature plasma cells, known as plasmablasts, are capable of antigen presentation until they mature and become fully differentiated plasma cells (126). Mature naïve B cells are known to encounter antigen in the lymphoid follicles. T cells in the germinal centers help B cells in the process of activation, which is followed by class switching, somatic hypermutation and finally clonal differentiation (127). There is not much known about which subtype(s) of B cells can present antigen through the MHC I pathway, as traditionally B cells are known to present through the MHC II pathway to CD4 T cells, and most research has focused on that. But based on the observation that MHC I is ubiquitously expressed while MHC II is expressed (128), it is likely that all B cell subtypes discussed above that can present through MHC class II pathway should also be capable of presenting antigen to CD8 T cells via MHC I pathway.

Even though B1 B cells are mostly thought to function independently of T cell signals/antigens, there have been reports that B1 B cells can also present antigen to T cells *in vitro* (82) and *in vivo* (80, 84, 85). After adoptive transfer of OVA-peptide pulsed B1a B cells along with CFSE (Carboxy-Fluorescein Succinimidyl Ester) labeled OVA antigen-specific CD4 T cells in mice, Margry et al., showed that B1a B cells were able to induce proliferation of antigen-specific CD4 T cells (80). This fact is also supported by the fact the B1 B cell subsets can constitutively express markers associated with antigen presentation and co-stimulation, such as MHC II, CD80 and CD86, upon stimulus (82). Moreover, reports from Zimecki et al., indicate that B1 B cells may be superior to conventional B2 cells in terms of antigen presentation, as they elicit greater proliferation of antigen-specific T cells (84, 85).

Antigen processing and presentation by B cells

Activation of B cells as APCs occurs via two major pathways: (i) through the B-cell receptor (T-cell dependent), or (ii) through germline encoded PAMP (Pathogen Associated Molecular Patterns) receptors (T-cell independent). PAMP receptors for macromolecules like polysaccharides, lipopolysaccharides, and other nonprotein antigens, have been shown to induce antigen presentation in B cells. The BCR specifically interacts with its cognate antigen, inducing a signaling cascade that stimulates internalization of the antigen, leading to B-cell activation and proliferation. Once stimulated, the B cell exhibits a membrane spreading and contraction response that assists with antigen aggregation and results in the formation of an immunological synapse with antigen-specific T cells (18, 119, 129-132). Unique to B cells, antigen uptake and processing is initiated by Cbl and Cbl-b (Cbls), which belong to the superfamily of E3 ubiquitin ligases. Cbls

promote naive B cell conversion into mature antigen presenting B cells and are essential for interaction between naïve B cells and cognate T cells (133).

As discussed earlier, the BCR plays a vital role during the development of B cells but is also crucial when presenting antigens. When the BCR encounters its cognate antigen, B cells are signaled to proliferate and differentiate into traditional antibody-secreting plasma cells and long-lived memory cells. Upon antigen binding, BCR oligomers are formed and the affinity of the antigen towards the BCR regulates the strength of BCR signaling (134). Tsourkas et al., demonstrated that as the affinity increases, the size and rate of oligomer formation as well as the number of antigens collected by the BCR, increases (135). BCRs also mediate endocytosis of the antigen by receptor internalization, for further processing and presentation to helper T cells. Signals through the BCR are a driving factor for B-cell survival, activation, maturation, migration, and differentiation into various developmental stages (136). Variations in BCR signaling have also been shown to affect the expression levels of co-stimulatory molecules on antigen-activated B cells.

Antigen Uptake and Processing

Professional APCs typically use one of the three major pathways for antigen internalization: endocytosis, pinocytosis, or phagocytosis (20). Generally, naïve B cells do not have phagocytic capabilities, but B1-B cells have been shown to phagocytose particles, including bacteria (89). Another recent report demonstrated that follicular B cells can mediate phagocytosis of particulate antigens through the BCR (137). However, the majority of antigen acquisition by B cells occurs through endocytosis for antigens smaller than 0.2μm (22). We have also demonstrated that B cells

can acquire antigen through fluid phase pinocytosis when plasmid DNA is used as an antigen (138). Antigenic peptides generated after either of these internalization pathways can be presented but can have different efficiencies, depending upon a range of factors such as antigen type, internalization mechanism used for antigen uptake, and APC subset (B cells, DCs, macrophages) involved in antigen presentation.

Processed antigens are presented on major histocompatibility complexes (MHC) which are assembled in the endoplasmic reticulum and functionally matured in the endosomal compartments (139). As previously discussed, there are two types of MHC molecules; class I and class II; MHC I complexes are generally loaded with antigenic peptides that results from proteasomal degradation of cytosolic proteins. In this pathway, cytosolic protein could be a self-protein, a viral protein (from a virus that has infected the cell) or an exogenous protein resulting from retro-translocation after phagocytosis of a pathogen. After proteasomal degradation, the peptides are transported to the ER through TAP, then processed by ERAP to be loaded on MHC molecules, and finally transported to the cell surface for presentation (140). Most exogenous proteins get processed and loaded on MHC II complexes in late endosomal compartments (141). Some cytosolic proteins are also processed through this pathway during autophagy (142).

Complement-coated antigens presented to B cells remain on the cell surface, bound by complement receptors, whereas IgG-coated antigen received by FcγRIIB receptor gets internalized in neutral endosomes and recycled to the surface either for presentation to DCs (cross presentation) or to T cells by MHC II (143). B cells function as antigen transporters and can carry complement-coated antigens to follicular DCs in the spleen or lymph nodes. This transportation is not mediated

by BCRs, so the B cells need not be antigen specific. Complement receptors, CR1 and CR2, act as transporters in this situation (144).

When BCR is involved in antigen detection and capture, antigen processing takes place through the traditional MHC II loading pathway. There are some unique features that have only been reported when B cells present antigen through the MHC II pathway. According to some reports, MHC II localizes to unique MHC class II vesicle compartments that are distinct from lysosomes and endosomes (145). Upon ligation of BCR by cognate antigen, MHCII dimers are redistributed to LAMP1-positive multivesicular bodies (132). BCR-antigen complexes are then transported through the endocytic pathways to MHC II-rich regions where ubiquitinylation occurs. E3 ligase "Itch" mediates ubiquitinylation of Igβ (146), which is important for sorting the complexes to LAMP1-positive compartments. In these compartments HLA-DM mediates loading of antigenic peptide by removal of CLIP peptide from MHCII (147) and distinguishes between strong versus weak binders from the peptide repertoire. Preferential loading of peptides that fit more tightly ultimately generates a highly specific response (148). Another report demonstrated that for B cells this activation is reversible after 24 hours, as assessed by the expression of cell surface markers that define B cell maturation and activation (149).

Expression of HLA-DO in B cells also regulates antigen presentation (150) by inhibiting expression of HLA-DM (151). Generally, germinal center B cells that are competent APCs have diminished expression of HLA-DO (152). B cells downregulate the expression of HLA-DO only when MHC II localizes in acidic compartments, which allows for HLA-DM-dependent peptide loading. This also maintains specificity for peptide loading of processed antigen that are internalized through the BCR (153).

Apart from presentation through MHC II complex, B cells can also process and present antigen through MHC I complexes, but less is known about this. There are several reports that show antigenic-peptide pulsed B cells are capable of presenting antigen to CD8 T cells but are not as efficient as dendritic cells when it comes to activation and proliferation of antigen-specific CD8 T cells (154, 155). The specific details of the mechanism by which B cells process and load antigens onto MHC I complexes, rather than onto MHC II complexes, are not clearly understood.

Activation and co-stimulatory signals

Generally, three signals are required to activate T cells (156), the first of which is in the form of the antigen being presented by the APC. Interaction of antigen-loaded MHC with the T-cell receptor initiates signaling that activates both the APC and T cell for their proliferation and differentiation (157). Secondly, the T cell needs to receive a costimulatory signal, which occurs primarily by CD80/CD86 on the APC binding to CD28 on the T cell. This second signal stabilizes the immunological synapse between APC and the T cells and induces the expression of other activation markers. Upon activation, B cells express MHC class I and II, CD80, CD83, CD86, CD40 and other costimulatory and adhesion molecules that support and strengthen antigen presentation (158). Finally, the T cells receive the third signal in the form of cytokines that activate them and polarize them towards an effector phenotype. In the case of B cells as the APC, the secreted cytokines can include IL-2, IL-4, IL-6, and/or IFNγ (159).

Many of the costimulatory and/or activation signals promote differentiation, survival and proliferation of both B cells and T cells. But some of these are unidirectional and act on either B

cells or T cells alone upon encounter with their ligand. Most important of all costimulatory/activation interactions is CD80/CD86 (APCs) and CD28 (T cells), as this interaction is involved with establishment of the immune synapse (160). Other cell surface interactions take place between APCs and T cells and occur after the presentation of antigen to T cells. For example, 4-1BB, ICOS and OX40 all become expressed on T cells after T-cell activation, whereas their ligands are present on activated APCs along with CD80/CD86 even before T-cell activation (161).

The roles of CD80 and CD86 have been extensively studied in the context of antigen presentation and it has been shown that increased expression of either of these make B cells potent APCs, as they provide stronger intercellular interaction and a more stable environment for T cell activation (160). CD80 and CD86 also play a significant role in antibody secretion; one study demonstrated that an antibody targeting CD80 in LPS activated B cells can suppress IgG secretion whereas an antibody targeting CD86 promotes antibody secretion (162). Signaling through CD83, on the other hand, is less understood. The identification of CD83L on T cells has been controversial. A few studies propose that a receptor for CD83 is expressed on human and murine CD4 and CD8 T cells (163). Expression of CD83 on B cells marks their activation, especially during germinal center reaction (164). It was also demonstrated by Akauliya et al., that antibody responses to influenza infection were significantly lower in CD83 KO mice compared to wild type mice, implying a role of CD83 in modulation of antibody responses, but this could also be attributed to reduced numbers of CD4 T cells in CD83KO mice (165). In another study using CD83 knock out mice, Krzyzak et al., reported defects in MHC II and CD86 expression upon stimulation, results in modulations in germinal center composition (more B cells in dark zones) and an enhanced IgE response (164).

Other costimulatory molecules expressed on B cells include ICOSL, CD134L (OX40L) and CD137L (4-1BBL) (Table 3). Each ligand interacts with its specific receptor and contributes towards B-cell and/or T-cell co-stimulation. ICOS (inducible co-stimulator) binds with ICOSL (ICOS ligand) and provides both positive and negative co-stimulatory signals to B cells. Their interaction promotes B-cell activation and differentiation. More specifically, it has been demonstrated that ICOS signaling promotes antibody-secreting B cells (166). It has also been shown that in ICOS-deficient mice, its absence impairs germinal center formation and causes defects in contact-dependent isotype class switching (167). Similarly, interaction between OX40 and OX40L promotes B-cell activation, proliferation, survival, and cytokine production (168, 169), but if disrupted/inhibited it can cause reduction in production of class-switched immunoglobulin isotypes (170). In contrast, CD137-CD137L interaction stimulates T cells only, as CD137 is only expressed on activated T cells (171, 172). There has not been any evidence that suggests a critical role of 4-1BB-4-1BBL signaling in B-cell activation or development.

One of the most studied costimulatory interactions between B cells and T cells is CD40-CD40L. The receptor and ligand are each expressed by both cell types. Most of the effect resulting from this interaction occurs in B cells, as it can promote activation, cytokine production, proliferation, antibody secretion and upregulation of several surface molecules involved in antigen presentation (173, 174). Evidence shows that CD40-CD40L signaling also regulates class switching, formation of germinal centers and humoral memory response (175). Most studies that have evaluated the role of CD40 signaling in B cells have shown that activation of CD40 plays a critical role in presenting antigen and activation of antigen-specific T cells. CD40 activation also results in improved survival of B cells through CD40-induced phosphoinositide 3-kinases (176).

Adhesion molecules such as ICAM-1 and LFA-1 (Table 3) are also expressed on B cells during antigen presentation; their interaction ensures increased stability of the synapse and results in amplification of activation signals (177). Specifically in the case of B cells, this signaling has been shown to promote antigen presentation by B cells by cooperating with CD40 signaling (178). Another adhesion molecule, CD22, belonging to the immunoglobulin superfamily, is also expressed on B cells. Several natural ligands, specifically N-linked oligosaccharides, are known to interact with CD22, many of which can be expressed on T cells and can potentially interact with CD22 (179). The exact nature of this interaction is not clearly understood as specific receptor-ligand pairs between B cells and T cells that involve CD22 are not known. Another surface molecule, CD81, which belongs to the tetraspanin family, has also been reported to contribute towards B-cell adhesion and T-cell dependent activation (180), but its natural ligand(s) are yet to be discovered.

External agents that can affect antigen presentation

CD4 T cells

In addition to effects mediated by co-stimulatory and adhesion molecules, B-cell activation and function can also be modulated by other environmental factors such as CD4 T cells, cytokines and TLRs. The interaction between B cells and CD4 T cells is very well studied, but mostly in the context of antigen presentation through the BCR and adaptive immunity (32, 181, 182). This interaction results in affinity maturation and differentiation of B cells into plasma cells and memory cells, leading to antibody secretion and at the same time regulating the development of memory CD4 T cells (182, 183). One of the most important interactions between CD4 T cells and

B cells is the CD40-CD40L (B cell – CD4 T cell) interaction, which plays a crucial rule in activation of B cells. As discussed earlier, activation of the CD40 pathway in B cells leads to a multitude of responses that are required by B cells for survival, activation, and proliferation (183, 184). Upon stimulation by B cells, CD4 T cells start secreting IL-2, which primarily acts as an autocrine differentiation and proliferation factor and promotes the development and maintenance of Tregs (185, 186). However, IL-2 has also been shown to affect B cell proliferation, specifically in humans (187), and induce differentiation of activated B cells into plasma cells (188).

A subset of CD4 T cells, called the T follicular helper cells, promote B cell proliferation and effector function through the production of IL-4 and IL-21 (189, 190). Studies have shown that knockout of either of these two cytokines leads to diminished B cell responses, which are further diminished with a combined deficiency of both (191). More specifically, the IL-4 pathway is primarily involved in the formation of germinal centers in type 2 immune responses (127, 192), whereas IL-21 influences the differentiation of B cells into Ig-secreting plasma cells (193, 194).

Cytokines

Like all immune cells, B cells are influenced by external stimuli provided by cytokines. Pro-inflammatory cytokines support antigen presentation through B cells by upregulating the co-stimulatory molecules. IL-4 induces a 10-fold increased expression of class II MHC antigen on B cells, and stimulation with IL-21 alone, or IL-21 and IL-2, upregulates CD86 expression on B cells (195, 196). Other studies have demonstrated the importance of IL-4 and/or IL-21 in promoting antigen presentation, specifically in B cells. IFN-γ is also thought to facilitate the antigen-

presenting activity of B cells; although some studies report that IFNγ downregulates the cell surface expression of MHC II on B cell in PBMC (197) and cell lines (39), while upregulating MHC I expression. Treatment with IFN-γ can also regulate the proliferation and differentiation of B cells (198, 199). IFN-γ has both positive and negative effects on B cell proliferation depending upon the stage of antigen presentation. Before antigen encounter, and in the later stages of antigen presentation, IFN-γ inhibits proliferation. However, during the early proliferative response upon antigen encounter, IFN-γ promotes B cell division. IFN-γ has also been reported to mediate and regulate antibody class switching on B cells. Interestingly, some anti-inflammatory cytokines can also support antigen presentation activity of B cells. IL-13 has been reported to enhance the expression of MHC II on B cells, and TGF-β also modestly increased the expression of MHC class II on B cells (200, 201).

Toll-like Receptors (TLRs)

In addition to cytokines, the antigen presentation activity of B cells is also influenced by TLRs. TLRs are type I transmembrane receptors which sense molecules containing PAMPs or Damage Associated Molecular Patterns (DAMPs). These receptors are expressed in B cells and affect their antigen presentation activity (202, 203). It was shown that TLR9 stimulation facilitates B cell antigen presentation with the upregulation of MHC class II, CD40, and CD80. Similarly, TLR2 and TLR4 stimulation also increase the expression of CD86 and MHC II in B cells (204-206). TLR7/8 ligands have also been reported to upregulate CD80 expression on B cells (207). TLR9 has been of most interest in the context of stimulating B cells for antigen presentation, and many studies show that TLR9 stimulation by CpG ODN (oligodeoxynucleotides) leads to increased

activation of B cells that promotes both innate and adaptive immune responses (38, 208-211). Jiang et al., reported that stimulation of naïve B cells with CpG ODN rescued them from apoptosis, caused proliferation and enhanced the expression of CD40, CD80, HLA-DR on their surface (212). They further demonstrated that these B cells do not mature into memory cells, but rather have increased ability to activate allogeneic CD4 and CD8 T cells (212). This can also be further enhanced when B cells are treated with a combination of TLR9 agonist and other stimulating agents (213-215). As reported by Giordani et al., IFN-alpha amplifies the effects of TLR9-mediated activation of naïve B cells. They demonstrated that there was increase in B cell activation, Ig production and frequency of CpG-induced memory B cells (214).

Other proteins

BAFF (B cell-activating factor) is fundamental for B cell survival and maturation (216). It has recently been found to upregulate B cell expression of CD40 and enhance B cell antigen-presentation to CD4+ T cells through increased expression of MHCII (217). In a mouse melanoma model, treatment with recombinant BAFF promoted central memory phenotype of T cells in vaccine-draining lymph nodes, along with an increase in the number of B cells with upregulated costimulatory molecules (218). Treatment with BAFF led to downstream T-cell activation and increased anti-tumor immunity, demonstrating one method of converting B cells into highly effective APCs. Along with T cell mediated anti-tumor effects, BAFF also induced CD4+FoxP3+ Treg population in the spleen and tumor microenvironment (218). However, overexpression of BAFF has been associated with autoimmune diseases in mice and humans, by allowing the emergence of autoreactive B cells (219, 220).

Outcome of antigen presentation

The outcome of antigen presentation on T cells, in terms of their phenotype and effector function, can vary vastly based on the antigen internalization pathway, the form of antigen presented (DNA/RNA/protein/peptide), the APC subtype involved, and the activation/developmental stage of the APC, as any of these factors can impact the efficacy of antigen processing and presentation. Consequently, here we discuss what is known about the effects on T cells activated by antigen presenting B cells, specifically related to CD8 T cells, how B cells may differ from other professional APCs, and what implications this has on use of B cells as APCs in immunotherapy.

Direct and cross presentation

One study, conducted in a Salmonella infection model, showed that antigen-specific B cells are capable of cross-presenting antigen to CD8+ T cells, and this cross-presentation is partially dependent on proteasomes; CD8+ T cells demonstrated decreased degranulation, as measured by CD107a expression, when CD8+ T cells were primed by proteasome-inhibited B cells (221). Salmonella-infected B cells were shown to promote CD8 T-cell proliferation with the help of CD4 T cells, and the resulting CD8 T cells were cytotoxic and secreted IFN-γ. Wit et al., also demonstrated that Salmonella-infected B cells could activate both central and effector memory CD8 T cells (221). Two other studies have demonstrated enhanced B cell cross-presentation when antigen is delivered with adjuvants. B cell cross-presentation to cytotoxic CD8+ T cells was enhanced when antigen was co-delivered with CpG-DNA (38) or a TLR2 agonist (222).

A lesser known and understudied function of B cells is the direct presentation of antigen to CD8 T cells on MHCI. Zentz et al., demonstrated that CD40-activated B cells (via co-culture with CD40L-expressing irradiated fibroblasts) could strongly and specifically expand rare populations of antigen-specific CD8 T cells from the PBMC of healthy donors. Epitope-specific (HPV-16, E7) CD8 T cells could be selectively expanded in 6 of 6 healthy donors with initial frequencies of less than 1 in 20,000 antigen-specific CD8 T cells. The authors observed up to a 106-fold expansion of antigen-specific CD8 T cells, and the resulting cultures contained up to 88% antigen-specific CD8 T cells (223). CD40-activated B cells (CD40-B cells) have similarly been used by many groups as a readily available source of highly efficient APC and have been shown to be capable of priming Th1 type anti-tumor responses (34, 208, 224, 225). In a murine study assessing the use of CD40-B cells as an anti-cancer vaccine, vaccination of wild-type mice with LCMV antigen-pulsed CD40-B cells significantly reduced growth of LL-LCMV subcutaneous tumors by direct and indirect activation of CD8 T cells, but antigen-pulsed LPS-activated B cells did not (226). Furthermore, using CD40L-expressing feeder cells for activation, vaccination with tumor antigen-pulsed CD40-B cells resulted in significantly delayed growth in both B16 melanomas and E.G7 lymphomas (227).

In related studies, CD40-B cells transduced with tumor antigen-encoding RNA or DNA have been demonstrated to prime tumor-specific cytotoxic CD4 and CD8 T cells *in vivo*. Fujiwara and colleagues generated a eukaryotic expression vector which contained three leukemia-specific antigens, primary granule protein proteinase 3, human neutrophil elastase, and cathepsin-G, inserted into the pcDNA3.1 plasmid. PBMC from five HLA-A2+ leukemia patients were cultured with plasmid-transduced CD40-B cells. The transduced B cells were able to stimulate both CD4

and CD8 responses against all three antigens. Furthermore, when CD3 cells isolated from PBMC were stimulated with DNA-transfected autologous CD40-B cells, the investigators were able to culture CD4 and CD8 T-cell lines that produced IFN-γ upon stimulation with autologous leukemia cells (228). Coughlin et al., similarly, demonstrated that CD40-B cells transfected with RNA could serve as a vaccine for tumor antigens. RNA transfected CD40-B cells induced IFNγ+ cytotoxic T cells which could be identified with tetramers and lysed neuroblastoma cell lines (229). Taken together, these studies indicate that CD40-activated B cells can express, process and present antigens on both MHCI and MHCII when transduced with tumor antigen encoded by DNA or RNA.

Comparison of B cells to other APCs

Activated B cells have many similarities with DC in terms of APC function. For example, after three to four weeks of CD40 activation, B cells were shown to express elevated levels of HLA class I and the costimulatory molecules CD80 and CD86 (228). However, CD40-activated B cells differ from DCs by displaying a rapid migratory pattern and undergoing highly dynamic, short-lived, and sequential interactions with T cells (230). Previous work from our group indicates that short APC to T-cell contact times can stimulate T cells with transient PD-1 expression, while longer (>15min) contact times resulted in persistent PD-1 expression and attenuated anti-tumor responses (231). Taken together these data suggest that T cell/B cell interactions could prove advantageous over T cell/DC interactions when initiating a cytotoxic response against PD-L1+ target cells (e.g., in many solid tumors) by stimulating T cells that may be less susceptible to PD-1 ligation. This remains to be demonstrated, but conceptually B cells may differ from DC in

inducing expression of other T cell checkpoint molecules on activated T cells. Furthermore, the tumor microenvironment is filled with other immunosuppressive tumor-derived factors, such as, prostaglandin E2 (232), TGF-β (233), VEGF (234) and IL-10 (235) which act in part by inhibiting DC differentiation, maturation, trafficking, and antigen presentation (236). Activated B cells, on the other hand, are relatively resistant to inhibition by tumor-associated immunosuppressive factors such as IL-10, TGF-β and VEGF. In *in vitro* studies, neither migration nor activation of CD40-B cells was inhibited by these immunosuppressive factors, nor did they influence the ability of CD40-B cells to induce proliferation of CD4+ or CD8+ T cells (237). This may in part explain some observations that tumor-infiltrating B cells have been associated with a better outcome (9-11), as they may provide better APC function in an immunosuppressive tumor microenvironment.

Kamphorst et al., have reported that if an antigen is processed after phagocytosis, conventional DCs are best suited for antigen presentation (154). They also demonstrated that B cells and DCs have similar efficacies if the antigen is acquired after receptor mediated endocytosis, but not if antigen enters by bulk phase endocytosis. These authors conclude that DC can process and present antigen similarly, irrespective of the antigen entry mechanism, whereas in the case of non-DC APCs, the entry mechanism can have a profound effect on antigen presentation (154). While in this report the authors studied antigen in protein form, antigen presentation by other antigen forms, for example encoded by DNA or RNA, could be different. In a study by Colluru et al., it was demonstrated that if DNA plasmid was passively delivered to APC, it was taken up by B cells by pinocytosis and the encoded antigen could be transcribed and translated. On the other hand, other professional APC subtypes (DCs and macrophages) failed to process the DNA and degraded it before it could be processed (138). In a recent study of an mRNA-based vaccine and its effects on

APC, Liang et al., demonstrated that multiple APC subsets could translate the vaccine mRNA *in vivo*. They showed that monocytes and DCs at the site of injection and in draining lymph nodes were mostly involved in translation of an mRNA vaccine. They also reported that B cells in draining lymph nodes could translate the mRNA vaccine. This was true for both intramuscular and intradermal injection routes, demonstrating that most professional APC subtypes can process RNA into antigen *in vivo* (238). In this study, the authors reported that DCs and monocytes preferentially presented the mRNA vaccine, relative to B cells, as there was an abundance of circulating DCs and monocytes at the site of injection.

In contrast to protein or nucleic acid sources of antigen, when presentation capabilities of different APC subsets were compared by Kamphorst et al. using peptide-loaded APCs, they reported that there were no significant differences between the APC subsets (154). Rosalia et al., have also shown that B cells and dendritic cells can present small synthetic peptide antigen to generate a similar T-cell response, as demonstrated by their ability to initiate proliferation of antigen-specific T cells (155). They also showed that if long synthetic peptides are delivered to APCs, which require subsequent processing by proteasomes and TAP-mediated MHC loading, then DCs are superior APCs, compared to B cells or macrophages (155). As described earlier, it remains possible that there could be other differences in the phenotype, or effector or memory function of T cells activated by different APC types, although this has not been extensively studied.

Use of B cells in immunotherapy

Various groups have studied ways of harnessing B cell APCs to improve immunotherapy (239). Our group has shown that B cells can serve as primary APCs in the context of DNA vaccines (138). Other groups have looked at ways of enhancing the APC function of human and murine B cells via *ex vivo* stimulation with CD40, IL-4, or IFN-γ. These agents have been shown to increase MHC I and II expression as well as CD80/86 expression on B cells. The activated B cells were able to stimulate CD4+ and CD8+ T cells leading to increased proliferation in an antigen-specific manner (225, 227, 240, 241). When activated B cells were adoptively transferred into tumor-bearing mice, they caused superior control of tumor growth. Lee-Chang et al., showed that activated B cells that are adoptively transferred also produce tumor-specific antibodies that contribute to the anti-tumor response (241). These studies highlight the potential value of B cell APCs as therapeutic agents and provide a compelling reason as to why their function as APCs needs to be better understood.

DCs have been extensively studied as a cellular immunotherapy approach, typically by loading DC with protein/peptide/nucleic acid antigen as a vaccine. In contrast, there have been relatively few studies exploring B cells as APC vaccines. However, B cells may have clear advantages as APC cellular immunotherapies. As described above, CD40-B cells are technically easy to generate in large numbers, tolerate cryopreservation, and thus could potentially be used at a markedly reduced cost when compared to DC (242). One study assessed the efficacy of CD40L- and IL-2-expressing autologous CLL B cells. The authors enrolled nine CLL patients out of which three patients showed >50% reduction in the size of affected lymph nodes and produced leukemia specific immunoglobulins (243). They also observed increased numbers of Treg cells before,

during and after the treatment of these patients, suggesting that their presence may be the reason behind the transient response they observed and that their removal could be the key to augmenting and prolonging responses. Another study evaluated B cells as a cancer vaccine, using CD40-activated B cells in combination with chemotherapy in dogs with non-Hodgkins lymphoma. The authors observed improved second clinical remission and survival following this combination treatment (244). Two other clinical studies in human patients utilized allogeneic B cells from healthy donors fused with autologous tumor cells. In a trial of renal cell carcinoma patients that the received B cell-based vaccines, two complete and two partial remissions were observed out of 11 total patients enrolled (245). In a trial of metastatic melanoma patients, one complete remission, one partial remission and five patients with stable disease were reported out of 16 patients enrolled (246). While these were small studies, clearly the increased interest in B cells as vaccines or vaccine adjuvants will lead to further development over the next decade (247).

Summary

While the general role of B cells in tumor immunity has been extensively investigated, there are still gaps in knowledge that restrict our understanding of B cells as APCs. This has become increasingly important given that several studies have demonstrated that the presence of tumor-infiltrating B cells results in better prognosis, and these B cells can function as APCs. Several studies have demonstrated that stimulated B cells can elicit a similar, or in some cases a better, T cell-mediated anti-tumor response than other APC subsets. External stimuli such as TLR ligands, cytokines, and other costimulatory molecules (such as CD40/CD40L and BAFF) have been evaluated for the ability to augment the antigen presentation capabilities of B cells. Some groups have attempted to use B cells as cellular vaccines, with early limited success. It is evident that a deeper understanding of B cell biology is necessary to more effectively harness the potential of B cells for cancer immunotherapy. In particular, capability of B cells to process plasmid DNA upon passive uptake could be of potential interest given the ongoing investigations to improve the immunogenicity of plasmid DNA vaccines. Based on this we evaluated the antigen presentation function of B cells when processing plasmid DNA and if B cells can be used as a therapeutic medium for improving the efficacy of DNA vaccines *in vivo*.

Doctoral Thesis Objectives

For my doctoral thesis I propose to investigate the mechanism of antigen presentation of DNA plasmid through B cells. This will test the hypothesis that direct presentation of DNA vaccines through professional antigen presenting cells will result in stronger immunogenicity, and that B cells serve as a viable alternative to DCs when considering their capability to activate CD8 T cells. To test this hypothesis, I will evaluate three specific aims:

1. To elucidate the mechanism of antigen presentation of plasmid DNA through professional antigen presenting cells *in vitro*.
2. To evaluate and compare the phenotype of CD8 T cells resulting from priming through antigen loaded B cells or DCs *in vitro*.
3. To target plasmid DNA vaccine to professional APCs *in vivo*.

Figures and Tables

Figure 1: ***Differentiation and development of B cell subsets.***

(A) B1 cells primarily originate in fetal liver, but also in fetal bone-marrow. They differentiate from fetal liver HSCs into Pro-B cells, Pre-B cells and immature B cells. After maturation they differentiate into either B1a or B1b cells. B1 cell are also generated after birth in the bone marrow. They then mature in spleen and primarily reside in serous cavities. (B) B2 cells are the conventional B cells that originate in the bone marrow, where they undergo differentiation and develop an antigen-specific functional BCR. They also undergo negative and positive selection to avoid autoimmunity and non-specific antigen responses. They then transition to the spleen where they mature into either follicular or marginal zone B cells. (C) Bregs are less understood when it comes to their development and differentiation. B cells can differentiate into Bregs upon induction with TLR agonist, CD40 agonist or cytokines such as IL-21, IL-6, IL-35 and IL-1β. It has been shown the B cells can transition into Bregs from various developmental stages, including plasma B cells, but the mechanisms for this are not completely understood.

Figure1: Differentiation and development of B cell subsets.

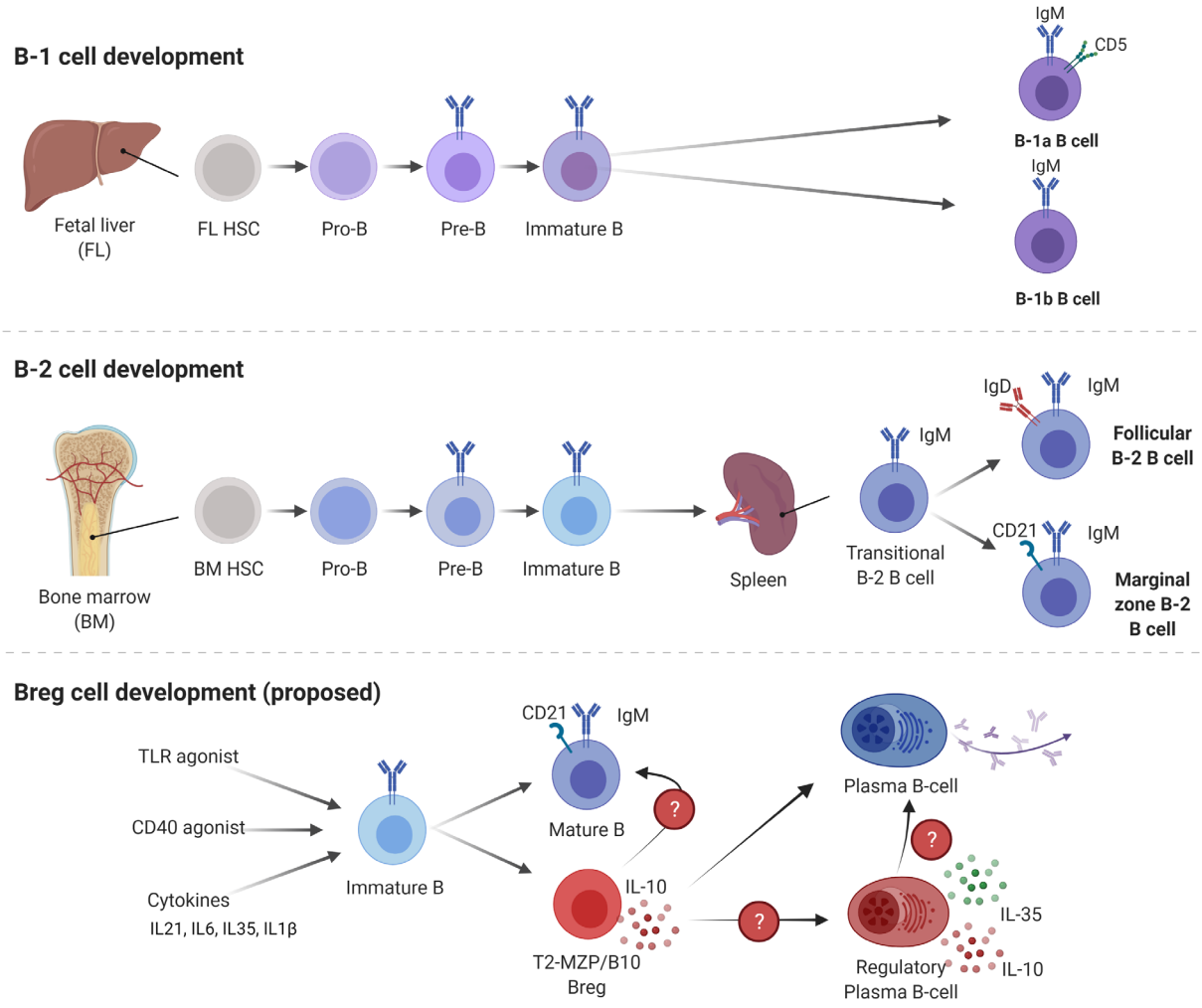

Figure 2: Phases of B cell development that form the adult peripheral B cell pool.

The first phase of B cell development has been reported with presence of B1 cells in yolk sac or aorta-gonad-mesonephros in mice. A dedicated HSC or common lymphoid progenitor (CLP) have not yet been identified for this population, so their development is not yet understood, and if they make it to the adult peripheral B cell pool is also not known. If a similar population of B1 cells arises in human embryo as well that is not known yet. The earliest recorded B cell population in humans have been in fetal liver at 6 pcw (post conception weeks). This is where the second phase of B cell development initiates in both humans and mice. In this phase most of the B cells that are generated fall in to B1 cell category and a few B2 cells are generated. Finally, the third phase of B cell development takes place after-birth in bone-marrow (mice and humans). During this phase most of the B cell population that is generated is B2 cells and very few B1 cells are also developed. B1 and B2 cells that arise during the second phase make it into the adult peripheral pool, but their population significantly drops after birth and only a few surviving cells remain. Most of the adult peripheral B cell pool consist of the cells that arise from the third phase of B cells development and consists mostly of B2 cells.

Figure 2: Phases of B cell development that form the adult peripheral B cell pool.

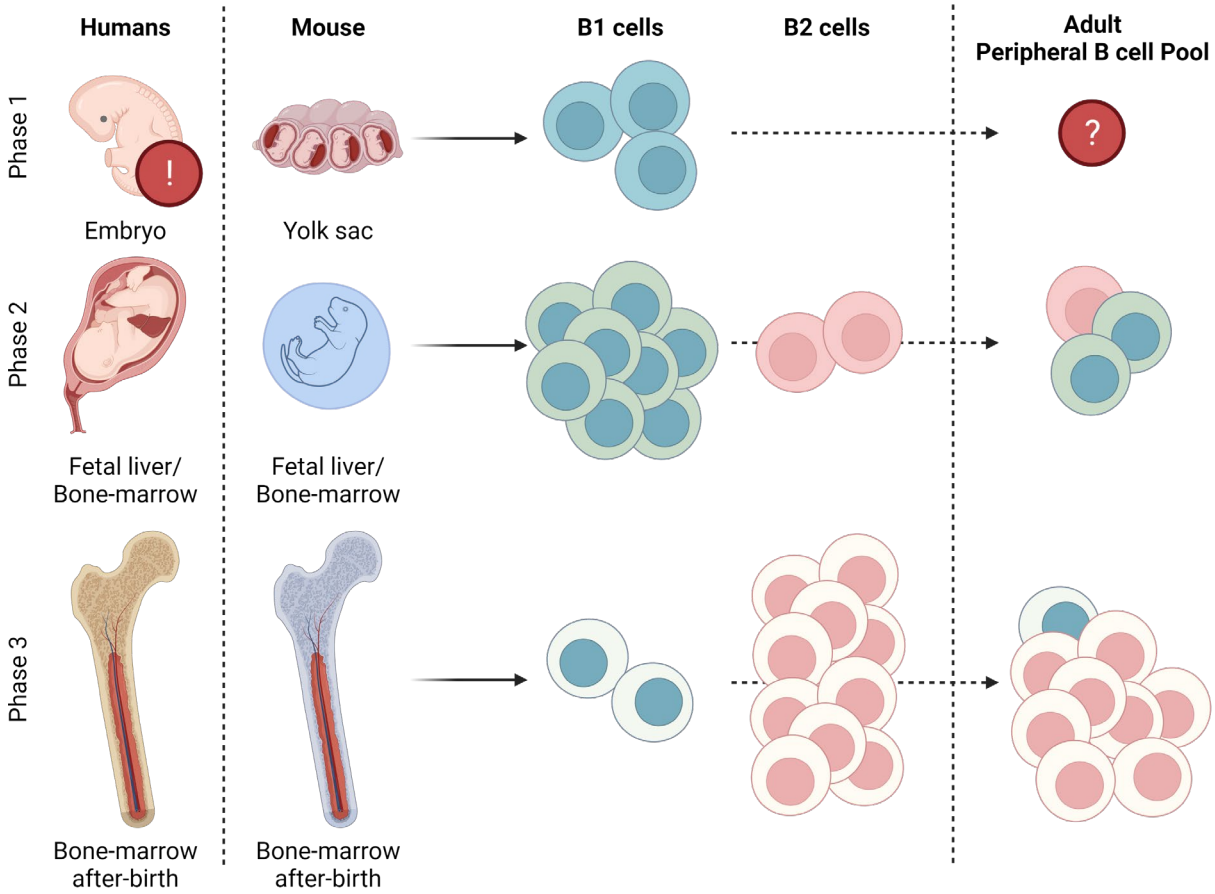

Table1: Expression profile of cell surface and intracellular markers characteristic of developmental stages of B cells in humans and mice.

Cell surface markers for identification of several B cell subsets are shown for both mouse and humans. For CLP, BLP, B1 and B2 progenitor cells: Lin- is CD3- CD4- CD8- Gr-1- CD11b- TER-119-. For PrePro B, Pro B and Pre B cells: Lin- = CD3- Gr-1- CD11b- TER-119-. B cell subsets for which cell surface expression profiles are either not known or not clearly defined are not shown and their respective columns are left blank with a hyphen.

Table 1: Expression profile of cell surface and intracellular markers characteristic of developmental stages of B cells in humans and mice.

Developmental Stage	Mouse	Humans
Common Lymphoid Progenitor	Lin- CD117- Ly6- Ly6D- IL-7 Ra+ Flt-3+	CD10+ CD34+ Pax5+
B lymphocyte Progenitor	Lin- CD117- Ly6- Ly6D+ IL-7 Ra+ Flt-3+	CD10+ CD34+ Pax5+
B1 Progenitor	Lin- CD45R low CD19+ CD93+	-
B2 Progenitor	Lin- CD45R+ CD19- CD93-	-
Pre-Pro B cell	Lin- CD45R+ CD19- CD24 low CD43+ CD93+ CD117- CXCR4+ FLt-3+ IL-7Ra+ IgM-	CD117 low CD10+ CD34+ CD38+ Pax5+
Pro-B cells	Lin- CD45R+ CD19+ CD24 + CD43+ CD117 low IL-7Ra+ IgM-	CD117 low CD10+ CD19+ CD20+ CD24+ CD34+ CD38+ CD93+ IL-3R+ IL-7 Ra+ Pax5+
Pre-B cells	Lin- CD45R+ CD19+ CD24 + CD43- IL-7Ra+ IgM-	CD117- CD10+ CD19+ CD20+ CD24+ CD34- CD38+ CD93+ IL-3 R+ IL-4 Ra+ IL-7 Ra+ Pax5+
Immature B cells	CD45R+ CD19+ CD23- CD24+ CD43- CD93+ IgD- IgM+	CD117- CD10+ CD19+ CD20+ CD21+ CD24+ CD27- CD38+ CD40+ CD93+ IL-4 Ra+ IL-7 Ra-
B1a Cells	CD1d mid CD5+ CD19 high CD23- CD43+	-
B1b cells	CD1d mid CD5- CD19 high CD23- CD43+	-
Transitional B cells	CD45R+ CD19+ CD24+ CD43- CD93+ IgM+ IgD low/+ (T1/T2)	CD10 low CD5+ CD19+ CD20+ CD21+ CD23+ CD24+ CD27- CD38+ CD93+ TACI+
Marginal Zone B cells	CD45R+ CD1d+ CD19 mid CD21 high CD23- CD43- CD93- IgM high IgD low	CD1c+ CD19+ CD20+ CD21+ CD27+ FCRL3+ TACI+
Follicular B cells	CD45R+ CD1d mid CD19 mid CD21 low CD23+ CD43- CXCR5+ IgM low IgD high	CD10- CD19+ CD20+ CD21+ CD22+ CD23+ CD24 low CD27- CD38 low CXCR5+ TAC+ MHC II+
Activated Germinal Center B cells	CD45R+ CD19+ CD40+ MHC II+	CD19+ CD20+ CD27+ CD38+ CD40+ CD83+ TACI+ MHC II+
Memory B cells	CD45R low CD19+ CD21+ CD27 mid/+ CD40+ MHC II+	CD19+ CD20+ CD21+ CD27+ CD93- TACI+

Plasmablast	CD45R low CD19+ CD27 high CD38+ CD138+	BCMA+ CD19 low CD27 high CD38+ CD93+ CD138-/low
Plasma cells	CD45R low BLIMP1+ CD19- CD27 high CD38 low CXCR4 high CD138+ MHC II -/low	BCMA+ BLIMP1+ CD19 low CD20-/low CD27 high CD38 high CD138+ CXCR4+ MHC II low
Regulatory B cells	CD1d+ CD5+ CD19+ CD23-/low CD24+ CD93-/low TIM-1+ IL-10+ IL-35+ TGF-β+	CD1d+ CD5+ CD19+ CD21+ CD24+ IL-10+ IL-35+ TGF-β+

Table2: Expression of cell surface markers in different phenotypes of Breg cells and their function in mice and humans.

A list of different B cell subset that have been identified to have regulatory B cell functions is shown. In table 2a, a list of Breg types that have been identified in mice, their expression profiles, location, and functions are listed. Similarly in table 2b, a list of Breg types that have been identified in humans, their expression profiles, location, and functions are listed.

Table 2: Expression of cell surface markers in different phenotypes of Breg cells and their function in mice and humans.

2a. Mice

Breg Type	Expression Profile	Location	Function
T2-MZP cells	CD19+ CD21 high CD23 high CD24 high	Spleen	IL-10 production, induction of Tregs, suppression of CD4 and CD8 T cells
MZ cells	CD19+ CD21 high CD23-	Spleen	IL-10 production, induction of Tregs, suppression of CD4 and CD8 T cells
B10 cells	CD5+ CD1d high	Spleen	IL-10 production, induction of Tregs, suppression of CD4 T cells, monocytes and DCs
Plasma cells	CD138+ MHCII low B220+	Spleen	IL-10 and IL-35 production, suppression of NK cells and effector CD4 T cells
Tim-1+ B cells	Tim-1+ CD19+	Spleen	IL-10 production and suppression of effector CD4 T cells
Plasmablasts	CD138+ CD44 high	Draining Lymph Nodes	IL-10 production and suppression of effector CD4 T cells and DCs

2b. Humans

Breg Type	Expression Profile	Location	Function
B10 cells	CD24 high CD27+	Blood	IL-10 production, suppression of DCs, monocytes, and effector CD4 T cells
Plasmablasts	CD119+ CD24 high CD27 int	Blood	IL-10 production, suppression of DC and effector CD4 T cells
Immature cells	CD19+ CD24 high CD38 high	Blood and site of inflammation	Production of IL-10, induction of Treg, suppression of Th1 and Th17, support iNKT homeostasis
Br1 Cells	CD19+ CD25 high CD71 high	Blood	Production of IL-10 and IgG4

Table 3: Expression of co-stimulatory and activation molecules on B cells during antigen presentation.

A list of activation molecules and other co-stimulatory molecules that are expressed on B cells during antigen presentation is shown. Their known ligands and their functions upon interaction are also listed. These interactions are critical for generating a robust immune response and successful activation of antigen-specific T cells.

Table 3: Expression of co-stimulatory and activation molecules on B cells during antigen presentation.

Receptor	Ligand	Function
CD80/86	CD28 and CTLA4	T cell activation and survival (CD28), inhibitory regulation of activated T cells (CTLA4)
CD83	CD83L	Prolonged expansion of CD8 T cells
CD278L (ICOSL)	CD278 (ICOS)	Stimulation and proliferation of T cells
CD134L (OX40L)	CD134 (OX40)	Stimulation and promotion of IgG response
CD137L (4-1BBL)	CD137 (4-1BB)	Stimulation of effector T cells
CD40/CD40L	CD40L/CD40	Activation, maturation, differentiation, proliferation, isotype switching, survival, cytokine production, memory response development
CD22	N-linked oligosaccharides	Adhesion and BCR signaling modulation
CD81	No natural ligands known yet	Credited towards adhesion and T cell-dependent B cell activation
CD11a-CD18/CD54 (LFA1/ICAM1)	CD54/CD11a-CD18 (ICAM1/LFA1)	Cell adhesion and enhanced activation & antigen presentation
CD72	CD100	Enhanced antigen presentation, development of B1b cells and production of high affinity IgG response

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Chapter 2

B cells licensed by DCs are the primary antigen presenting cells for plasmid DNA vaccine

Most of the data that forms this chapter has been presented as a poster either at annual meeting of "Society of Immunotherapy in Cancer" - 2019 or at annual meeting of "The American Association of Immunologists (AAI)" - 2022.

The work in this chapter has also been prepared as a manuscript and is currently under process of submission for publication in "Journal of Immunotherapy in Cancer." All the experiments that form this chapter were performed and analyzed by me.

Abstract

DNA vaccines have been an attractive approach for treating cancer patients, however, have demonstrated modest immunogenicity in human clinical trials. Dendritic cells (DC) are known to cross-present DNA-encoded antigens expressed in bystander cells. However, we have previously reported that B cells, and not DC, serve as primary antigen-presenting cells (APCs) following passive uptake of plasmid DNA. Here we sought to understand the requirements for B cells to present DNA-encoded antigens, to ultimately increase the immunogenicity of plasmid DNA vaccines. OT-1 CD8+ T cells and isolated APC populations were used to study antigen-presentation and T-cell activation. T-cell activation was measured by IFNγ release and proliferation was assessed by flow cytometry. Cell-cell interactions were studied using trans-well plate studies, and RNA-seq was used to evaluate transcriptional changes in B cells. We demonstrated that, following passive uptake of plasmid DNA, B cells, but not DC, can translate the encoded antigen. However, CD8 T cells were only activated by B cells when they were co-cultured with DCs. We demonstrated that a cell-cell contact is required between B cells and DCs. Using MHCI KO and re-purification studies, we demonstrated that B cells were the primary APCs and DCs serve to license this function. We demonstrated that there is a large difference in the gene expression profiles of B cells that have been licensed by DCs, compared to the B cells that have not, and that these share similar gene expression signatures to B cells activated with a TLR7/8 agonist. Our data demonstrate that B cells transcribe and translate antigens encoded by plasmid DNA following passive uptake, however, require licensing by live DC to present antigen to CD8 T cells. Further study of the role of B cells as APCs will be important to improve the immunological efficacy of DNA vaccines. Future investigations could result in the development of B cell-centered novel therapeutic approaches for the treatment of cancer patients.

Introduction

Given the current success of mRNA vaccines that have been developed for COVID-19, there has been increased interest in understanding the mechanism of action of nucleic acid vaccines (1-4). Nucleic acid vaccines, using either mRNA or DNA, essentially work on the same principle, that they require pre-processing by antigen-presenting cells (APCs) to translate the encoded antigen into a protein. That protein is then either directly presented or cross-presented by professional APCs to activate antigen-specific T cells (5). While mRNA vaccines and DNA vaccines are similarly appealing as potential therapeutic strategies for cancer treatment, DNA vaccines in particular have demonstrable safety, easy manipulation, scalability, stability, and economical manufacturing (6). However, while a DNA vaccine has been approved for canine melanoma (7), early phase clinical studies in humans have been generally disappointing (8). Further studies to understand their mechanism of action, in order to improve their immunogenicity, are therefore needed.

Current delivery approaches use intradermal or intramuscular injections, with or without adjuvants, and often with or without electroporation. The majority of administered DNA is encountered by local non-professional APCs such as dermal cells and myocytes (9). Some of the tissue-resident professional APCs, such as B cells, dendritic cells (DC) and macrophages, can also encounter the DNA vaccine. Studies in murine models have demonstrated that DC are required, but they function primarily to cross present antigens produced by bystander cells that have taken up and expressed DNA-encoded antigens (10). In fact, studies using DNA plasmids encoding antigens under a DC-specific promoter failed to elicit immune responses in murine studies (11, 12).

These observations led us to explore whether subsets of professional APCs could serve as primary APC. The identification of primary APC could be advantageous. We have previously reported that upon passive uptake, professional APC subsets process plasmid DNA differently (13). We have reported that DCs and macrophages capture the plasmid DNA by phagocytosis after which it undergoes endosomal/lysosomal degradation. On the contrary, B cells capture the DNA by macro-pinocytosis and translocate it to the nucleus where the encoded antigen is transcribed (13). This implies that only B cells can effectively process the naked plasmid DNA amongst the professional APC subsets. This suggests that targeting DNA vaccines to B cells specifically and understanding the requirements for antigen presentation by B cells, could be important to improve the immunogenicity of DNA vaccines.

In this chapter we analyzed B cells and the requirements for their antigen presentation capability following passive uptake of plasmid DNA. We demonstrate that B cells transcribe, translate, and present encoded antigen to CD8+ T cells, but require DCs to license their antigen-presentation capacity via cell-cell interaction(s). Following DNA uptake and exposure to DC, the phenotype of B cells changed dramatically, with gene expression signatures similar to those of B cells activated by TLR7/8 agonists and through the B-cell receptor. Future studies will explore the specific receptors on B cells that become activated by DCs, as this may enable next generation DNA vaccine approaches using DNA-loaded autologous B cells as APCs.

Results

B cells translate antigen encoded by plasmid DNA when co-cultured with DCs

We previously reported that if purified B cells, DCs or macrophages isolated from C57Bl/6 mice spleens were individually incubated with plasmid DNA encoding ovalbumin or SSX2, only B cells were able to transcribe the encoded antigen (13). Based on these results, we wished to evaluate the requirements for B cells to present antigen to CD8 T cells, and whether known B cell activating agents might further augment antigen presentation. After overnight incubation of B cells with OVA DNA, either CD8 T cells from OT-1 mice or B3Z (T cell Hybridoma, specific for OVA) were added to the culture with or without activation agents. These agents included CD40L, anti-CD40 mAb, BAFF, IL4 and IL2. As shown in Figure 1A, surprisingly, B cells were unable to activate antigen-specific CD8 T cells, as demonstrated by the absence of β-gal activity from B3Z cells or secreted IFN-γ from OT-1 CD8 T cells (Fig 1A).

Because we did not detect CD8 T-cell activation, despite upregulation of APC machinery, we next evaluated whether the antigen encoded by DNA was translated in different APC subsets. We cultured B cells and DCs, either individually or together, and incubated with DNA encoding GFP. As shown in Figure 1B, we identified a very small percentage of B cells that expressed GFP, exclusively in the group where B cells and DCs were in co-culture. On the contrary, DCs did not express GFP, either when cultured alone or with B cells. Collectively, our results indicated that upon passive uptake of plasmid DNA, B cells were the only subset of professional APCs that would transcribe and translate the encoded antigen. However, for translation of the encoded antigen, B cells required co-culture with DCs.

DNA loaded B cells activate antigen-specific CD8 T cells when co-cultured with DCs

As demonstrated above, translation of encoded antigen occurred only when B cells and DCs were in co-culture. Hence, we next tested this co-culture for *in vitro* antigen presentation using a plasmid DNA encoding ovalbumin. Purified B cells and DCs were incubated with OVA plasmid DNA either individually or in co-culture. The following day, ovalbumin-specific PKH67-labeled CD8 T cells (from OT1 mice) were added to the culture. As in Figure 1B, we analyzed CD8 T cells for activation and proliferation. Absence of secreted IFN-γ (Fig 2A) and proliferation of CD8 T cells (data not shown) signified that neither individual culture nor co-culture of B cells and DCs could elicit CD8 T cell activation after passive uptake of DNA in this *in vitro* system. In addition, we also examined if inclusion of OVA specific CD4 T cells (OT2 mice) and/or GM-CSF to this co-culture could augment antigen presentation through B cells. Both of them were added along with PKH67-labeled CD8 T cells. GM-CSF was added to maintain and promote survival of DCs *in vitro*, whereas CD4 T cells were included to support antigen presentation through B cells. Neither CD4 T cells nor GM-CSF was able to promote activation of antigen-specific CD8 T cells, as demonstrated by absence of IFN- γ (Fig 2B) and CD8 T cell proliferation (data not shown).

Reasoning that DCs might outcompete B cells for DNA uptake, we next tested providing DNA to B cells prior to culture with DCs. Specifically, DCs were added to the culture 24 hours after B cells had been loaded with OVA DNA. As shown in Figure 2C, we observed proliferation and activation of antigen-specific CD8 T cells, but only when B cells were cultured with DC. Similarly, we also observed an increased amount of secreted IFN-γ from the supernatant of B cell and DC co-culture, compared to individual cultures (Fig 2C). Importantly, addition of CD4 T cells appeared to be required in this co-culture, since we observed a loss in CD8 T cell proliferation and

IFN-γ secretion in the absence of CD4 T cells (Fig 2C). Individual cultures of B cells or DCs, each loaded with DNA, did not display proliferation or activation of CD8 T cells, with or without addition of CD4 T cells (Fig 2C).

CD4 T cells and IL4 each promote B cell activation and survival to support *in vitro* antigen processing and presentation

We next tested whether the CD4 T cells needed to be antigen-specific in this *in vitro* system. B cells were loaded with DNA as before and cultured with DCs and antigen-specific (from OT2 mice), or antigen non-specific (from C57Bl/6 mice), CD4 T cells. Both, CD4 T cells from C57Bl/6 mice and from OT2 mice were able to similarly activate CD8 T cells as demonstrated by levels of secreted IFN-γ by the two groups (Fig 3A). This suggested that CD4 T cells were playing a helper cell role in this co-culture, potentially by release of a cytokine. Others have demonstrated that IL4 can substitute for the helper function of CD4 T cells (14, 15), and hence we specifically evaluated IL4. As shown in Fig 3B and 3C, we found that the requirement for CD4 T cells with DNA-loaded B cells and DCs to activate CD8 T cells, and lead to IFN-γ release, could be replaced by co-culture with IL-4. We further analyzed the activation status of B cells following culture with either CD4 T cells and/or IL4. We observed increased expression of CD83, CD86, and MHCII, and a greater number of live B cells, after culture with CD4 T cells or IL4 (Fig 3D), in B cells and DCs co-culture groups. This demonstrated that either CD4 T cells or IL4s activated B cells and promoted their survival.

B cells licensed by DCs are the primary antigen presenting cells for plasmid DNA

As co-culture of DNA-loaded B cells and DCs was required for activation and proliferation of antigen-specific CD8 T cells, there was a possibility that DCs were either cross dressing or cross presenting the antigen generated by the B cells. To address this, we first re-purified DNA-loaded B cells and DCs after three days of co-culture using magnetic bead selection. These re-purified B cells and DCs were then individually cultured with PKH67-labeled CD8 T cells in the presence of GM-CSF and IL4. As shown in Fig 4A and 4B, only re-purified B cells, and not re-purified DC, were able to activate CD8 T cells, leading to their proliferation and release of IFN-γ. To further address the APC cell type directly activating CD8 T cells in this system, we performed similar studies using B cells and DCs from MHCI knockout (MHCI-KO) mice. We found that MHCI-KO DCs did not affect the activation and proliferation of CD8 T cells, or the levels of secreted IFN-γ. On the contrary, use of MHCI-KO B cells negatively impacted activation and proliferation of CD8 T cells and resulted in significantly lower levels of secreted IFN-γ (Fig 4C and 4D). Collectively, these data demonstrate that B cells were the primary antigen presenting cells in this co-culture and interacted directly with CD8 T cells. DCs, on the other hand, functioned as helper cells that enabled and licensed B cells to process the antigen encoded in plasmid DNA for presentation through MHCI.

Cell-cell interaction between B cells and live DCs is essential for licensing of B cells by DCs

We next sought to understand the nature of interaction between B cells and DCs. Our first approach was to test if protein(s) expressed on the surface of DCs or factor(s) secreted by DCs were essential

in licensing of B cells. For this, we prepared (1) lysates from DCs, and (2) supernatant from cultured DCs. We utilized these fractions in lieu of whole live DCs either alone or in combination. Neither of these DC fractions were able to satisfy the requirement of whole live DCs by DNA-loaded B cells, as demonstrated by the loss in CD8 T cell proliferation and secreted IFN-γ (Fig 5A-B). Similarly, paraformaldehyde-fixed DCs and heat-killed DCs were not able to replace live DCs in the *in vitro* system (data not shown). We further tested if there was requirement of physical interaction between B cells and DCs using trans-well plates. Each one of the physical separations of DNA-loaded B cells, DCs, or CD8 T cells resulted in loss of CD8 T cell proliferation and loss in secreted IFN-γ. It was only when all the three cell types; B cells, DCs and CD8 T cells were in the same well and interacted physically, we observed CD8 T cell proliferation and IFN-γ secretion (Fig 5C-D). These results suggested that a membrane-bound factor on live DC was required to license B cells to present a DNA-encoded antigen.

Cell-cell interaction between B cells and live DCs results in release of pro-inflammatory cytokines and chemokines

We next wished to determine functional changes that occurred in DNA-loaded B cells and DC following co-culture. We analyzed the supernatants from DNA-loaded B cells cultured with DCs for changes in secreted cytokines and chemokines. This was evaluated in the presence or absence of GM-CSF and IL-4. In line with our previous results, increased expression of cytokines such as IFN-γ, TNF-α, IL-1Ra, TIMP1 and RANTES were observed in the co-culture group (Fig 6). ICAM1 (soluble CD54) was also found at increased concentrations in the co-culture group (Fig 6). In terms of chemokines, we observed increased concentrations of MIP1α, and MIP1β in the co-

culture group, which was dependent on the presence of GM-CSF and/or IL4 (Fig 6). Furthermore, we observed increased expression of other chemotactic proteins, CCL2, CXCL2, CCL17, CCL12 and IL16 in the co-culture group (Fig 6). These finding suggest that the interaction of DNA-loaded B cells with DC results in the production of several cytokines and chemokines that (1) promote antigen presentation, and (2) promote inflammatory responses and (3) promote chemotaxis of immune populations.

Distinct Gene Expression Patterns are observed in DNA-loaded B cells that are licensed by DCs

Finally, we wished to understand the changes occurring in B cells at the gene expression level, following their interaction with DCs. DNA-loaded B cells were cultured for 3 days with DCs and then separated into individual populations by flow cytometry. B cells were then analyzed by RNA-seq. Upon principal component analysis, all the biological replicates formed tight groups demonstrating minimal variance, however large variation was observed between B cells cultured with DCs and those not cultured with DC (Fig 7A). This was indicative of vastly different gene expression signatures. This was confirmed by MA plot showing log fold change (M) of each gene plotted against its mean average intensity/expression (A) (Fig 7B). There were 6845 genes that were significantly ($p<0.05$, adjusted for multiple comparisons) differentially regulated between the two groups. The top upregulated genes in B cells after co-culture with DCs were clustered based on their molecular function and biological processes using gene ontology. Most of these genes classified under the category of cytokine and chemokine related to immune system responses, more specifically related to inflammation type responses (Fig 7C, Fig 8). We then

performed gene set enrichment analysis (GSEA) (16, 17), to match our gene data set against prior defined B cell related gene-sets. Based on the enrichment scores and the relevance to APC function of B cells, we identified two prior defined gene sets most associated with DC-licensed B cells: B cells cultured with TLR7 agonist (imiquimod) versus TLR4 agonist (monophosphoryl lipid A) (Fig 7D), and B cells simulated through IgG (Fig 7E). Together, these gene sets suggested that the B cells licensed by DC displayed a more activated phenotype, associated with antigen presentation, and activation by TLR and/or the B cell receptor.

Based on the findings from the GSEA analysis, we investigated the effect of a TLR7 agonist on the APC function of B cells directly. This was performed with TLR7 agonist alone or in combination with other B cell activation agents such as CD40, CD40L and TLR9 agonist CpG. None of these treatments induced DNA-loaded B cells to activate CD8 T cells in the absence of DCs (Fig 9). We also surveyed the literature for known cell surface interactions between B cells and DCs. We tested the possible role of the most prominent interactions associated with antigen presentation by using blocking antibodies targeting CD23-IgE or CD70-CD27. In addition, we evaluated the CD40-CD40L interaction using APC from CD40 KO mice. Blockade of the CD70-CD27 interaction did not affect CD8 T cell activation or levels of secreted IFN-γ (Fig. 10B). On the other hand, blockade of CD23, or blockade of CD40-CD40L by use of B cells and DCs from CD40-KO mice, impeded but did not entirely abrogate CD8 T cell proliferation and secretion of IFN- γ (Fig. 10A, 10C).

Taken together our data suggest that possibly multiple cellular interactions between B cells and DC, including CD40-CD40L and CD23-IgE, lead to activation of B cells that have increased antigen presentation function. Our future studies will be focused on understanding these specific

interactions between B cells and DCs and how this leads to changes in B cell antigen presentation function.

Discussion and Conclusion

In this chapter, we demonstrated that B cells that have taken up DNA by passive transfer can translate the encoded antigen but require co-culture with DCs to present the encoded antigen to activate CD8 T cells. This presentation to CD8 T cells is by B cells, and not via cross-presentation by DCs. Further, we demonstrated that this is due to a cell-cell interaction that requires CD4 T cells or IL4, and this encounter results in an inflammatory response with release of multiple cytokines and cell attractant chemokines. In addition, culture of DNA-loaded B cells with DCs results in a dramatic change in B cell phenotype as evidenced by changes in gene expression profiles. As such, we provide the first evidence, to our knowledge, of DCs providing a licensing function to B cells, facilitating their function as APCs. The role of B cells as APC has been largely understudied relative to their role in humoral immunity. Our findings may be relevant to the anti-tumor role of B cells in tumors and are certainly of relevance to studies to enable the use of B cells as APCs for anti-tumor vaccines.

To our knowledge, this is the first evidence of B cell licensing through DCs. The precise signaling between these cells type for this licensing remains unknown. One known natural interaction between B cells and DCs is when germinal center B cells capture antigen complexes from the surface of follicular DCs (18). Apart from BCR stimulation by the antigen complex on DCs, other cell surface molecules like ICAM-1, VCAM-1 and BAFF are expressed on DCs that interact with their ligands LFA-1, VLA-1, and BAFF-R respectively on B cells, hence these are potential ligand-receptor interactions (18). Interactions involving integrins ICAM-1 and VCAM-1 have been shown to facilitate B cell survival (19). Furthermore, Carrasco. et. al., have demonstrated that ICAM-1/LFA-1 interaction promotes B cell adhesion and synapse formation by lowering the

antigen threshold for B cell activation (20). On the other hand, stimulation through BAFF has been known to generally promote B cell survival, activation and maturation (21). More specifically BAFF signaling has been demonstrated to promote maintenance of germinal centers (22). In our cytokine array, we found upregulation of soluble ICAM-1 in the co-culture groups. This suggests that ICAM-1 expressed by DCs in this co-culture, and the ICAM-1/LFA-1 interaction, could play a role in promoting antigen processing and presentation by B cells. Currently, we are uncertain about the exact interactions that are occurring during this co-culture. It could also be a combinatorial effect of cell surface interaction(s) and secreted cytokines during the co-culture. We could not identify any specific interaction from our RNA-seq data analysis. However, from our blocking and knockout studies, we know that there could be several possibilities. Understanding how each of these interactions is important for APC function of B cells is one of our future directives.

In response to co-culture with DCs, we found that B cells changed their transcriptional phenotype dramatically. From our RNA-seq analysis, we showed that this new phenotype is representative of B cells that have been stimulated through BCR and TLR7/8. It has been known that signaling through BCR is critical for B cell activation and differentiation upon interaction with antigen (23). Internalization of antigen by BCR is the primary mode of antigen processing and presentation by B cells. This leads to BCR oligomerization and subsequently presentation of peptide through MHC (24). It is unknown whether activation of the BCR occurs following DC co-culture, or whether the gene expression profile of DC-licensed B cells is just similar to those of BCR-activated B cells. Similarly, it is currently not understood if the DC-licensed B cells are activated through TLR7/8 signaling or if their profile resembles that of TLR7/8 activated B cells. TLR7/8 activation has been

shown to promote B cell proliferation, induce expression of co-stimulation molecules and augment antigen-specific immunoglobulin production (25). Notwithstanding, these findings support the observation of a change in phenotype to cells with antigen-presentation capacity

The role of B cells as APC has been largely understudied. This has now become of more relevance, as recent reports have demonstrated that presence of tumor infiltrating B cells correlate with better prognosis of the disease. In particular, an increased number of tertiary lymphoid structures, where these B cells reside, have been associated with increased survival of cancer patients. Although their specific role in the tumor microenvironment in not clear, there are indications that these B cells are capable of presenting antigen, as demonstrated by increased expression of antigen presentation related surface markers. This makes understanding the role of B cells as APCs of paramount importance, and whether they are functionally different from antibody-producing B cells or regulatory B cells requires further investigation. Moreover, it has been previously shown by us that upon priming of CD8 T cells through peptide loaded B cells or DCs, there were differences in the resulting checkpoint marker expression (26). Therefore, understanding the differences between B cells and other professional APC subsets in context of their capacity to activate antigen-specific T cells is also of importance.

Overall, our findings demonstrated that B cells are the primary professional APC that can directly process and present plasmid DNA to activate CD8 T cells. While our intent has been to focus on passive delivery of DNA to APC, relevant to most immunization methods, a limitation of our studies is that these findings may not be relevant for other methods of DNA delivery, including the use of nanoparticles, other transfection reagents, or electroporation. In fact, these other delivery methods may be desirable to bypass DNA degradation and promote direct presentation by DC.

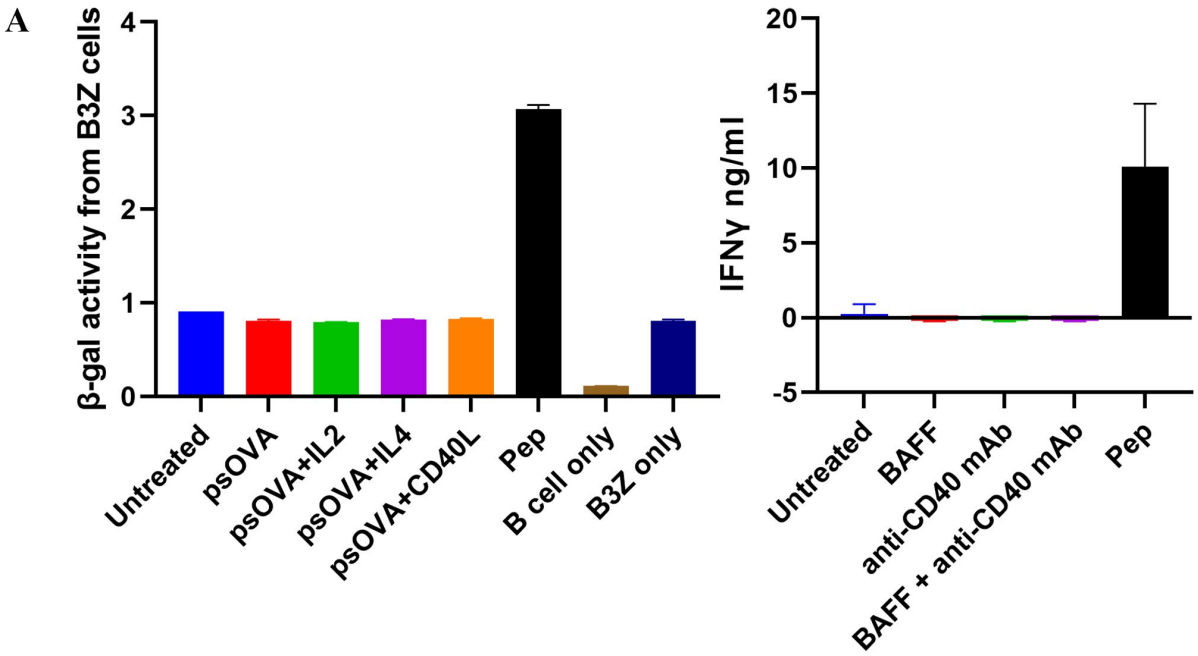
This will be a focus of future studies. In addition, future studies aimed at determining the precise signaling provided by DC to license B cells will be important to develop novel methods of vaccination. In particular, we expect these studies could target nucleic acids specifically to B cells or use DNA-loaded B cells for delivery as a cell-based therapeutic vaccine.

Figures and Tables

Figure 1: B cells translate antigen encoded by plasmid DNA when co-cultured with DCs.

B cells from C57Bl/6 mice were isolated from spleens using negative selection and incubated with ovalbumin-expressing plasmid DNA for passive uptake. Activation agents (BAFF, anti-CD40 mAb, CD40L, IL2, and IL4) and CD8 T-cells from OT-1 mice or B3Z cells (T cell hybridoma) were added to B cells the following day. After three to five days of incubation (A) β-galactosidase (β-gal) activity from B3Z cells was measured or IFN-γ secreted from OT-1 CD8 T cells was measured by ELISA. (B) B cells and DCs either alone or in combination were incubated with GFP plasmid DNA. After three days of culture cells were analyzed using Amnis imaging flow cytometer for expression of GFP.

Figure 1: B cells translate antigen encoded by plasmid DNA when co-cultured with DCs.



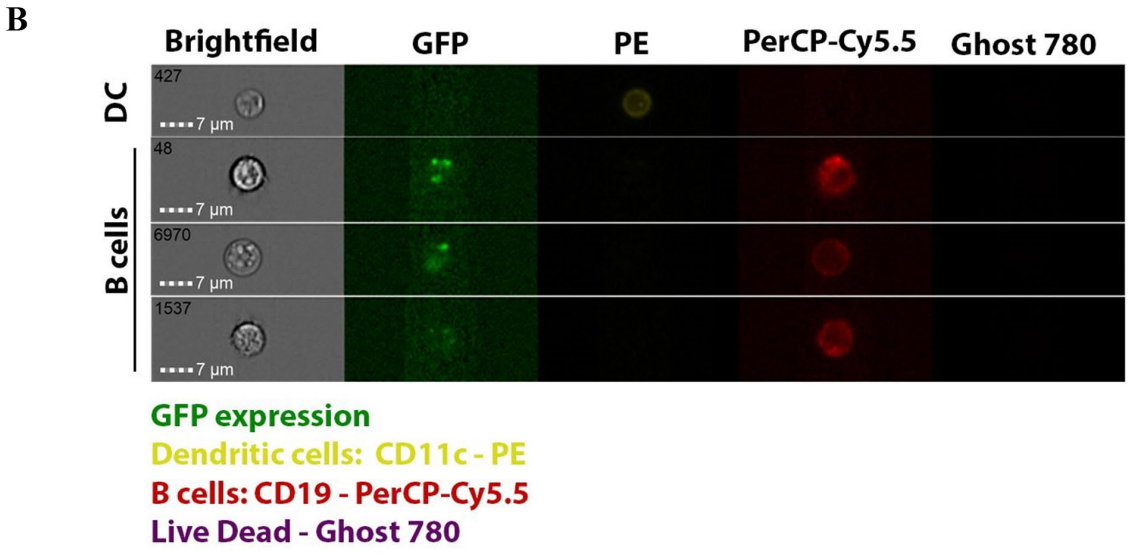


Figure 2: DNA loaded B cells activate antigen-specific CD8 T cells when co-cultured with DCs.

B cells and DCs were isolated using negative selection from C57Bl/6 mouse splenocytes. B cells and DCs were then incubated with OVA plasmid DNA either alone or in combination for passive uptake. CD4 T-cells negatively selected from OT2 mice spleens and GM-CSF were added to the culture on the following day along with PKH67-labeled CD8 T-cells negatively selected from OT1 mice spleens. (A, B) After five days of culture, supernatants were analyzed for IFN-γ secretion via ELISA. (C) Cell culture was performed similarly, except DCs were added the day following DNA loading of B cells, along with CD4 and CD8 T cells. Similarly, after five days of culture CD8 T cells were analyzed for proliferation and IFN-γ secretion was measured by ELISA. Asterisks (***) indicate $p<0.0001$, with comparison made between B cells and DCs co-culture groups. Results are from one experiment, with samples assessed in triplicate, and are representative of five similar, independent experiments.

Figure 2: DNA loaded B cells activate antigen-specific CD8 T cells when co-cultured with DCs.

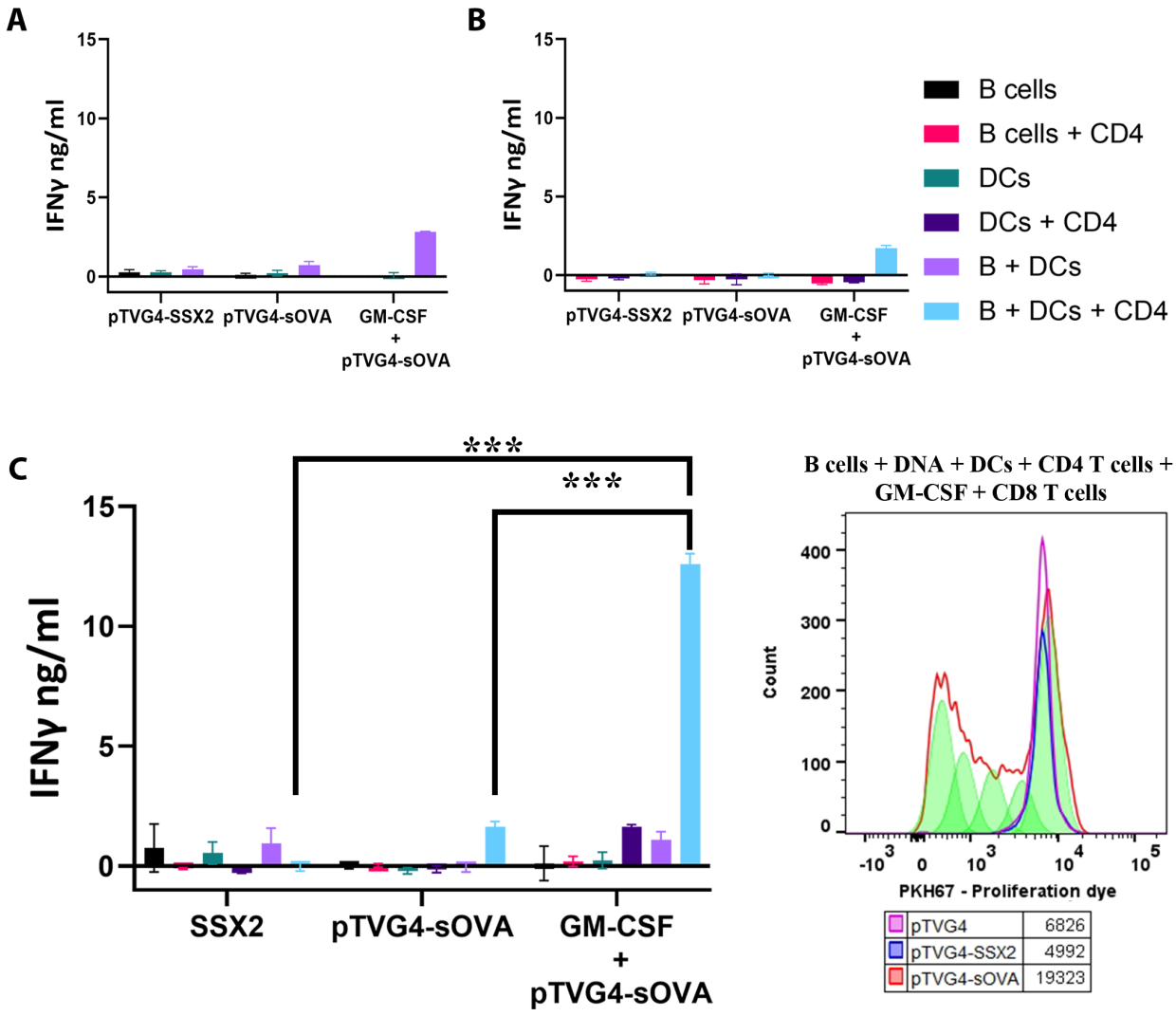


Figure 3: CD4 T cells and IL4 each promote B cell activation and survival to support *in vitro* antigen processing and presentation.

In vitro antigen presentation assay was set up as in Fig 2B, however, CD4 T cells were either isolated from C57Bl/6 mice spleens or OT2 mice spleens. (A) IFN- γ secretion as measured by ELISA. (B) CD4 T –cells were replaced by addition of IL4 to the co-culture, IFN- γ secretion and (C) proliferation of CD8 T-cells were measured. (D) Counts of live B cells and MFIs of CD83, CD86 and MHC II as recorded after two, four or six days of incubation with either CD4 T-cells, GM-CSF, IL4, and DCs either alone or in combination, by flow cytometry. Asterisks (****) indicate $p<0.0001$, results are from one experiment, with samples assessed in triplicate, and are representative of three similar, independent experiments.

Figure 3: CD4 T cells and IL4 each promote B cell activation and survival to support *in vitro* antigen processing and presentation.

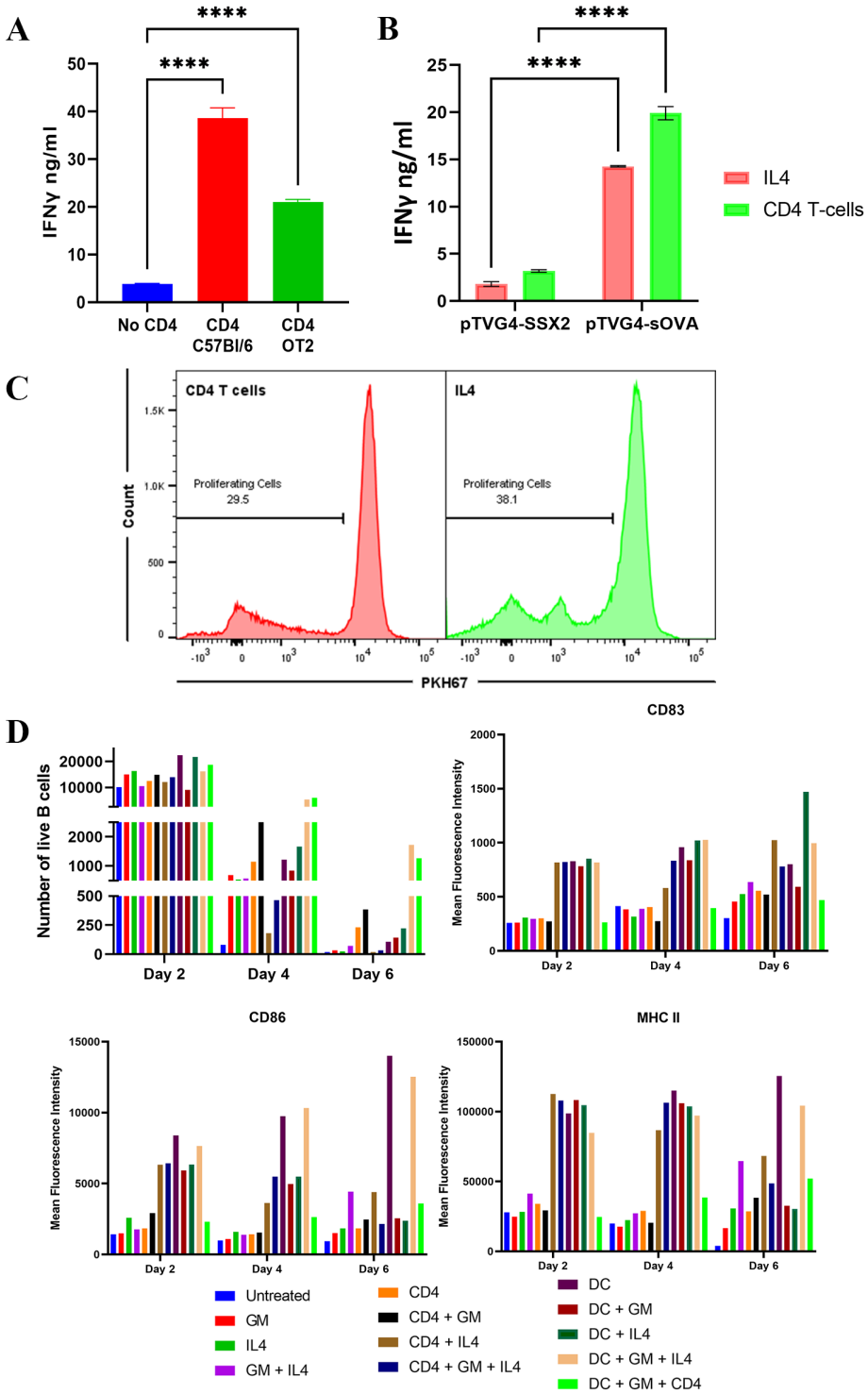


Figure 4: B cells licensed by DCs are the primary antigen presenting cells for plasmid DNA.

OVA plasmid DNA-loaded B cells were co-cultured with DCs and then re-purified after three days of co-culture. PKH67-labeled CD8 T-cells were added to either re-purified B cells or DCs. After four days of culture, CD8 T-cells were analyzed for (A) IFN- γ secretion and (B) proliferation. *In vitro* assay was set-up as in Fig 2B, however B cells and DCs were isolated from either C57Bl/6 mice or MHCI-KO mice spleens. After five days of incubation with PKH67-labeled CD8-T cells, (C) IFN- γ secretion and (D) proliferation were recorded. Asterisks * = $p<0.05$, ** = $p<0.01$ and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in triplicate, and are representative of three similar, independent experiments.

Figure 4: B cells licensed by DCs are the primary antigen presenting cells for plasmid DNA.

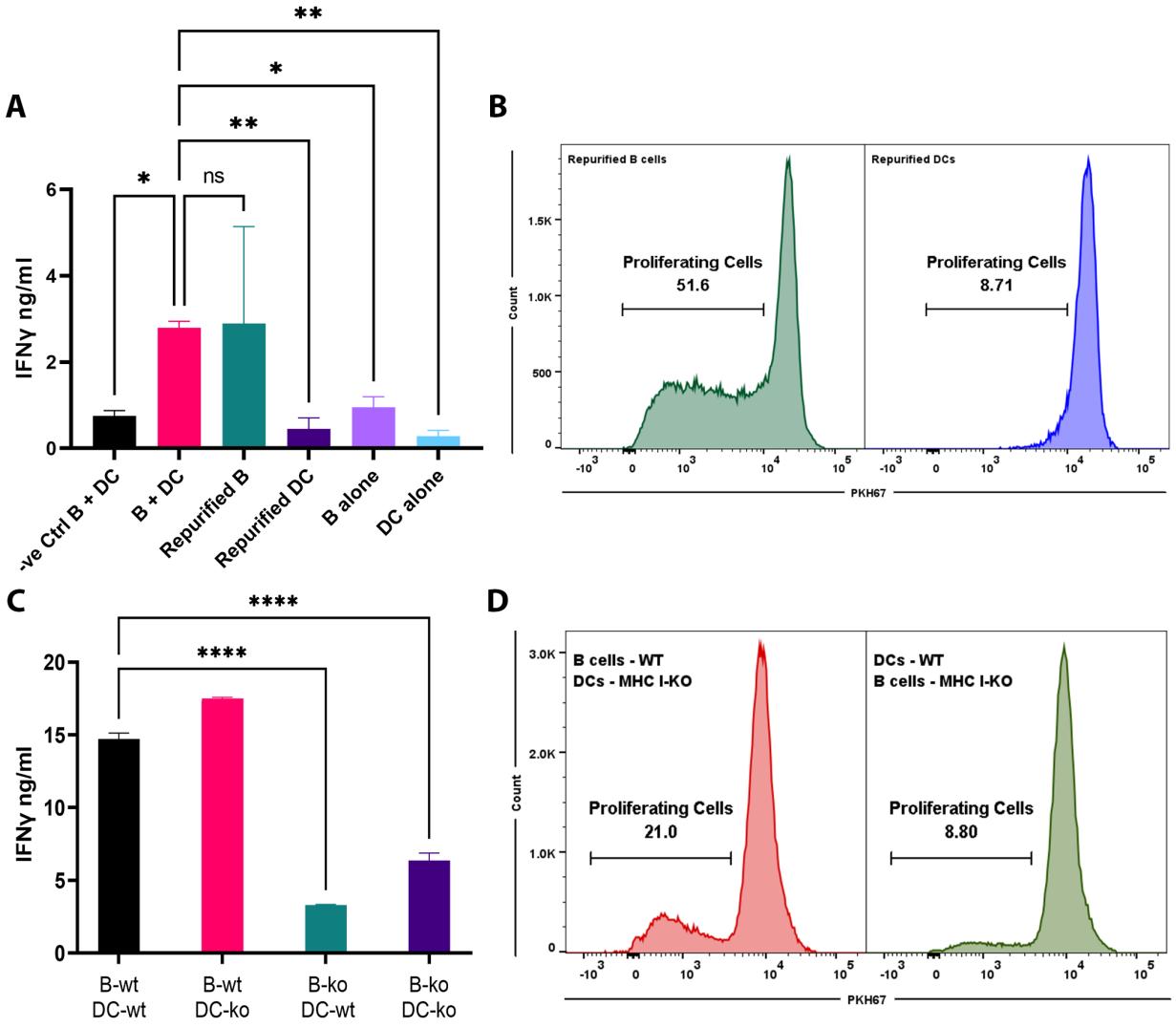


Figure 5: Cell-cell interaction between B cells and live DCs is required for licensing of B cells by DCs.

OVA DNA-loaded B cells and DCs were co-cultured as in Fig 2B but were separated by using trans-well culture plates. After five days of co-culture PKH67-labled CD8 T-cells were analyzed for (A) IFN- γ secretion and (B) proliferation. Using the similar *in vitro* antigen presentation set-up, we replaced live DCs with either lysates prepared from DCs by repeated freeze thaw followed by sonication or by supernatant collected from live DC cultures. (C) Secreted IFN- γ levels were measured by ELISA and (D) proliferation of PKH67-labeled CD8 T-cells were measured using flow cytometry. Asterisks ** indicate $p<0.01$ and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in triplicate, and are representative of three similar, independent experiments.

Figure 5: Cell-cell interaction between B cells and live DCs is required for licensing of B cells by DCs.

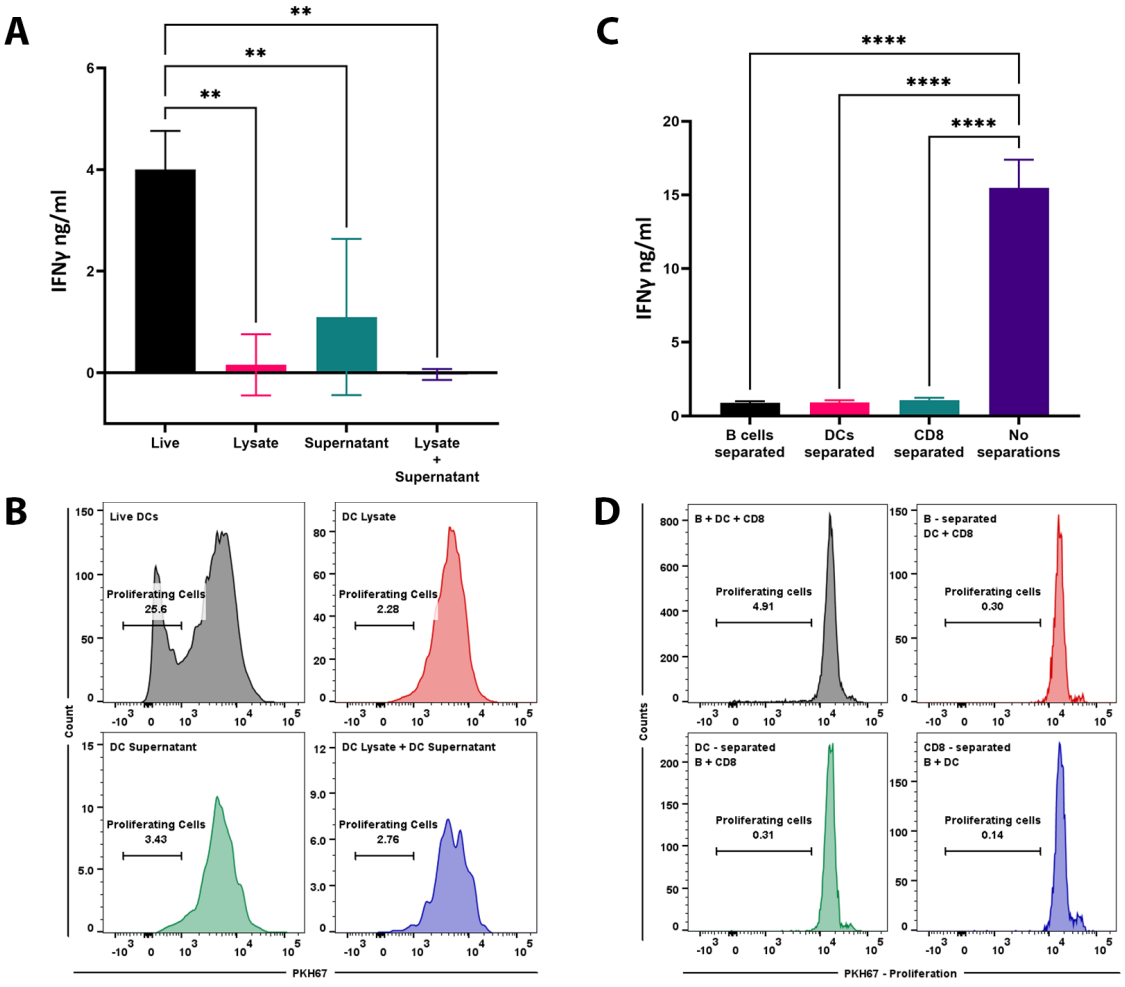


Figure 6: Cell-cell interaction between B cells and live DCs results in release of pro-inflammatory cytokines and chemokines.

In vitro antigen presentation assay was set up as in Fig 2B, and supernatants were collected after five days of culture. The supernatants were used for detection of immune-response related cytokines and chemokines using blot-based cytokine array. (A) Representative blots after exposure for ten minute using BioRad Chemi-Doc imaging system. (B) Relative expression was quantified by measuring the intensity of each band using NIH ImageJ software. Asterisks * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$ and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in duplicates, and are representative of three similar, independent experiments.

Figure 6: Cell-cell interaction between B cells and live DCs results in release of pro-inflammatory cytokines and chemokines.

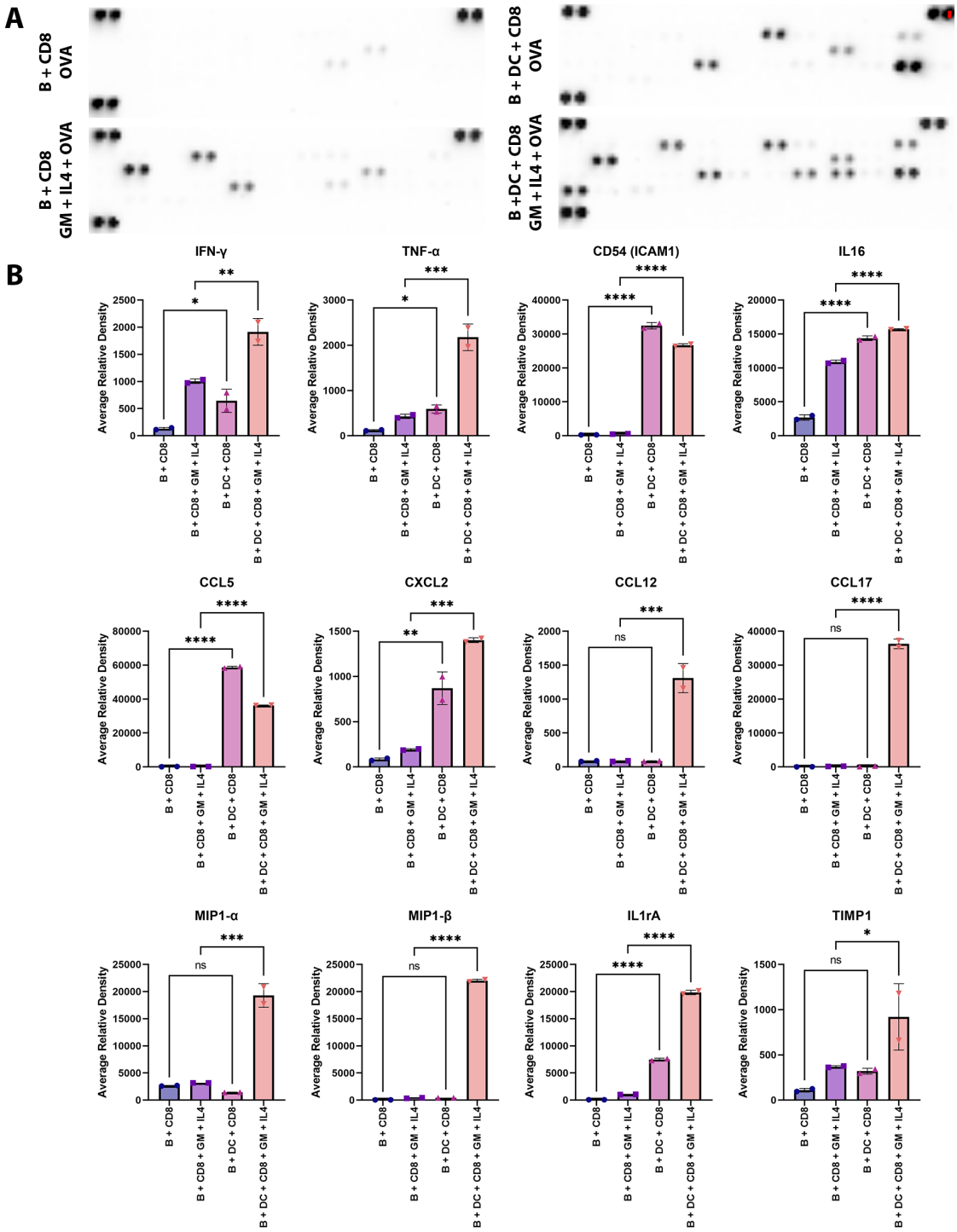


Figure 7: Distinct Gene Expression Patterns are observed in DNA-loaded B cells that are licensed by DCs.

DNA-loaded B cells were cultured, either alone or with DCs, with CD8 T-cells for three days. B cells were sorted using CD19 cell surface marker and processed for total RNA isolation. cDNA libraries were synthesized and indexed for sequencing. After analysis by using Project Galaxy, log2-fold changes in gene expression were calculated from both groups. (A) PCA and (B) MA plots were generated to demonstrate variance and significant changes in gene expression between the two treatment groups. (C) gProfiler, an online gene ontology tool, was used to categorize four hundred most upregulated genes in B cells after co-culture with DCs. (D) and (E) represent the two most prominent enrichment plots from GSEA analysis performed on the resulting gene list. Results are from one experiment, with samples assessed in six biological replicates.

Figure 7: Distinct Gene Expression Patterns are observed in DNA-loaded B cells that are licensed by DCs.

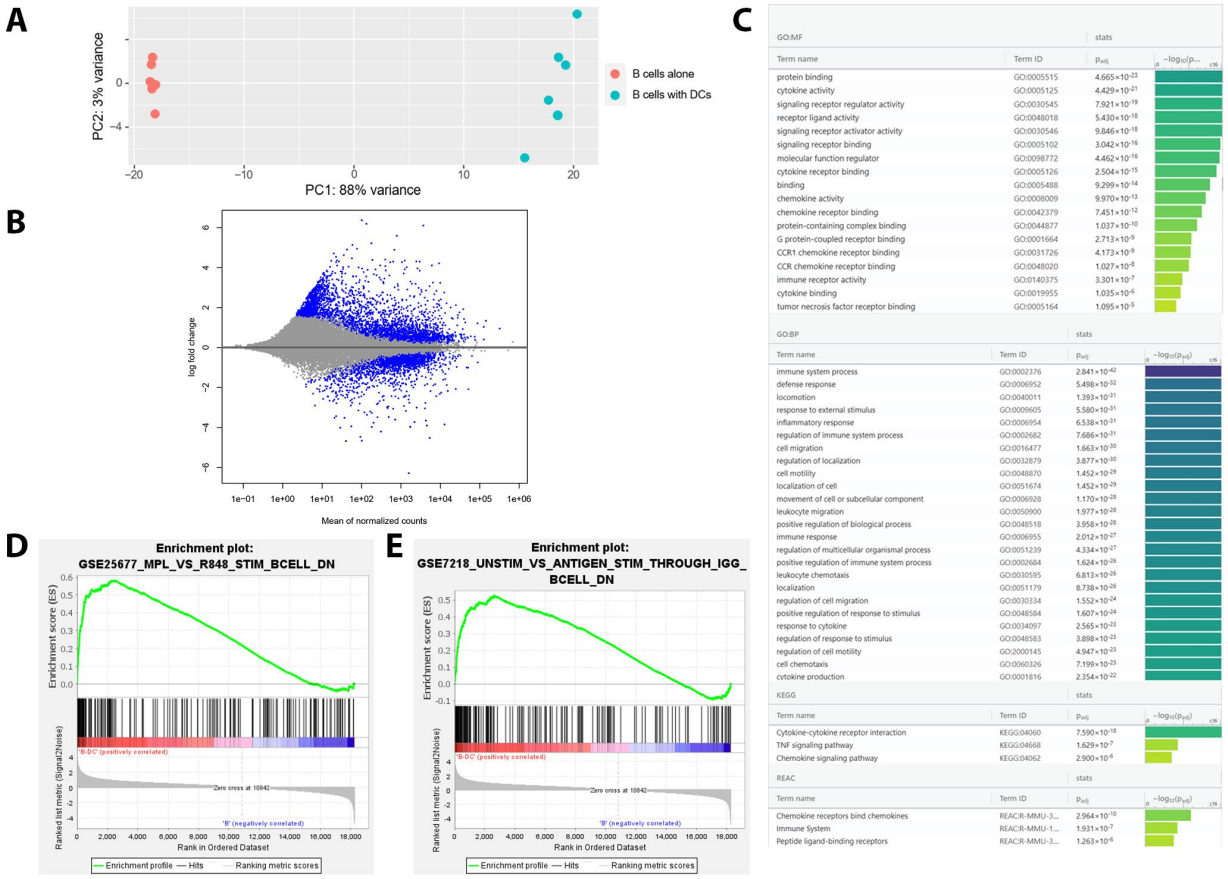


Figure 8: Top upregulated genes in B cells after co-culture with DCs represent increased immune-response related cytokine and chemokine signaling.

Four hundred topmost upregulated genes were categorized based on their involvement in MF (molecular functions), BP (biological processes) and pathways defined by KEGG and REAC databases. Genes are listed according to their adjusted p-value in descending order and with a heat-map that indicates the genes that are overexpressed in B cells after co-culture with DCs

Figure 8: Top upregulated genes in B cells after co-culture with DCs represent increased immune-response related cytokine and chemokine signaling.

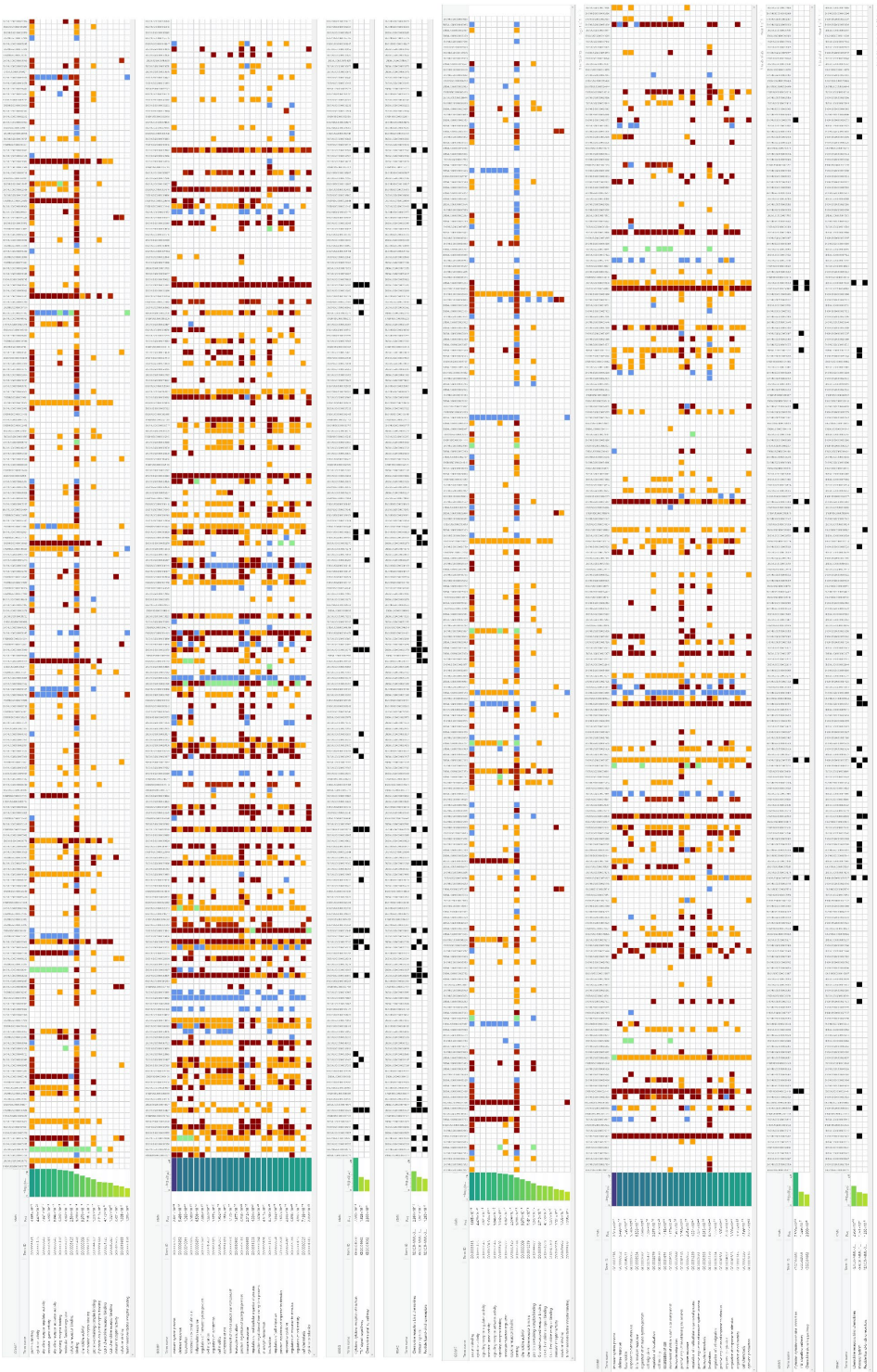


Figure 9: TLR agonists R848 and CpG alone or in combination with adhesion molecules LFA-1 and ICAM-1 are not sufficient for B cell licensing, in place of DCs.

OVA DNA loaded B cells were treated either individually or in combination with TLR7 agonist (Guardiquimod, Guardi), TLR7/8 agonist (R848) and TLR9 agonist (CpG). Similarly, OVA DNA-loaded B cells were also treated with anti-LFA-1 or ICAM-1 either alone or in combination with TLR7/8 agonist (R848). All treatments were added after overnight passive uptake of OVA plasmid DNA by B cells along with CFSE-labeled CD8 T-cells from OT1 mice spleen. (A) Levels of secreted IFN-γ were measured for each treatment group and compared with OVA DNA loaded B cells co-cultured with DCs. (B) Proliferation of CD8 T-cells was measured by loss of CFSE dye using flow cytometry. Results are from one experiment, with samples assessed in triplicates, and are representative of three similar, independent experiments.

Figure 9: TLR agonists R848 and CpG alone or in combination with adhesion molecules LFA-1 and ICAM-1 are not sufficient for B cell licensing, in place of DCs.

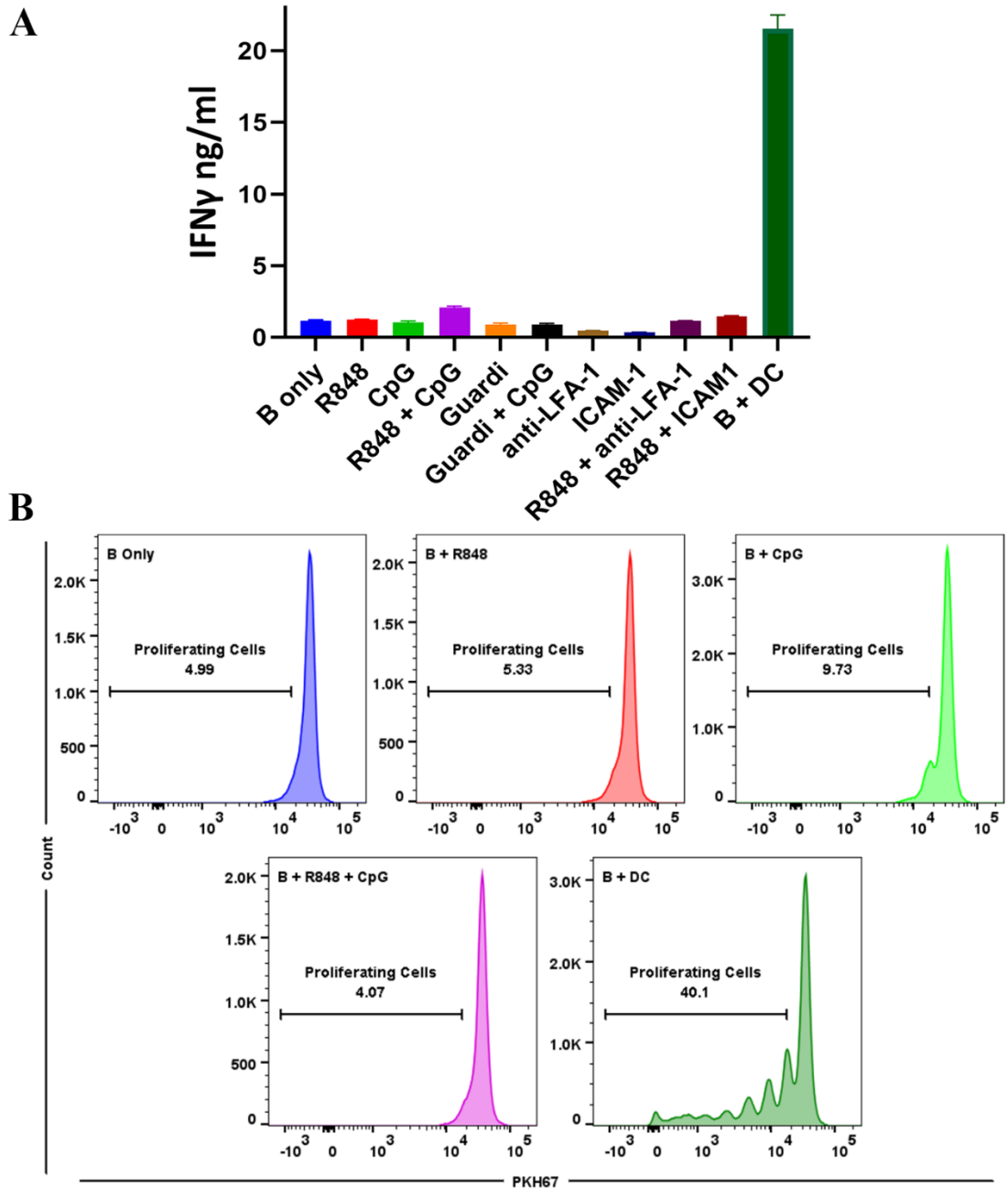
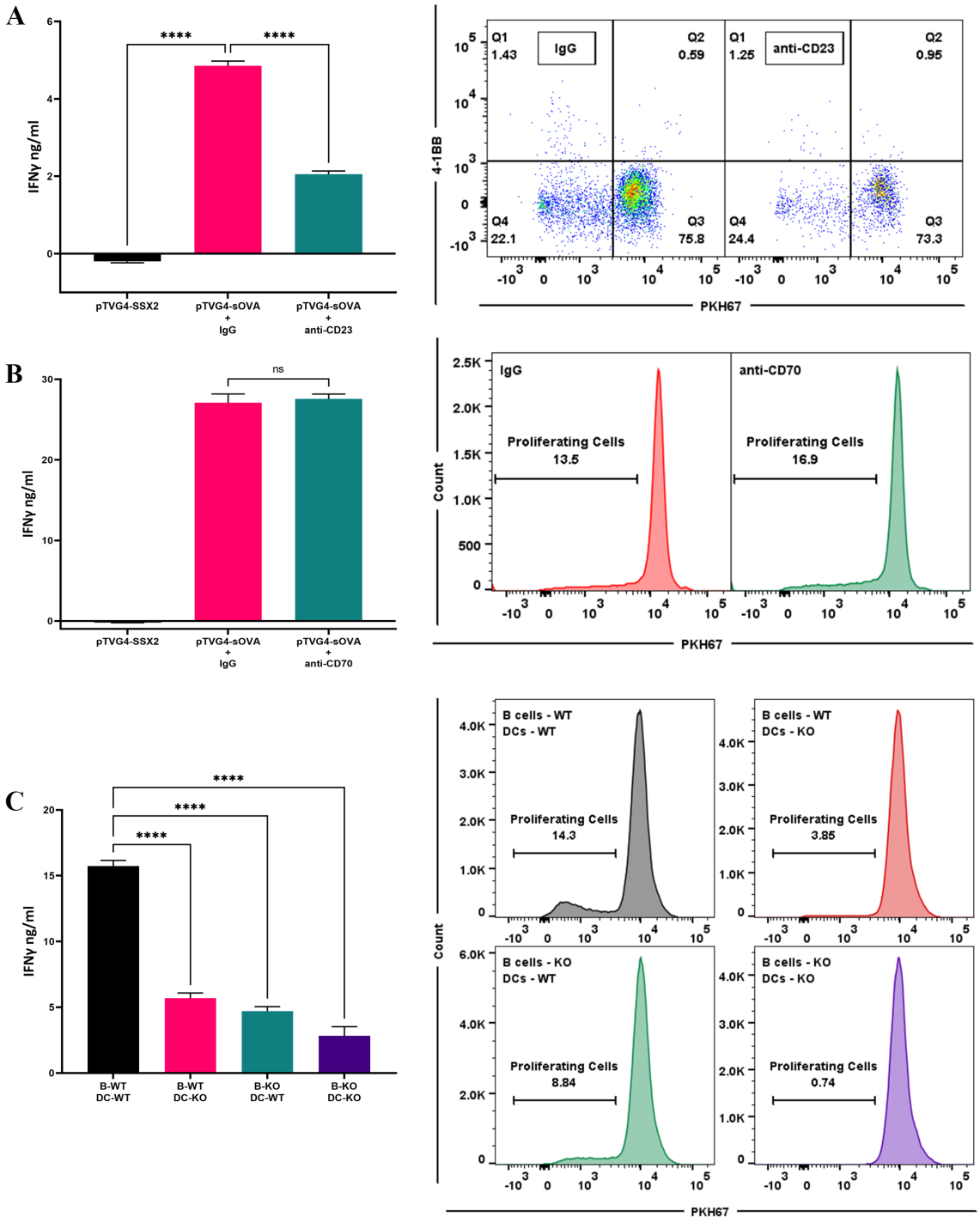


Figure 10: Blocking CD23-IgE and CD40-CD40L, but not CD70-CD27 interaction, negatively impacts B cell licensing.

In vitro antigen presentation assay was setup as in Fig 2B, however, blocking antibodies for CD23 and CD70 were added to the culture on the second day along with DCs and PKH67-labeled CD8 T-cells. For CD40-CD40L blockade, B cells and DCs were used from either C57Bl/6 mice spleens or CD40-KO mice spleens. After five days of culture, proliferation of CD8 T-cells was evaluated and IFN- γ secretion was measured (A) following anti-CD23 blockade treatment, (B) following anti-CD70 blockade treatment, and (C) using B cells and/or DCs from CD40-KO mice spleens. Asterisks ** indicate $p<0.01$ and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in triplicates, and are representative of three similar, independent experiments.

Figure 10: Blocking CD23-IgE and CD40-CD40L, but not CD70-CD27 interaction, negatively impacts B cell licensing.



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Acknowledgements

I would like to thank Dr. Jacques Galipeau and his lab member Pradyut Paul for their insights on B cell specific activation agents. A special agent developed by their lab “mGIFT4” was also tested on B cells for inducing APC function.

Chapter 3

Both B cells and DCs can similarly activate CD8 T cells and result in similar anti-tumor responses when CD8 T cells are primed through peptide-loaded APCs

Work in this chapter is currently being formatted as a manuscript for publication at "*Journal of Immunotherapy in Cancer*." All the experiments that form this chapter were performed and analyzed by me.

Abstract

T cell mediated cytotoxicity and immunity is the primary response from nucleic acid or antigenic material-based vaccination approaches. However, there could be intrinsic differences in the CD8 T cells when primed through different APC subsets. Moreover, exogenous peptide loaded APC vaccines have been under investigation as a therapeutic approach for the treatment of cancer patients. However, APC vaccines have generally demonstrated limited efficacy in clinical trials. To date, only one APC based vaccine, sipuleucel-T has been approved by FDA for the treatment of metastatic castration-resistant prostate cancer. DCs, best known for their cross-priming ability, have been the ultimate choice for APC based vaccine research. However, B cells which can also function similarly to activate CD4 and CD8 T cells remain largely understudied as APC for vaccines. Here we compare the phenotype and function of activated T cells that result from epitope specific priming through either B cells or DCs.

We isolated B cells and DCs from C57Bl/6 mouse splenocytes, which were either treated or not with LPS for maturation. These cells were then either loaded or not with SIINFEKL peptide (Ovalbumin specific antigen) for priming CD8 T cells from OT-1 mice (ovalbumin specific). The resulting T cells were analyzed for their phenotype, function, and anti-tumor efficacy, using flow cytometry, ELISA, and E.G7 ovalbumin expressing murine tumor model, respectively.

We report that both immature B cells and immature DCs are similarly capable of activating antigen-specific CD8 T cells. However, in certain instances mature DCs generate a stronger CD8 T cell activation profile when compared to mature B cells. In agreement with the activation marker expression, we report that both B cells and DCs result in similar expression of exhaustion and

checkpoint related markers on activated CD8 T cells. Furthermore, we report similar expression of pro-inflammatory and cytotoxicity related cell surface proteins and intracellular cytokines, in B cells and DCs. Lastly, we report that immature B cells, mature DCs and immature DCs, all generate a similar anti-tumor response upon adoptive transfer of primed CD8 T cells in tumor bearing mice.

Collectively, our data indicated that both B cells and DCs are equally capable of activating CD8 T cells and generating an anti-tumor response. Additionally, recent reports have proposed that B cells play an APC function in the tumor microenvironment. This, and the fact that B cells are relatively easier to culture and expand when compared to DCs, warrants further investigation into APC function of B cells and their potential use as APC-based vaccines.

Introduction

As we demonstrated in chapter 1, B cells are the primary APCs for presentation of plasmid DNA upon passive uptake. We wished to develop and evaluate the efficacy of B cell-centered immunization strategies. One of the primary outcomes of immunizations is generation of antigen-specific CD8 T cells. These cells result in targeted cytotoxicity and generate inflammation related immune responses. To analyze the phenotype of resulting CD8 T cells we either primed them through peptide pulsed B cells or DCs and compared their function. Moreover, the approach of generating antigenic peptide loaded APCs has been evaluated by many groups as a vaccination strategy for treatment of cancer patients. So, the findings from this chapter can also be relevant in development of similar B cell focused strategies.

APC based vaccines can be a direct way of eliciting an antigen-specific anti-tumor response, by activating tumor-specific CD8 T cells. The conceptual idea behind APC vaccines, is to load tumor-antigen-specific peptide(s) on professional APC subsets (PBMCs or lymphocytes) that would result in primed T cells for anti-tumor inflammatory and cytotoxic responses (1). Among the groups of APC subsets, dendritic cells (DCs) have been of greater interest for development of these cell-based vaccines (2-5). Over several years, DCs have been demonstrated as the primary cross-presenter of the antigenic epitopes to activate CD8 T cells (6). Moreover, they have been accredited with most cross-priming and cross-dressing that occurs in humans, to elicit antigen-specific immune responses (7, 8). However, other APC subsets, such as B cells and macrophages have been less studied for their use as APC vaccines (9).

Currently, sipuleucel-T is the only vaccine that has been approved by FDA for treatment of metastatic castration-resistant prostate cancer (10). It comprises of patient blood cells that are enriched for APCs, favoring maturation of DCs by GM-CSF treatment *in vitro* (11). Other DC-based vaccines that are either currently in development or have failed in clinical trials include use of *in vitro* generated monocyte derived DCs, exosomes derived from DCs, *in vitro* generated DCs from CD34+ hematopoietic precursors, naturally circulating blood DCs, allogeneic plasmacytoid DC cell line and exosomes derived from plasmacytoid DCs. However, very few B cell or macrophage-based vaccination strategies have been examined (9). One such example was use of B cells electroporated with ovarian cancer specific mRNA antigen to promote expansion of antigen-specific tumor-infiltrating T cells *in vitro* (12). Some groups have also demonstrated that use of activated macrophages can result in inhibition of tumor growth in murine models (13) (14). A few clinical trials have reported use of macrophage based vaccine for treatment of metastatic forms of colorectal cancer, non-small cell lung cancer, ovarian cancer, and renal cell carcinoma (14). Results from these trials demonstrated that macrophage-based vaccines failed regardless of the variations in the dosage, scheduling, mode of administration and technology used to generate these vaccines (15).

Recent studies by other groups and us have demonstrated that B cells are capable of functioning as APCs (16-19). It has also been reported that unlike DCs, B cell based vaccines could be resistant to immune suppression by cytokines like IL10, TGF-β and VEGF (9, 20). Moreover, use of B cells as APC vaccine can be relatively economical and easier, as high number of circulating B cells can be purified from blood and expanded *ex vivo* for use as APCs (21, 22). Taken together, all these factors suggest that B cells could be an excellent alternative to DC/macrophage-based vaccines.

Although B cells have been largely understudied for their APC function, recent evidence provides a strong rationale to better understand B cells as APCs.

To directly compare B cells and DCs in their mature and immature forms, we performed a side-by-side comparison of peptide-pulsed B cells and DCs. This approach eliminates the differences in the capabilities of antigen uptake and processing by different APC subsets but rather tests them directly by their ability to activate CD8 T cells through epitope-specific priming. We demonstrated that even though CD8 T cells primed through B cells and DCs had different expression profiles of activation, checkpoint, and cytotoxic markers, they had similar effector and central memory phenotypes and resulted in similar anti-tumor efficacy in a murine tumor model. We showed that immature B cells, immature DCs and LPS-matured DCs performed similarly, whereas LPS matured B cells failed to generate an anti-tumor response in tumor bearing mice. Our future studies are to evaluate mature B cells and how the resulting T cell phenotype is different from CD8 T cells primed through immature B cells. We will also evaluate if different modes of activation could have varying effects on the APC function of B cells, as CD40 activated B cells have been shown to perform as potent APCs (22).

Results:

Epitope-specific priming through DCs resulted in similar or stronger activation marker expression on CD8 T cells, compared to priming through peptide-pulsed B cells

B cells have been reported by many to be capable of activating CD8 T cells. Current interest in APC based vaccines led us to perform a side-by-side comparison of B cells and DCs in their mature and immature states. Mature APCs were generated by LPS treatment for 24 hours and non-LPS treated APCs were used as immature forms of B cells and DCs (Fig 1). In addition, we evaluated the effect of GM-CSF and IL4 on epitope-specific priming of CD8 T cells, as it has been previously reported by us and others that APC function of B cells and DCs could be augmented in their presence. More specifically, we showed that B cells had increased survival and activation in presence of IL4, whereas DCs demonstrated increased survival and activation in presence of GM-CSF (Fig 1). First, we evaluated the expression of cell surface activation markers on CD8 T cells following priming through LPS-matured or immature B cells/DCs. As shown in Fig 2 and Fig 3, expression of OX40 and CD69 (Fig 2A), CD28 (Fig 2C), KLRG1 (Fig 3A), and FASR (Fig 3C) were similar between CD8 T cells primed with B cells or DCs. However, priming through peptide loaded LPS-matured DCs resulted in increased expression of CD25 (Fig 2A) and CD40L (Fig 2D). The percentage of CD8 T cells expressing CD25 and CD69 (Fig 2B), CD28 (Fig 2D), KLRG1 (Fig 3B) and FAS-R (Fig 3D) was also similar between epitope-specific priming through B cells and DCs. On the contrary, the percentage of CD8 T cells that expressed OX40 (Fig 2B) and CD40L (Fig 2D) was increased when primed through DCs. Furthermore, expression of CD27 (Fig 2C) and Ki67 (Fig 3A) was increased on CD8 T cells when primed through LPS-matured B cells, whereas immature B cells demonstrated the opposite (Fig 2C). Similar was true for the percentage of CD8

T cells positive for CD27 (Fig 2D) and Ki67 (Fig 3B) expression. Lastly, we observed increased expression of 4-1BB (CD137) on CD8 T cells primed through immature and LPS-matured B cells, more prominently at 48-hour time point (Fig 3A). However, percentage of 4-1BB positive CD8 T cells was increased at 24-hour time point but similar at 48 hour time point (Fig 3B).

Both B cells and DCs generated similar percentages of effector and central memory CD8 T cells and resulted in CD8 T cells proliferation

We next wished to evaluate how LPS-matured and immature, B cells or DCs, differed in generation of effector and central memory phenotypes upon epitope-specific priming of CD8 T cells. As shown in Fig 4, both B cells and DCs resulted in similar percentages of central (CD44+ CD62L+) (Fig 4A) and effector (CD44+ CD62L-) (Fig 4B) memory CD8 T cells, in most treatment groups. However, priming through LPS-matured B cells in presence of GM-CSF and IL4 led to an increased percentage of central memory CD8 T cells and a decreased percentage of effector memory CD8 T cells, when compared to their respective DC treatment groups after 48 hours. We also evaluated the ability of peptide-loaded B cells and DCs in their mature and immature forms to induce CD8 T cell proliferation. We demonstrated that both B cells and DCs induced CD8 T cell proliferation similarly at 48-hour time point (Fig 1C). Proliferation was not observed at 24-hour time point (data not shown). Collectively, our data suggested that even though priming through both B cells and DCs generated different activation profiles they result in similar percentages of memory phenotypes and proliferating antigen-specific CD8 T cells.

Priming through DCs resulted in similar or increased expression of checkpoint and exhaustion related markers on CD8 T cells, when compared to priming through B cells

We then evaluated the expression of exhaustion and checkpoint related markers on primed CD8 T cells. As shown in Fig 5 and 6, we observed similar expression of CD96 (Fig 5C), CD160 (Fig 6A), and VISTA (6C) on CD8 T cells when primed through B cells or DCs. However, we observed increased expression of CD272 (BTLA) (Fig 5C) and EOMES (Fig 6A) when CD8 T cells were primed with LPS-matured or immature DCs. Whereas expression of CD244 (Fig 5C), ICOS and TIM3 (Fig 6C) were increased only when priming occurred through LPS-matured DCs. However, expression of CD244 was upregulated only at 24-hour time point and was similar between all groups compared at 48 hour time point. On the contrary, expression of LAG3 (Fig 5A) and TIGIT (Fig 6A) were increased on CD8 T cells when primed through LPS-matured or immature B cells. Expression of PD1 (Fig 5A) was found to be similar throughout different comparisons, except for when CD8 T cells were primed through LPS-matured B cells at 48-hour time point. Interestingly, expression of PD1 was generally lower in all groups when treated with GM-CSF and IL4. Lastly, we demonstrated that expression of CTLA4 (Fig 5A) was increased in CD8 T cells when primed through immature B cells and LPS-matured DCs, not treated with GM-CSF and IL4.

We also demonstrated that percentages of CD8 T cells positive for expression of PD1 and LAG3 (Fig 5B), TIGIT (Fig 6B), and ICOS (Fig 6D) were similar between all compared groups. Furthermore, we observed that percentages of CD8 T cells that expressed CD272 (BTLA) (Fig 5D) and TIM3 (Fig 6D) were increased when primed through LPS-matured or immature DCs but only at 24-hour time point. Whereas increased percentages of CD96 and CD244 (Fig 5D), and EOMES (Fig 6B) were observed on CD8 T cells primed through LPS-matured DCs. On the

contrary, percentages of CD8 T cells that were positive for expression of CD160 (NK1) (Fig 6B), and VISTA (Fig 6D) were found to be increased when primed through LPS-matured B cells. Lastly, percentages of CD8 T cells that expressed CTLA4 (Fig 5B) were increased when primed through immature B cells and LPS-matured DCs, not treated with GM-CSF and IL4, following the same trend as their mean fluorescence intensity.

Priming through both B cells and DCs resulted in similar expression of cytotoxicity related markers on CD8 T cells

We next wished to characterize the CD8 T cells based on the expression of cytotoxicity related markers. As shown in Fig 7 and Fig 8, we observed similar expression of FAS-L and TRAIL (CD253) (Fig 7A) on CD8 T cells that were primed with LPS-matured and immature B cells/DCs. However, expression of TNF-α (TNF-a) (Fig 8A) and Perforin (Fig 8C) were observed to be increased when primed through DCs but only at 24-hour time point. Whereas expression of IFN-γ (IFN-g) (Fig 8A) was increased only when CD8 T cells were primed through DCs in presence of GM-CSF and IL4. On the other hand, expression of CD107a (LAMP1) (Fig 7A) and Granzyme-B (Grz-B) (Fig 8A) were increased when primed through LPS-matured B cells, specifically at 48-hour time point.

We also demonstrated that percentages of CD8 T cells that expressed FASL, CD107a, and TRAIL (Fig 7B), IFN-γ (Fig 7B) and Perforin (Fig 8D) were increased when primed through DCs. However, CD107a and IFN-γ expressing CD8 T cells upon priming through DCs were significantly increased only at 24-hour time point. Whereas percentages of TNF-α expressing CD8

T cells were similar between all compared groups (Fig 8B). Conversely, percentage of CD8 T cells expressing Grz-B was increased when primed through LPS-matured or immature B cells, but only at 24-hour time point.

Lastly, we wished to quantify the levels of secreted IFN-γ following priming of CD8 T cells. We found that CD8 T cells primed by peptide-pulsed DCs led to increased levels of IFN-γ after 24 hours of co-culture. However, we observed that levels of IFN-γ were similar at 48-hour time point between all compared groups (Fig 8E).

Priming through peptide-pulsed LPS-matured B cells resulted in increased expression of IL10

We next wished to analyze the expression of Th2 related markers on the resulting CD8 T cells, for this we measured the expression of IL2, IL4 and IL10 on the primed cells. As shown in Fig 9A, we observed that CD8 T cells expressed higher levels of IL2 when primed through DCs, but only at 24-hour time point. Similarly, percentage of IL2 expressing CD8 T cells was increased after 24 hours of priming through DCs, but not at 48 hours (Fig 9B). We also demonstrated that expression of IL4 was increased when CD8 T cells were primed through LPS-matured or immature DCs at 24-hour time point (Fig 9A). Whereas percentage of CD8 T cells positive for IL4 expression was increased when primed through immature DCs at 48 hours (Fig 9B). Lastly, we observed that CD8 T cells primed through LPS-matured B cells had increased expression of IL10 (Fig 9A) and the percentage of IL10 positive CD8 T cells was also found to be increased in the same group (Fig 9B), at 48-hour time point.

tSNE analysis revealed different phenotypes of CD8 T cells upon priming through LPS-matured B cells and LPS-matured DCs.

We then wished to perform tSNE analysis on primed CD8 T cells to identify and classify them into clusters based on their resulting phenotypes. For tSNE analysis and clustering of CD8 T cell populations, we used proteins that defined effector function of CD8 T cells. This list included EOMES, 4-1BB, Ki67, Grz-B, IFN-γ, TNF-α, Perforin, IL2, IL4 and IL10. As shown in Fig 10A, we demonstrated that priming through B cells or DCs resulted in significantly different CD8 T cell phenotypes as represented by the cluster formations after tSNE. We observed that population 1 (upper right, in magenta) was over-represented among CD8 T cells primed through B cells, whereas population 2 (lower left, in pink) was overrepresented when DCs primed CD8 T cells. Interestingly, population 3 (center, in blue) was equally represented when immature B cells or DCs primed CD8 T cells, but population 3 was represented more prominently when LPS-mature B cells were used to prime CD8 T cells compared to priming through LPS-matured DCs. Moreover, population 6 (center, in green) was equally represented among CD8 T cells from all the groups. Lastly, we observed population 8 (bottom right, in light green), was represented in higher numbers when immature DCs primed CD8 T cells, specifically when treated with GM-CSF and IL4. The phenotype of each population cluster is represented as a heat map (Fig 10B) and percentages of each population cluster among differently primed CD8 T cells are presented as bar charts (Fig 10C).

CD8 T cells primed through immature B cells, LPS-matured DCs, and immature DCs generated similar anti-tumor response

Lastly, we wished to understand the anti-tumor efficacy of CD8 T cells primed with peptide-loaded LPS-matured or immature B cells or DCs. We implanted E.G7-OVA cells subcutaneously in C57Bl/6 mice and adoptively transferred primed CD8 T cells. As shown in Fig 11B, we found that CD8 T cells primed by immature B cells, LPS-matured DCs and immature DCs generated a similar anti-tumor response. However, CD8 T cells primed through LPS-matured B cells failed to provide an anti-tumor response in tumor-bearing mice. A survival curve was plotted using Kaplan-Meier analysis (Fig 11C). Collectively, our data demonstrated that immature B cells, but not LPS-matured B cells, can be a potential alternative to DCs, when priming CD8 T cells.

Discussion and Conclusion

In this chapter, we demonstrated that immature B cells, immature DC, and LPS-matured DCs primed CD8 T cells similarly and generated comparable anti-tumor responses. However, LPS-matured B cells differed from LPS-matured DCs and failed to generate an antigen-specific CD8 T cell mediated anti-tumor response. We observed that activation profiles of CD8 T cells primed through immature B cell or DC were similar, whereas increased expressions of some proteins were recorded in the case of CD8 T cells primed through LPS-matured DCs. Similarly, we observed that expression of checkpoint and exhaustion related markers were similar among the CD8 T cells primed through immature B cells or DCs, but increased expression of some markers were observed on CD8 T cells primed through LPS-matured DCs. In addition, we demonstrated that priming through LPS-matured B cells resulted in increased IL10 expression in CD8 T cells. Lastly, our tSNE analysis showed different clusters of populations of CD8 T cells when compared between B cells and DC primed groups. To our knowledge this is the first study that directly compares both B cells and DCs for their ability to prime CD8 T cells *in vitro* and assess the anti-tumor efficacy of resulting CD8 T cells *in vivo*.

Immature B cells and DCs performed similarly when their anti-tumor responses were compared, whereas LPS-matured B cells generated an immunosuppressive response likely due to increased IL10 expression. Although clustering through tSNE did not classify any IL10 high T regulatory type cell population, flow cytometry data showed significant increase in IL10 expression in overall CD8 T cell population. Moreover, it has been previously reported that LPS-matured B cells can result in anergic CD8 T cells (23, 24), whereas CD40L activated B cells can trigger CD8 T cells for cytotoxic responses (25). This is in agreement with our data and suggests that a combination

of anergic CD8 T cells and IL10 producing CD8 T cells resulted from priming through LPS-matured B cells and led to poor anti-tumor efficacy. Taken together, this suggests that mere expression of activation markers on B cells is not sufficient for successful priming of CD8 T cells. However, the mode of activation of B cells also plays a significant role in development of their APC function. Speculatively, similar could be true for DCs as well, and therefore further investigations are required to understand the effects of commonly used activation agents on APC subsets.

Our data also demonstrated that priming of CD8 T cells through dendritic cells, specifically LPS-matured DCs, resulted in increased activation and exhaustion marker expression. While this could be advantageous briefly and could also provide greater immunogenicity, it could ultimately attract immunosuppression by tumor microenvironment. In fact, one of the anticipated reasons for the failure and limited success of DC based vaccines is VEGF mediated immunosuppression by tumors (26). Other factors including poor migration ability and poor lymphoid homing capability of DCs can contribute towards poor efficacy of DC vaccines in humans (27).

Moreover, *ex vivo* expansion of DCs is a cumbersome and expensive manufacturing process, as DCs lack proliferative potential (9). All these together have factored in as hurdles in development of clinically successful DC based vaccine, and it has now become necessary to explore other APC subsets as options for cell-based therapies. On the contrary, B cells are now gaining more interest with mounting evidence that demonstrated their capability to prime CD8 T cells and function as potent APCs. Through this study we provide evidence that CD8 T cells primed through immature B cells or DCs generated similar anti-tumor effects. Others have also reported that *ex vivo* expanded B cells can induce anti-tumor immunity (18, 19). Moreover, *in vivo* studies by some

groups have demonstrated that upon adoptive transfer of B cells that have been stimulated *ex vivo* with tumor antigens generated a protective effect against the tumor (25, 28, 29). As previously discussed, use of B cells as APC provides additional advantages over DCs such as easier expansion and economical manufacturing, along with clinical benefits such as resistance to immunosuppression by tumors.

The results of this study are limited to the use of antigenic peptide loaded APCs for priming of CD8 T cells. These results can be different if the antigen processing abilities of APCs are taken in to consideration, by introducing the antigen in other forms such as tumor lysates, protein, mRNA, or DNA. To address this, several research groups including ours are currently investigating APC function of B cells when stimulated with different antigen types. However, similar to our experimental set up, peptide pulsed DCs are being actively investigated as an immunotherapy aproach. One such recent study, tested multi-epitope pulsed autologous DCs for safety and immune responses in cancer patients with glioblastomas (30). They reported enhanced immune responses in 33% of patients, without any significant improvement in progression free survival or overall survival, which is comparable to other DC vaccine trials (30). On the other hand, clinical trials that tested B cells as vaccines have utilized CD40L or IL2 transduced chronic lymphocytic leukemia (CLL) cells in CLL patients, allogeneic B cells fused with autologous tumor cells in metastatic melanoma and renal cell carcinoma patients (29). However, human studies that evaluate peptide-pulsed B cells as a therapeutic approach have been absent.

Overall, our findings demonstrated that B cells can be just as competent as DCs in priming CD8 T cells that resulted in similar anti-tumor responses. Our future directions include testing effects of activation agents on peptide-pulsed B cells and how that changes the phenotypes and function

of resulting CD8 T cells. We expect that maturation through CD40L, or BAFF could result in more potent B cell APCs, which could be used for priming T cell and generate anti-tumor effects.

Figures and Tables

Figure 1: Treatment of B cells and DCs with LPS resulted in increased expression of maturation related markers.

B cells and DCs isolated from C57 BL/6 mice were either treated or not with LPS. Expression of activation and maturation markers such as MHCI, MHCII, CD80 CD83 and CD86 were quantified by flow cytometry. (A) Expression on B cells before and after LPS treatment. (B) Expression on DCs before and after LPS treatment.

Figure 1: Treatment of B cells and DCs with LPS resulted in increased expression of maturation related markers.

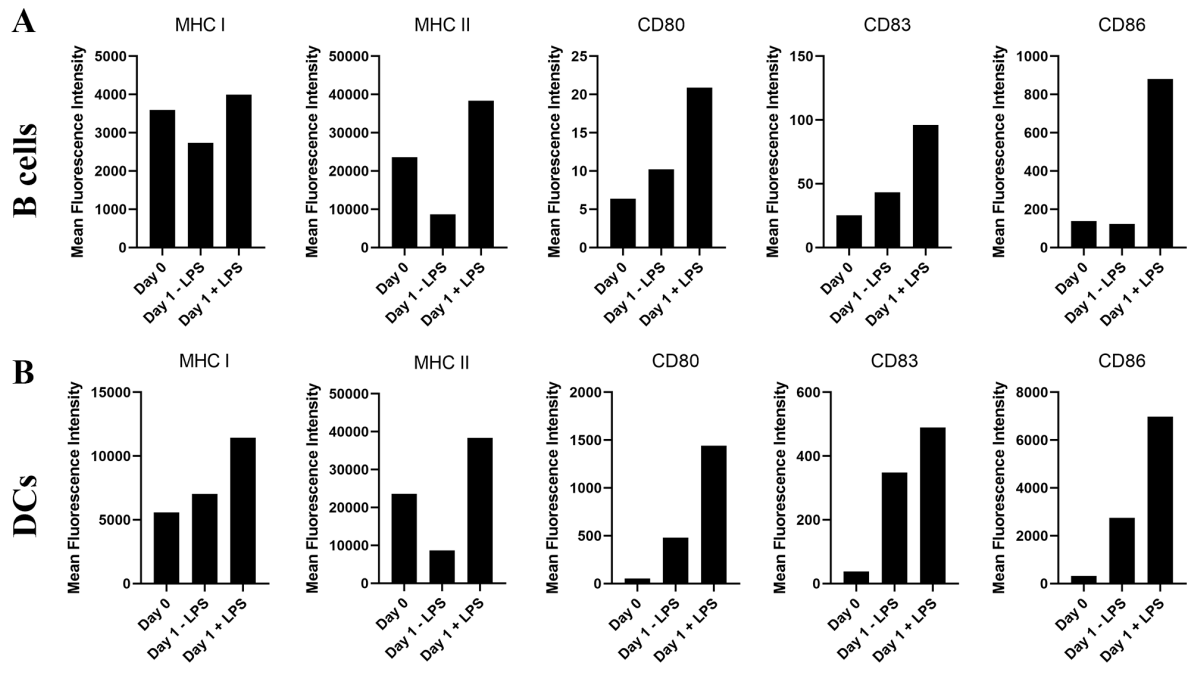

Figure 2: Epitope-specific priming through DCs resulted in similar or stronger activation marker expression on CD8 T cells, compared to priming through peptide-pulsed B cells

B cells and DCs isolated from C57Bl/6 spleen were either LPS treated or not and then loaded with SIINFEKL peptide. Non-peptide stimulated APCs and CD8 T cells alone groups were included to control for background and were used as negative controls. After 24 hours or 48 hours of incubation with CD8 T cells, flow cytometry was performed to measure expression of activation markers CD25, OX40, CD69, CD40L, CD28, and CD27. (A,C) Mean fluorescence intensities and (B,D) percentage of CD8 T cells positive (pos) for expression of the respective cell surface markers were plotted. MFIs and percentages were subtracted for background from their respective non-peptide stimulated groups and represented as single dots for each mouse replicate, with mean and standard deviation from five replicates. Asterisks * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$, and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in five biological replicates, and are representative of two similar, independent experiments.

Figure 2: Epitope-specific priming through DCs resulted in similar or stronger activation marker expression on CD8 T cells, compared to priming through peptide-pulsed B cells

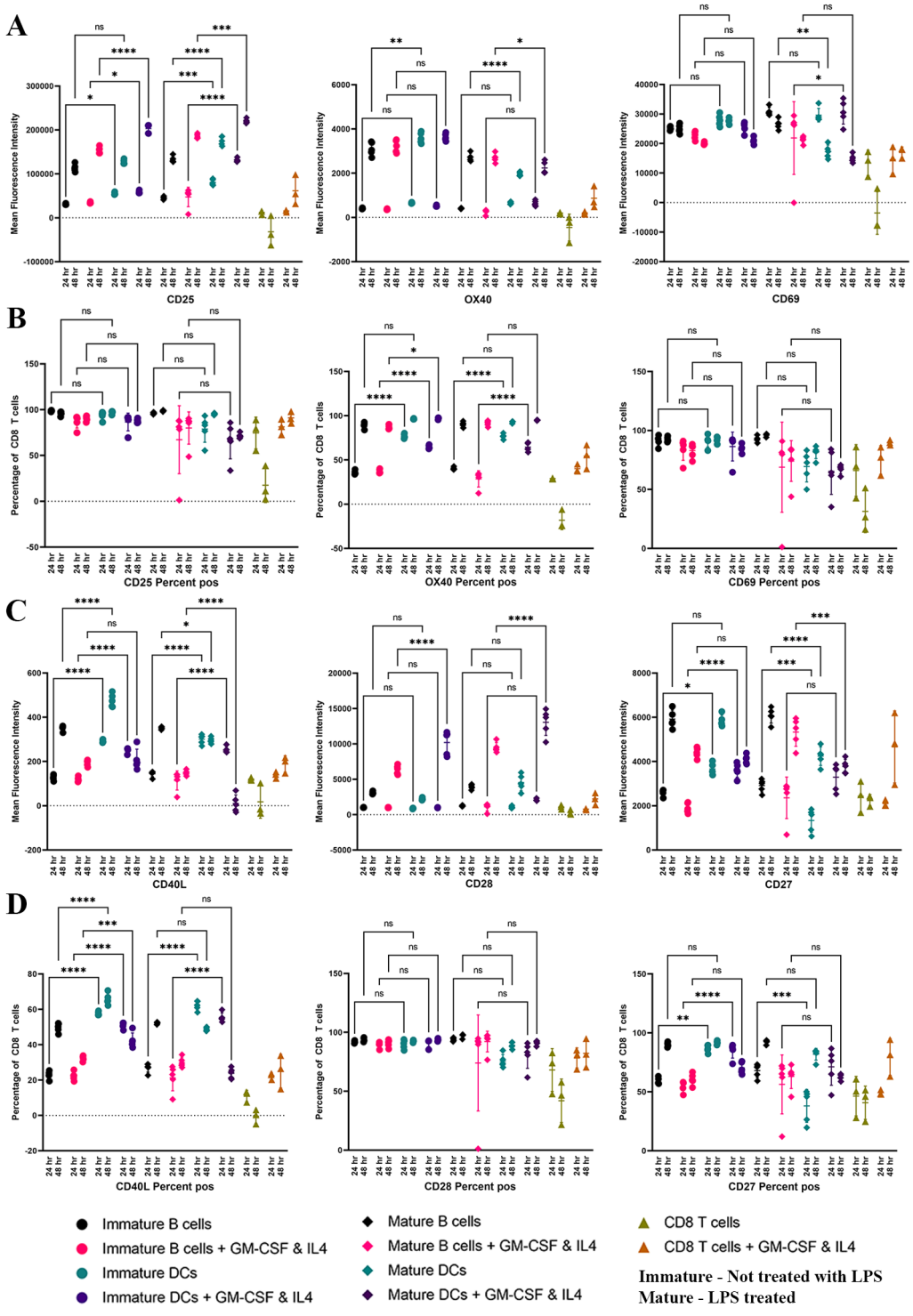

Figure 3: Epitope-specific priming through DCs resulted in similar or stronger activation marker expression on CD8 T cells, compared to priming through peptide-pulsed B cells

B cells and DCs isolated from C57Bl/6 spleens were either LPS treated or not and then loaded with SIINFEKL peptide. Non-peptide stimulated APCs and CD8 T cells alone groups were included to control for background and were used as negative controls. After 24 hours and 48 hours of incubation with CD8 T cells, flow cytometry was performed to measure expression of activation markers 4-1BB, Ki67, KLRG1, and FAS-R. (A,C) Mean fluorescence intensities and (B,D) percentage of CD8 T cells positive (pos) for expression of the respective cell surface markers were plotted. MFIs and percentages were subtracted for background from their respective non-peptide stimulated groups and represented as single dots for each mouse replicate, with mean and standard deviation from five replicates. Asterisks * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$, and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in five biological replicates, and are representative of two similar, independent experiments.

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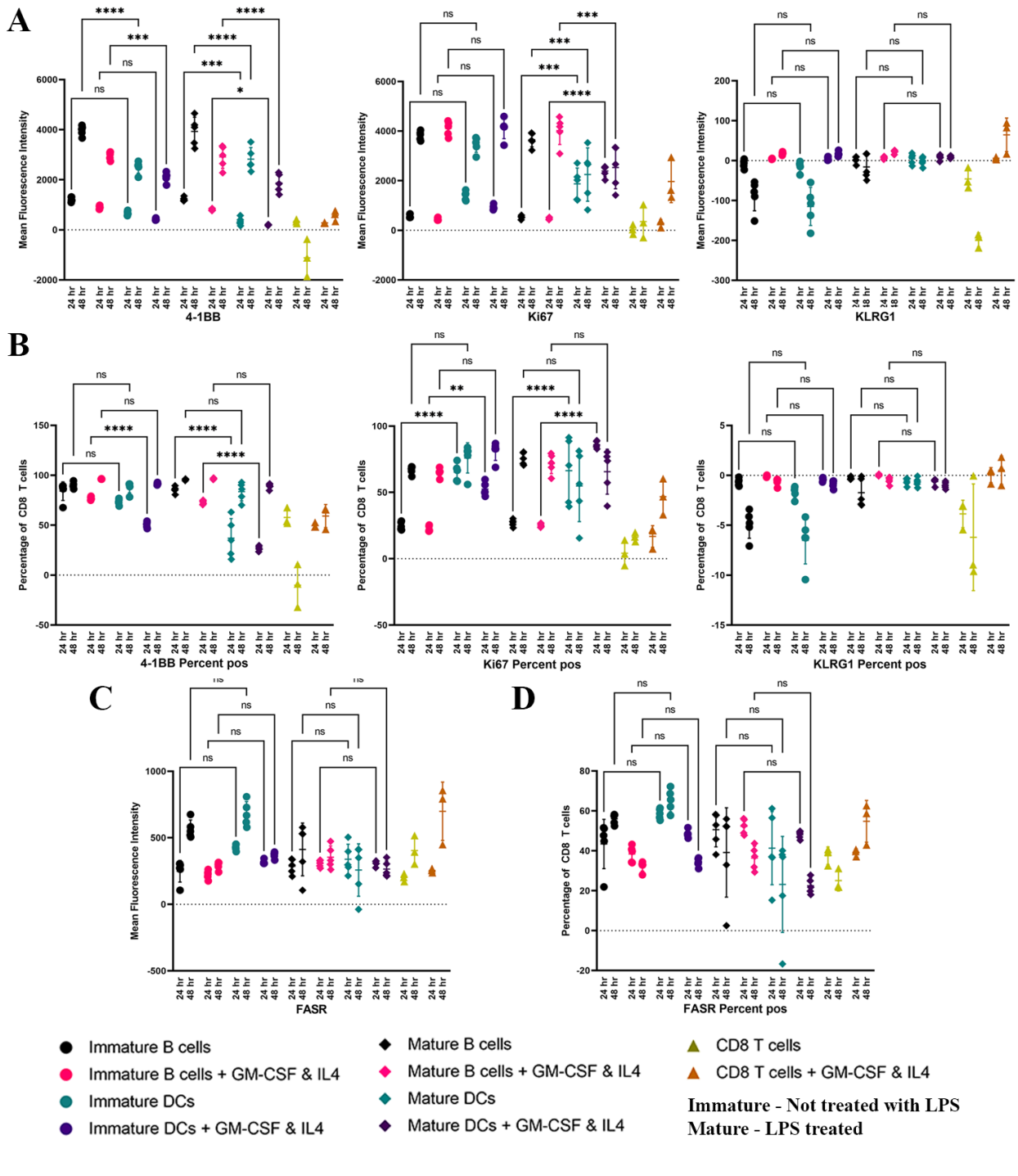


Figure 4: Both B cells and DCs generated similar percentages of effector and central memory CD8 T cells and resulted in CD8 T cells proliferation

B cells and DCs isolated from C57Bl/6 spleen were either LPS treated or not and then loaded with SIINFEKL peptide. Non-peptide stimulated APCs and CD8 T cells alone groups were included to control for background and were used as negative controls. After 24 hours (A) and 48 hours (B) of incubation with CD8 T cells, flow cytometry was performed to identify effector memory CD8 T cells (CD44+ CD62L-) and central memory CD8 T cells (CD44+ CD62L+). Percentages were subtracted for background from their respective non-peptide stimulated groups and represented as single dots for each mouse replicate, with mean and standard deviation from five replicates (C) Representative proliferation of CD8 T cells as measured by loss in CFSE. Asterisks * indicate $p<0.05$ and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in five biological replicates, and are representative of two similar, independent experiments.

Figure 4: Both B cells and DCs generated similar percentages of effector and central memory CD8 T cells and resulted in CD8 T cells proliferation

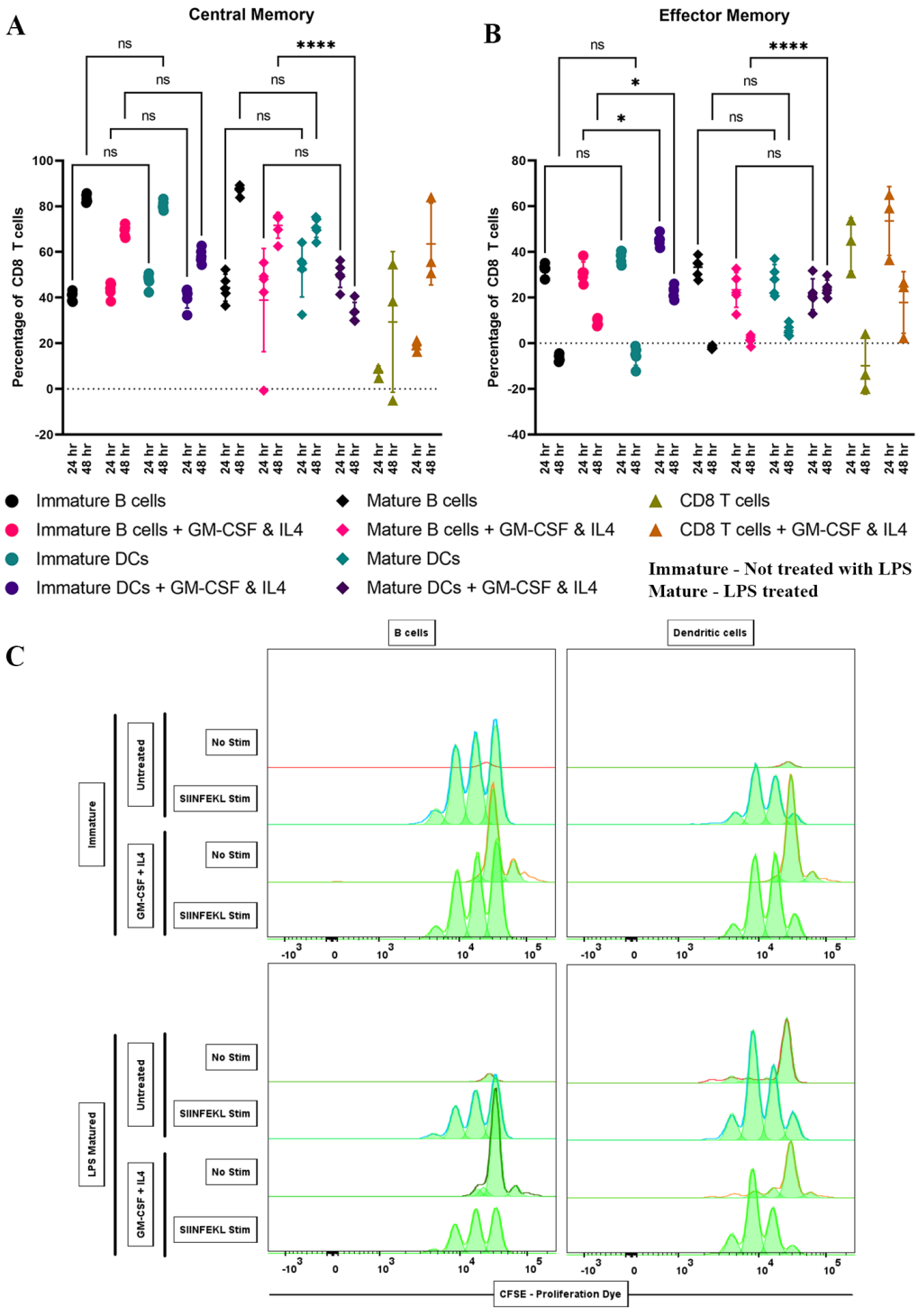


Figure 5: Priming through DCs resulted in similar or increased expression of checkpoint and exhaustion related markers on CD8 T cells, when compared to priming through B cells

In vitro assay was set up as in Fig 2; after 24 hours and 48 hours of incubation with CD8 T cells, flow cytometry was performed to measure expression of checkpoint and exhaustion markers PD1, LAG3, CTLA4, CD272, CD244, and CD96. (A,C) Mean fluorescence intensities and (B,D) percentage of CD8 T cells positive (pos) for expression of the respective cell surface markers were plotted. MFIs and percentages were subtracted for background from their respective non-peptide stimulated groups and represented as single dots for each mouse replicate, with mean and standard deviation from five replicates. Asterisks * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$, and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in five biological replicates, and are representative of two similar, independent experiments.

Figure 5: Priming through DCs resulted in similar or increased expression of checkpoint and exhaustion related markers on CD8 T cells, when compared to priming through B cells

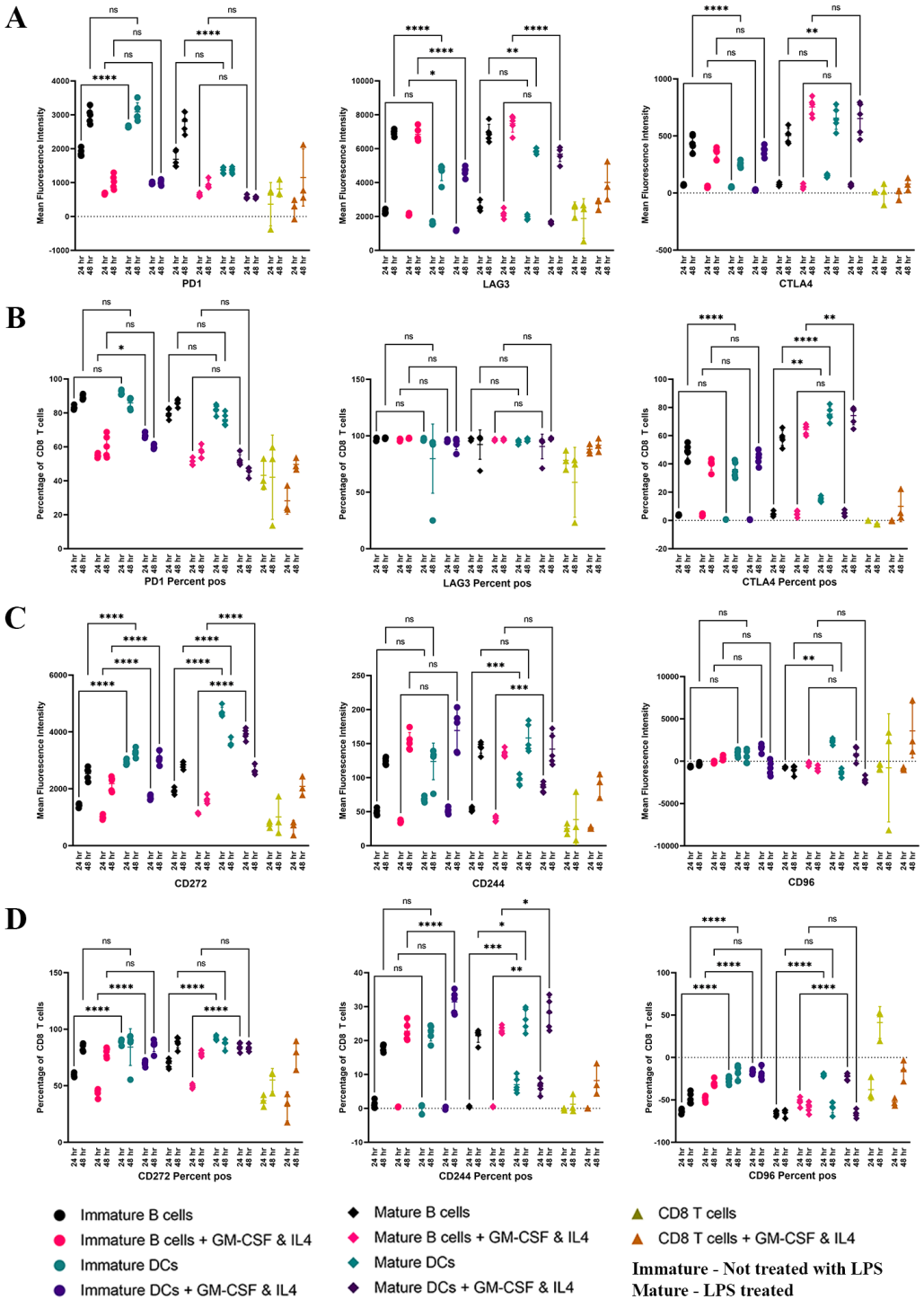


Figure 6: Priming through DCs resulted in similar or increased expression of checkpoint and exhaustion related markers on CD8 T cells, when compared to priming through B cells

In vitro assay was set up as in Fig 2; after 24 hours and 48 hours of incubation with CD8 T cells, flow cytometry was performed to measure expression of checkpoint and exhaustion markers EOMES, CD160, TIGIT, ICOS, VISTA, and TIM3. (A,C) Mean fluorescence intensities and (B,D) percentage of CD8 T cells positive (pos) for expression of the respective cell surface markers were plotted. MFIs and percentages were subtracted for background from their respective non-peptide stimulated groups and represented as single dots for each mouse replicate, with mean and standard deviation from five replicates. Asterisks * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$, and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in five biological replicates, and are representative of two similar, independent experiments.

Figure 6: Priming through DCs resulted in similar or increased expression of checkpoint and exhaustion related markers on CD8 T cells, when compared to priming through B cells

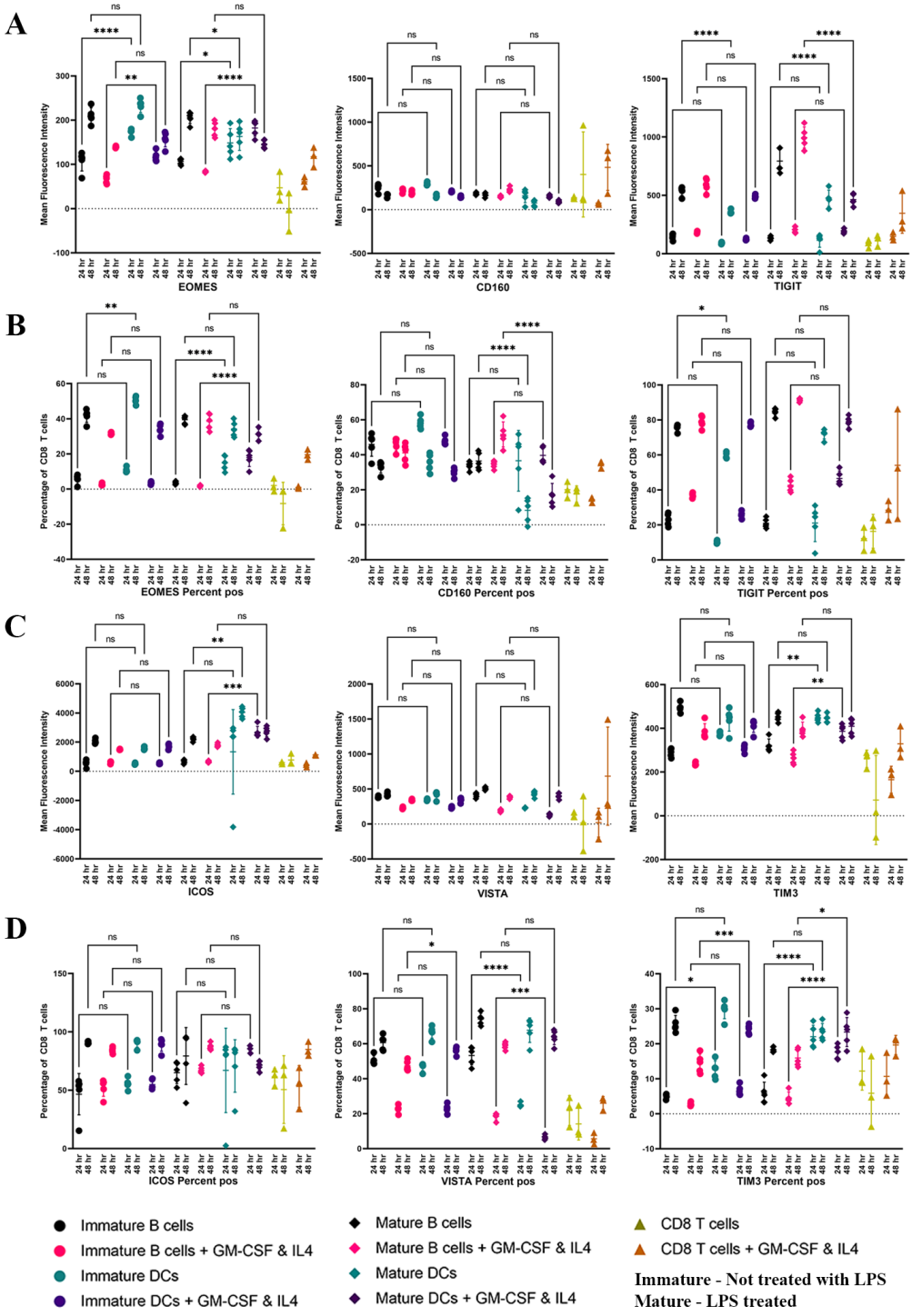


Figure 7: Priming through both B cells and DCs resulted in similar expression of cytotoxicity related markers on CD8 T cells.

In vitro assay was set up as in Fig 2; after 24 hours and 48 hours of incubation with CD8 T cells, flow cytometry was performed to measure expression of cytotoxicity related intra-cellular proteins. Expression of FAS-L (FASL), CD107a, and TRAIL were quantified. (A) Mean fluorescence intensities and (B) percentage of CD8 T cells positive (pos) for expression of the respective cell surface markers were plotted. MFIs and percentages were subtracted for background from their respective non-peptide stimulated groups and represented as single dots for each mouse replicate, with mean and standard deviation from five replicates. Asterisks * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$, and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in five biological replicates.

Figure 7: Priming through both B cells and DCs resulted in similar expression of cytotoxicity related markers on CD8 T cells.

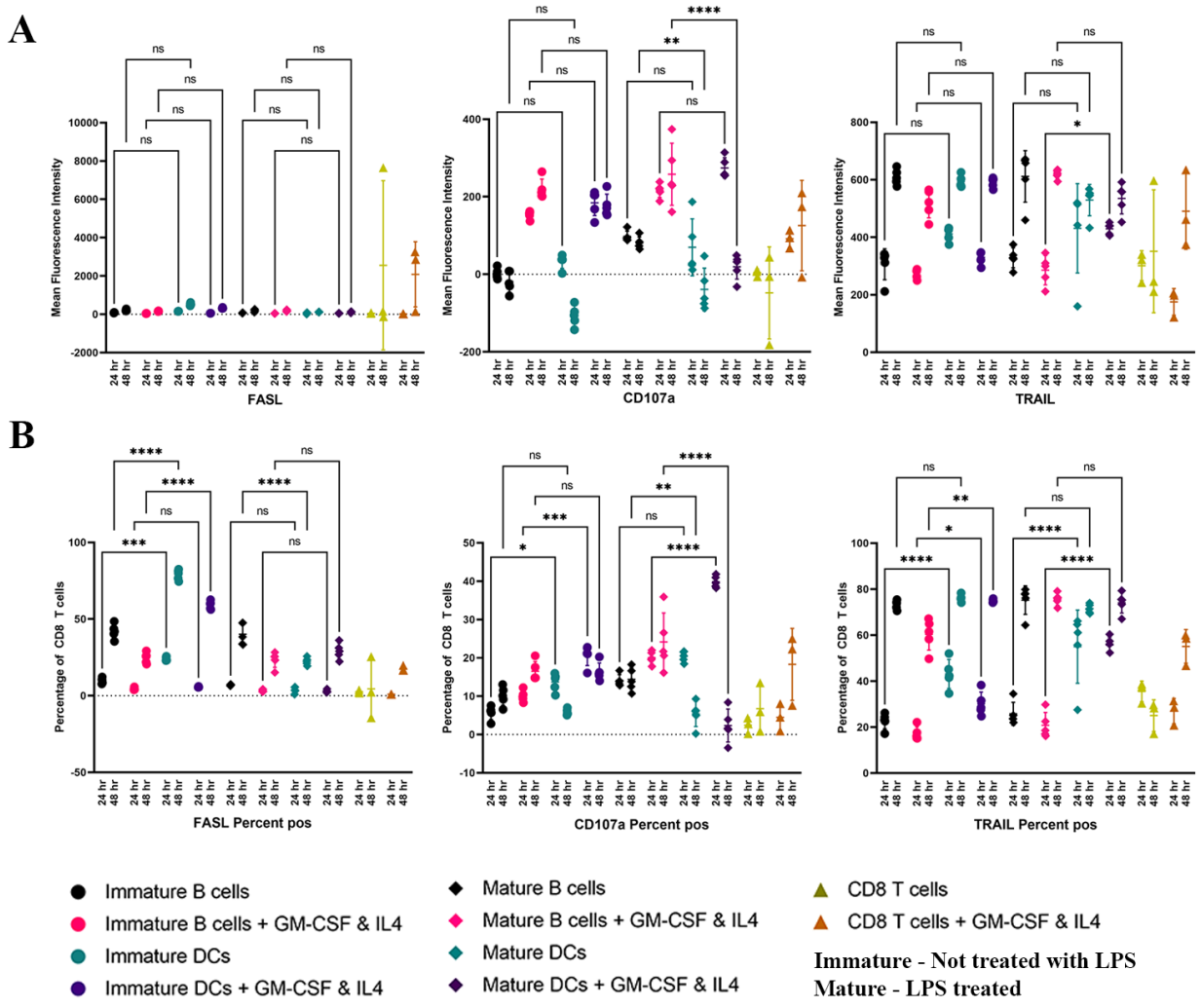


Figure 8: Priming through both B cells and DCs resulted in similar expression of cytotoxicity related markers on CD8 T cells.

In vitro assay was set up as in Fig 2; after 24 hours and 48 hours of incubation with CD8 T cells, flow cytometry was performed to measure expression of cytotoxicity related intra-cellular proteins. Expression of Granzyme B (Grz-B), Interferon-gamma (IFN-g), TNF-alpha (TNF-a), and Perforin was quantified. (A,C) Mean fluorescence intensities and (B,D) percentage of CD8 T cells positive (pos) for expression of the respective cell surface markers were plotted. MFIs and percentages were subtracted for background from their respective non-peptide stimulated groups and represented as single dots for each mouse replicate, with mean and standard deviation from five replicates. (E) Levels of secreted IFN-g were also quantified using ELISA after 24 and 48 hours. Asterisks * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$, and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in five biological replicates.

Figure 8: Priming through both B cells and DCs resulted in similar expression of cytotoxicity related markers on CD8 T cells.

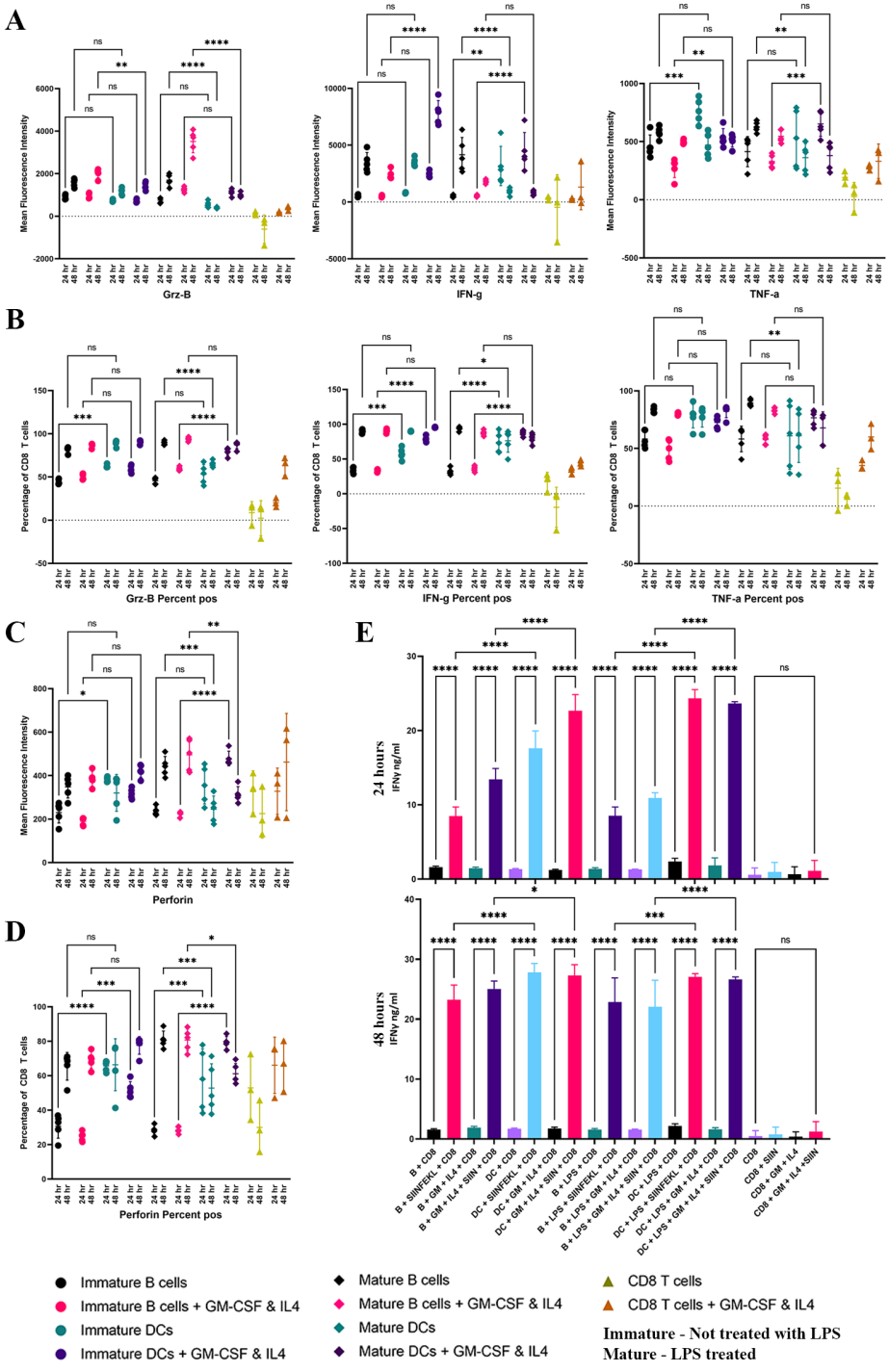


Figure 9: Priming through peptide-pulsed LPS-matured B cells resulted in increased expression of IL10.

In vitro assay was set up as in Fig 2; after 24 hours and 48 hours of incubation with CD8 T cells, flow cytometry was performed to measure expression of cytokines related to helper T cells via intra-cellular staining. Expression of IL10, IL4, and IL2 was quantified. (A) Mean fluorescence intensities and (B) percentage of CD8 T cells positive (pos) for expression of the respective cell surface markers were plotted. MFIs and percentages were subtracted for background from their respective non-peptide stimulated groups and represented as single dots for each mouse replicate, with mean and standard deviation from five replicates. Asterisks * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$, and **** indicate $p<0.0001$, results are from one experiment, with samples assessed in five biological replicates.

Figure 9: Priming through peptide-pulsed LPS-matured B cells resulted in increased expression of IL10.

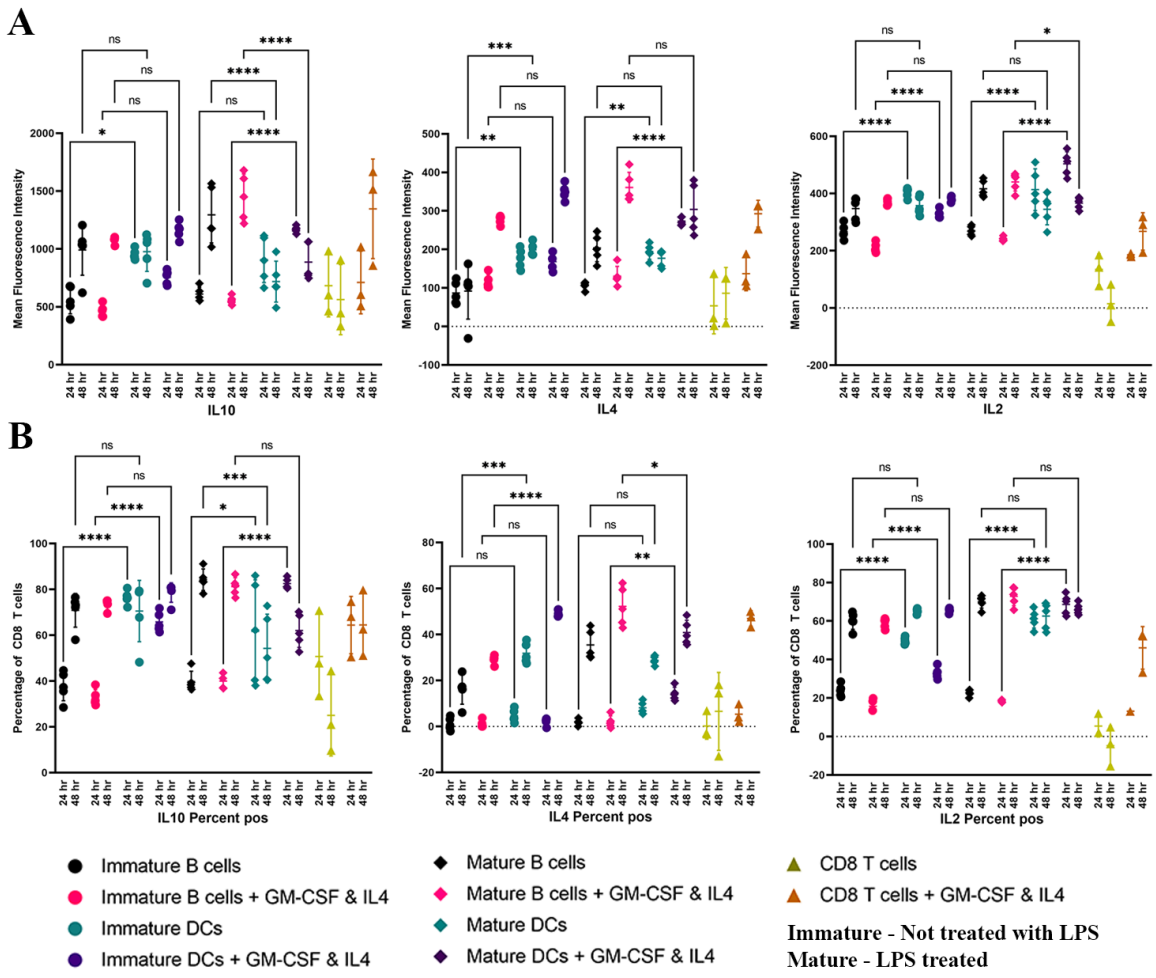


Figure 10: tSNE analysis revealed different phenotypes of CD8 T cells upon priming through LPS-matured B cells and LPS-matured DCs.

In vitro assay was set up as in Fig 2; after 24 hours and 48 hours of incubation with CD8 T cells, flow cytometry was performed to measure expression of cell surface markers and intracellular cytokines. tSNE analysis was performed using FlowJo (version 10.8), based on expression of 4-1BB, EOMES, Grz-B, IFN-g, IL10, IL4, IL2, Ki67, Perforin and TNF-a. (A) tSNE plots for each treatment groups and (B) heat-maps representative of each cluster defined after tSNE analysis were plotted. (C) Bar graph representing percentage of cells in each population cluster. Representative of one experiment with six biological replicates for each treatment group.

Figure 10: tSNE analysis revealed different phenotypes of CD8 T cells upon priming through LPS-matured B cells and LPS-matured DCs.

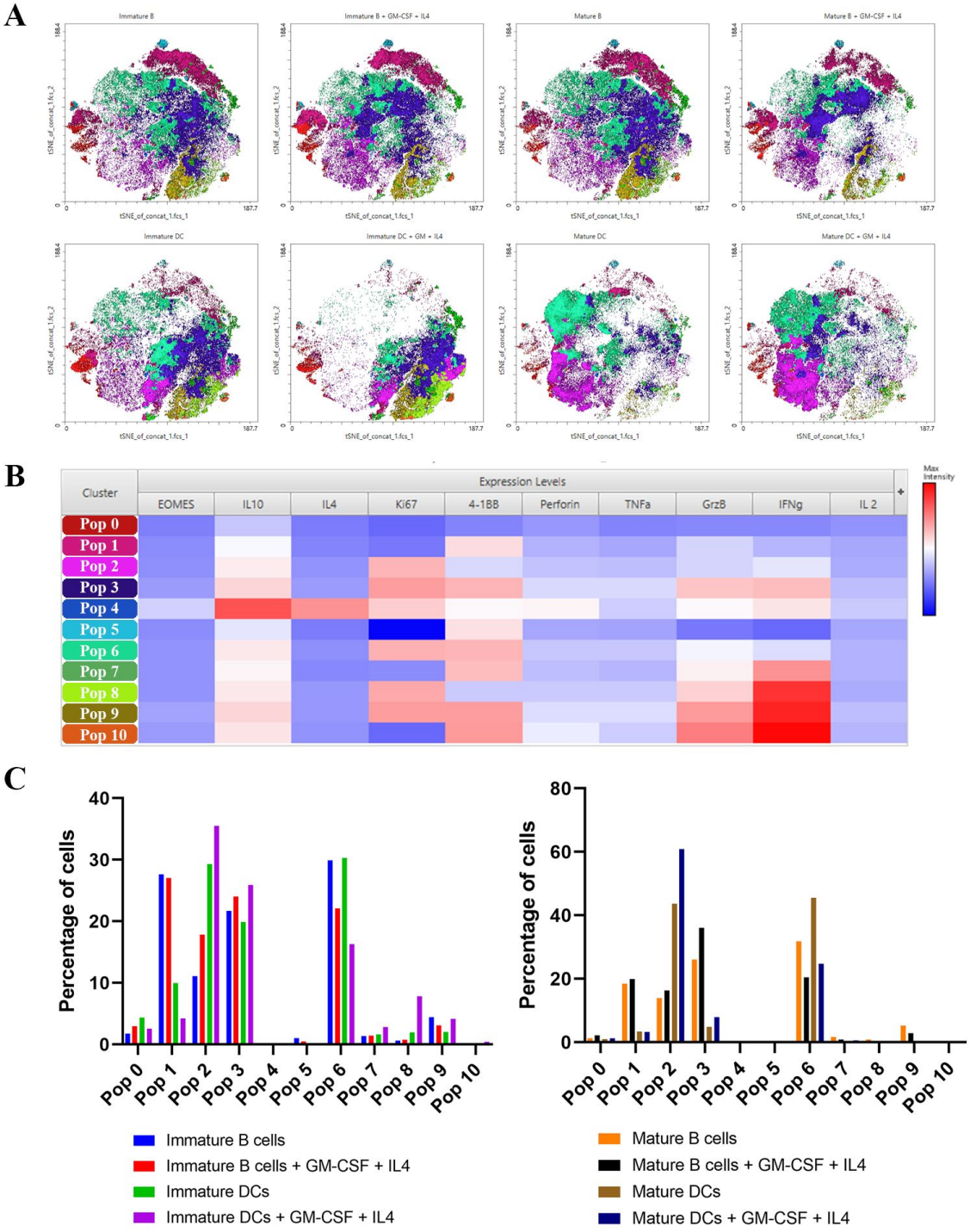
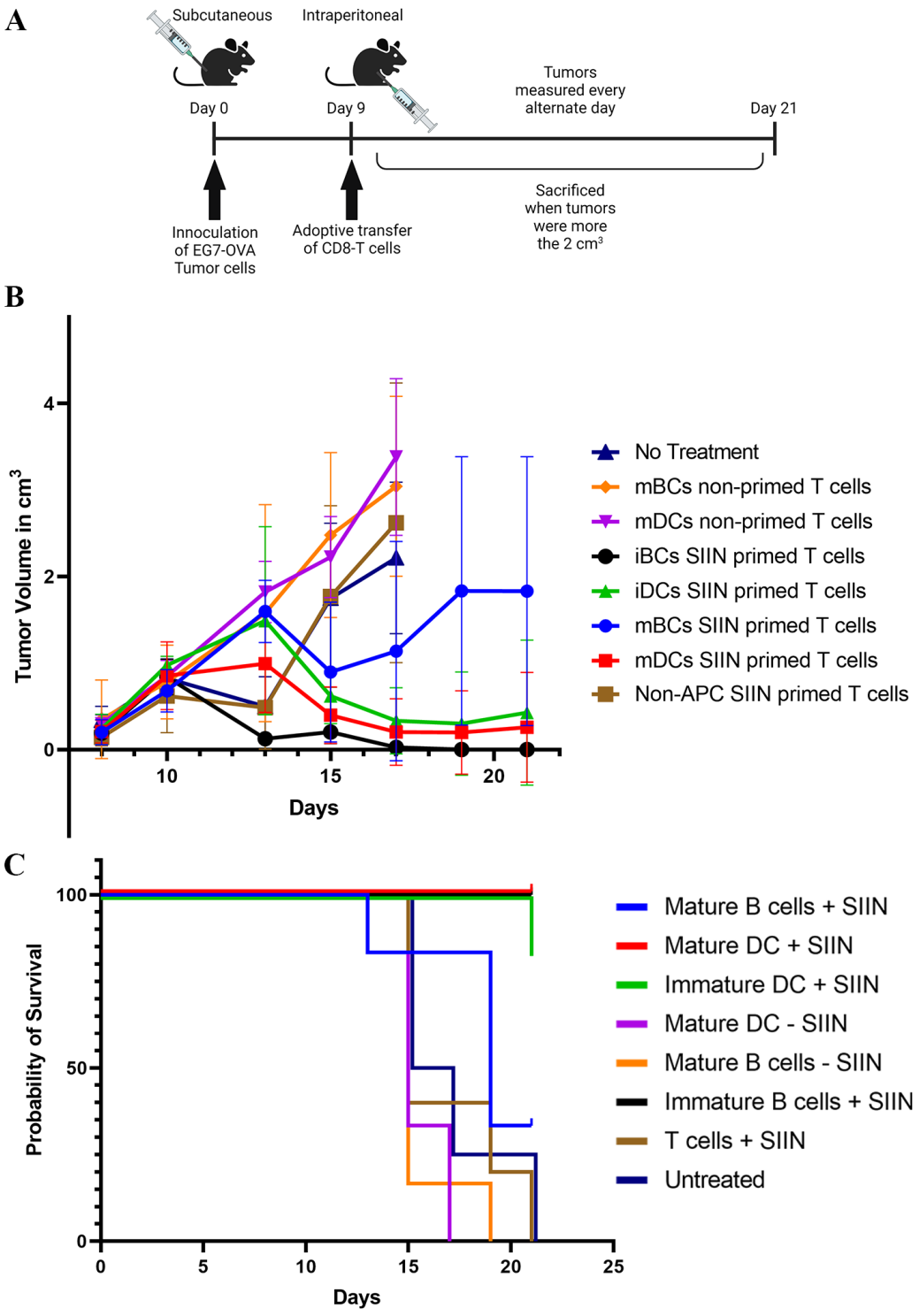


Figure 11: CD8 T cells primed through immature B cells, LPS-matured DCs, and immature DCs generated similar anti-tumor response upon adoptive transfer in murine tumor model.

C57Bl/6 mice were inoculated with E.G7-OVA tumors subcutaneously, with six mice per treatment group (except for negative control groups). CD8 T cells were primed *in vitro* as in Fig 2 and after 48 hours of incubation, CD8 T cells were re-purified using negative selection. Re-purified CD8 T cells were then adoptively transferred to tumor bearing mice on day nine, via intra-peritoneal injections. (A) Schematic and timeline of the tumor study. (B) Tumor volumes (cm^3) were measured every alternate day and (C) survival curve was also plotted; mice were marked as dead and euthanized once their tumor volumes reached 2 cm^3. Asterisks * indicate $p<0.05$, ** indicate $p<0.01$, *** indicate $p<0.001$, and **** indicate $p<0.0001$, results are from one experiment, for untreated and T cells only group, five and four mice were enrolled, respectively.

Figure 11: CD8 T cells primed through immature B cells, LPS-matured DCs, and immature DCs generated similar anti-tumor response upon adoptive transfer in murine tumor model.



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Chapter 4

Vaccination approaches utilizing B cells as antigen presenting cells for plasmid DNA can elicit immune responses *in vitro* and *in vivo*

All experiments presented in this chapter were performed and analyzed by me. Nanoparticles were provided by Guojon Chen from Dr. Sarah Gong's research group.

Abstract

Anti-tumor DNA vaccines are a relatively newer therapeutic approach for treatment of cancer patients. Preclinical studies in animal models have shown promising results, which proves the capability of DNA vaccines to successfully generate cellular as well as humoral response against the tumor. However, they have resulted in limited success when administered to humans as suggested by early-stage clinical trials. We believe promoting presentation of DNA vaccine by professional APCs will generate a stronger immunogenic response. Our previous findings have shown that amongst the professional APC subtypes, B-cells are the only cells, which can express the encoded antigen upon passive uptake of plasmid DNA. More recently, we have demonstrated using *in vitro* culture that B-cells are the primary antigen presenting cells following passive uptake of plasmid DNA, however they require licensing through DCs to activate antigen-specific CD8 T cells. Based on our findings, we hypothesize that either targeting DNA vaccine specifically to B-cells or delivering *ex vivo* prepared DNA-loaded autologous B cells would generate stronger and more robust immune response against the encoded antigen. We took advantage of an EBV protein, GP350 that specifically targets CD21+ B cells. We utilized either GP350-derived peptides for developing nanoparticles or GP350 expressing exosomes as vehicles for delivering DNA plasmid specifically to B cells. We also generated DNA-loaded B cells *ex vivo* and tested their immunogenicity *in vivo*. We demonstrated that nanoparticles were successfully generated using B cell specific peptide and were loaded with plasmid DNA. However, nanoparticles generated using GP350-derived peptides were not specific to B cells, when tested in mixed PBMC cultures. We also demonstrated that GP350 expressing exosomes can be loaded with DNA plasmid either by using electroporation or by use of a transfection reagent. We also demonstrated that adoptive

transfer of *ex vivo* generated DNA-loaded B cells resulted in an increase in antigen-specific CD8 T cells *in vivo*. Collectively, our findings show that there were several approaches that can be applied to develop a B cell-centered vaccination approach for treatment of cancer patients. However, further investigations are required to understand how each of these strategies can be fine-tuned for generating optimal immunogenicity using plasmid DNA vaccines. These strategies also allow us to manipulate the professional APCs and enhance antigen presentation through them to further augment the immunogenicity of DNA vaccines.

Introduction

Our group has been specifically interested in DNA vaccines as a means to activate tumor-specific T cells to treat prostate cancer. DNA vaccines, relative to other vaccine approaches, are advantageous in being relatively easy, rapid and inexpensive to manufacture, and "off-the-shelf" rather than individualized, allowing treatment across patient populations independent of MHC haplotype. However, while DNA vaccines have been explored in human clinical trials, they have generally demonstrated lower efficacy than other vaccine approaches. We have previously demonstrated that a DNA vaccine encoding prostatic acid phosphatase can elicit tumor antigen-specific CD8 T cells, and immune responses were associated with favorable changes in PSA doubling time in patients with recurrent prostate cancer. Taken together, these findings demonstrate that DNA vaccines, either alone or in combination with other therapies, are effective in humans, and that rational efforts to increase their immunogenicity should further improve their clinical efficacy. Furthermore, we have also demonstrated that B cells are the primary antigen presenting cells for plasmid DNA and therefore direct targeting of DNA vaccine to B cells or generation of autologous B cells for presentation of DNA vaccines can be potent vaccination strategies.

To evaluate direct targeting of plasmid DNA to B cells, we explored nanoparticles and exosomes as possible delivery systems. Recent studies have shown that nanoparticles and exosomes both can be potent drug delivery approaches and can be modified accordingly for targeted delivery of drugs (1-4). Both approaches are relatively new and are rapidly developing. Nanoparticles have been shown to improve the efficacy of previously existing drugs, sometimes by overcoming drug resistance (5). In cancer therapy nanoparticles have been utilized specifically for their targeting

characteristic, and to protect normal cells from cytotoxicity (6, 7). Moreover, it has been shown that when active targeting is employed, nanoparticles interact with cell surface receptors which leads to their internalization by endocytosis and ultimately release of the drug inside the cell (8). This makes them suitable for delivery of macromolecular drugs such as proteins and nucleic acid-based drugs (9). Similarly, use of exosomes is being actively investigated for targeted drug delivery as a cancer therapeutic (10). Exosomes, provide a natural alternative to other nano-molecule based approaches. Exosomes have been characterized to be non-cytotoxic, biocompatible, easy to generate and store, and have low immunogenic properties (10, 11).

For targeting either of these delivery vehicles to B cells, we utilized the properties of EBV protein GP350, which specifically targets CD21 expressed on surface of B cells (12). It has been previously shown that exosomes expressing the EBV protein GP350 can target B cells by specifically binding to human CD21 (13). Similarly, small peptides (RMWPSSTVNLSAGRR) derived from GP350 protein can be used to specifically target CD21 (14), these peptides can be a means to modify a prior existing nanoparticle system to introduce B cell specificity.

Another alternative for B cell centered vaccination approach, is generation of autologous B cells which can be optimally activated and loaded with plasmid DNA *ex vivo* for use as a therapeutic. Some groups have demonstrated that CD40L or anti-CD40 antibody activated B cells function as potent APCs and have also shown that CD40 stimulated autologous B cells result in efficient T cell activation, *in vitro* and *in vivo* (15-17). However, CD40 activated B cells have not been tested for delivery of nucleic acid or peptide based vaccines. As we had already demonstrated the importance of B cells in presenting DNA plasmid and their capability to elicit similar CD8 T cell mediated responses upon peptide pulsing when compared to DCs. Therefore, we had sufficient

evidence to examine potency of DNA loaded autologous B cells *in vivo*, alongside our B cell targeted approaches.

In this chapter, we investigated B cells targeted approaches that included nanoparticles and exosome-based delivery systems and tested DNA-loaded autologous B cells for their efficacy *in vivo*. We demonstrated that B cell specific nanoparticles can be easily generated, however, delivery of DNA plasmid may not be specific to B cells. Similarly, we demonstrated that GP350 expressing exosomes can be efficiently generated and loaded with plasmid DNA, however optimizations are required for efficient loading and content quantification of these exosomes. Lastly, we demonstrated that DNA-loaded B cells licensed by DCs can elicit *in vivo* immune responses, similar to that of naked plasmid DNA immunization. Taken together, these studies suggested that B cell-centered vaccination approaches, specifically for plasmid DNA requires further investigation and optimization.

Results:

Peptide derived from GP350 protein specifically targeted B cells

We previously demonstrated that B cells are the primary antigen presenting cells for DNA plasmid. In this chapter, we seek to determine if plasmid DNA can be targeted directly to B cells *in vitro* and *in vivo* without the need for ex vivo loading or manipulation of B cells. Our first approach required development of nanoparticles that would encapsulate the DNA and deliver to B cells specifically. For this, we synthesized two B cell specific small peptides tagged with FITC, FITC-CGGGGEDPGFFNVE (B1) and FITC-CGGGGKKKKKKKEDPGFFNVE (B2), based on previously published CD21 specific epitopes of GP350 protein (14), which are derived from GP350 protein of EBV and targets human CD21 (B-cell specific). As shown in Fig 1, we observed that B2 peptide (GP350 derived), specifically bound to B cells, whereas B1 peptide (GP350 derived) failed to do the same. Peptides B3 and B4 were scrambled and were non-specific for CD21, therefore did not demonstrate binding on any of the cell lineages tested.

Nanoparticles generated using B cells specific peptide were successfully loaded with plasmid DNA

B2 peptide that we found to be specifically targeting B cells were used for development of nanoparticles. B2 peptide (specific) or B3 peptide (non-specific) were linked with cationic block copolymers developed by Dr. Sarah Gong's lab. These polymer-linked peptides were then mixed with plasmid DNA to form nanoparticles (Fig 2). We demonstrated using gel retardation assay that plasmid DNA and copolymer formed nanoparticle complexes (Fig 3). Naked DNA and

nanoparticle loaded with plasmid DNA were ran alongside and the shift in their band patterns demonstrate, that there was not any free plasmid DNA or peptide after complexing reaction, and the calculated N to P ratio was optimal for generation of these B cell specific nanoparticles.

B cell specific nanoparticles were non-specifically captured by dendritic cells and macrophages

We next evaluated the specificity of these nanoparticles that used either CD21-specific peptide (B2) or scrambled peptide (B3), for their ability to target B cells *in vitro*. As shown in Fig 4A, we demonstrated that nanoparticles coated with B-cell targeting peptide show preferential uptake by B cells and can carry DNA to B cells (Fig 4B). However, we also found that this delivery approach was not entirely specific, and DNA was carried to other cell lineages such as macrophages and dendritic cells as well, which were present in the mixed PBMC cell populations.

Exosomes generated from iMEF cell line were detected using flow cytometry and quantified for protein content using Bradford assay

As an alternative approach to deliver DNA plasmid specifically to B cells we wished to evaluate the efficacy of GP350 expressing exosomes. For this, we first wanted to optimize detection, quantification, and plasmid DNA loading, following their collection from iMEF cultures. As shown in Fig 5A, we demonstrated that biotinylated CD9 antibody bound to streptavidin magnetic beads captured the exosomes from our culture media supernatants. These exosomes were then labeled with FITC exosome stain and were ultimately detected by flow cytometry. We also

demonstrated that protein content of these exosomes can be calculated using Bradford assay (Fig 5B), which could be used as a means for relative quantification of number of exosomes present in the media supernatant and use for normalization of exosome numbers between different exosome preparations.

Exosomes were loaded with plasmid DNA either by electroporation or transfection

We next evaluated different approaches to load the exosomes with plasmid DNA, with optimal efficiency. Electroporation has been used by many to permeabilize the membrane of exosomes and more recently transfection reagents have also been developed for this purpose. We wished to test both of these methods with native and GP350-expressing exosomes. As shown in Fig 6A, we were able to detect Cy5-labeled plasmid DNA in both native and GP350-expressing exosomes by electroporation. Approximately 76% of all exosomes were found to be positive for plasmid DNA for both types of exosomes. Similarly, we demonstrated that use of exosome transfection reagent (Exo-Fect) also resulted in plasmid DNA loaded exosomes (Fig 6B). However, efficiency of plasmid DNA loading was variable between the two types of exosomes. Approximately 97% of native exosomes were found to be positive for plasmid DNA, whereas only 57% of GP350 exosomes were found to be positive for plasmid DNA. Indicating that further optimizations are required for both approaches.

Antigen-specific CD8 T cells were detected *in vivo* after adoptive transfer of DC-licensed DNA loaded B cells

As we demonstrated using *in vitro* antigen presentation assay that B cells are the primary antigen presenting cells for plasmid DNA but required licensing from DCs to activate antigen-specific CD8 T cells. We wished to evaluate the efficacy of DC-licensed DNA-loaded B cells in generating an immunogenic response *in vivo*. We immunized C57Bl/6 mice twice, seven days apart with either plasmid DNA, B cells loaded with plasmid DNA, re-purified DC-licensed B cells loaded with plasmid DNA or DNA loaded B cells with DCs. As shown in Fig 7A and 7B, we demonstrated that DC-licensed OVA-DNA loaded B cells were able to elicit an immune response *in vivo* and resulted in generation of antigen-specific (SIINFEKL) CD8 T cells. We further demonstrated that these cells are activated as they had increased expression of 4-1BB (Fig 7C) and PD1 (Fig 7D). This suggested that B cell-centered vaccination strategy could be an alternative to current delivery approaches for plasmid DNA but require further optimizations.

Discussion and Conclusion

Overall, our data demonstrated that several approaches can be employed to develop a B cell centered therapeutic approach. However, a deeper understanding of each of these approaches is currently required. Nanoparticles were generated to be specific for B cells but were captured by other cell lineages nearby. Similarly, exosomes specific for B cells were successfully generated and loaded with plasmid DNA, but optimizations are required for efficient loading and quantification of these DNA loaded exosomes. Moreover, DC-licensed DNA-loaded B cells elicited an *in vivo* response, but the resulting immunogenicity was similar to naked plasmid DNA immunizations.

Delivery of DNA plasmid using nanoparticles proved to be challenging, as nearby cell populations like DCs and macrophages, which are known for their phagocytic activity captured the nanoparticles non-specifically. Nanoparticles have been previously tested for delivery of DNA vaccines (18, 19), however specific targeting of DNA vaccine to B cells using nanoparticles was a novel approach. Nanoparticles have been shown to improve immunogenic response by enhancing DNA uptake and nuclear delivery of DNA to APCs (20). This property of nanoparticles could be exploited when delivering plasmid DNA to DCs, as we have previously demonstrated that plasmid DNA gets degraded upon passive uptake by DCs (21). Therefore, non-specific uptake of DNA-loaded nanoparticles by DCs could ultimately have positive outcome and warrants further investigations.

Exosome-based delivery is a relatively newer area of research and is currently under active exploration. There has been growing interest in exosome related research, as their potential roles

in cell-cell communication specifically in diseases such as cancer are being reported (22). However, techniques and methods for their isolation, identification, detection, quantification, and analysis are under-developed (23). Generation of DNA-loaded exosomes has been attempted by some groups, who reported limited gene delivery (24, 25). We faced similar challenges while developing methods for generating DNA-loaded exosomes. Moreover, we now know that B cells require licensing through DCs to gain the antigen presentation function. Therefore, it would be more sensible to generate DNA loaded autologous B cells for our purposes, unless we deliver the co-factor(s) provided by cell surface interaction through DCs along with plasmid DNA, to induce APC function of B cells. Same would be true for application of nanoparticle-based delivery of plasmid DNA.

Use of autologous B cells as vaccines has been demonstrated to be advantageous by several groups (26). Manufacturing of these autologous B cells often require treatments with B cells activation agents such as CD40L (27), anti-CD40 agonistic antibodies (28), TLR9 agonist CpG (29), IL21 and IL4 (16), which act by inducing antigen presentation abilities of B cells. There is growing interest in use of B cells as immunotherapy, as recent reports demonstrate their ability to prime T cell responses and induce anti-tumor effects (26). Moreover, B cell APCs can evade immune suppression as they have been shown to be resistant to factors such as prostaglandin E2 (30), IL10 (31), VEGF (32) and TGF-β (33). However, application of DNA-loaded B cells as an immunotherapy approach is a novel idea and to our knowledge has not been tested before. Given the evidence from our *in vitro* studies that demonstrated crucial role of B cells in processing of DNA vaccines, our future studies will involve optimization of DC-licensed DNA-loaded B cell vaccination strategy.

Collectively, these findings have provided insight that targeting B cells *in vivo* through nanoparticles or exosomes may not be as effective as adoptively transferring B cells, and this is the approach that we are currently pursuing. Specifically, once we identify the factor(s) provided by DC that are required for B cell presentation, we expect to evaluate this vaccine approach in murine tumor models with an ultimate goal to develop a novel vaccine delivery approach as a treatment for cancer patients.

Figures and Tables:

Figure 1: Peptide derived from GP350 protein specifically targeted B cells.

PBMCs were plated at 0.4 x 106 cells per well in triplicates for each treatment group and incubated with 4µg/ml of peptide (B1, B2, B3 or B4). B1 and B2 were B cell specific peptides derived from GP350 protein, whereas B3 and B4 were non-specific scrambled peptides. B1, B2 and B3 were FITC labeled, and B4 was Cy5-labeled. PBMCs were evaluated using flow cytometry to determine peptide binding on T cells (CD4+ and CD8+), macrophages (CD11b+), DCs (CD11c+) and B cells (CD19+). Percentages of cells are presented as bar graphs from three replicates with mean and standard deviation. Data presented here is from one experiment and is representative of three similar experiments.

Figure 1: Peptide derived from GP350 protein specifically targeted B cells.

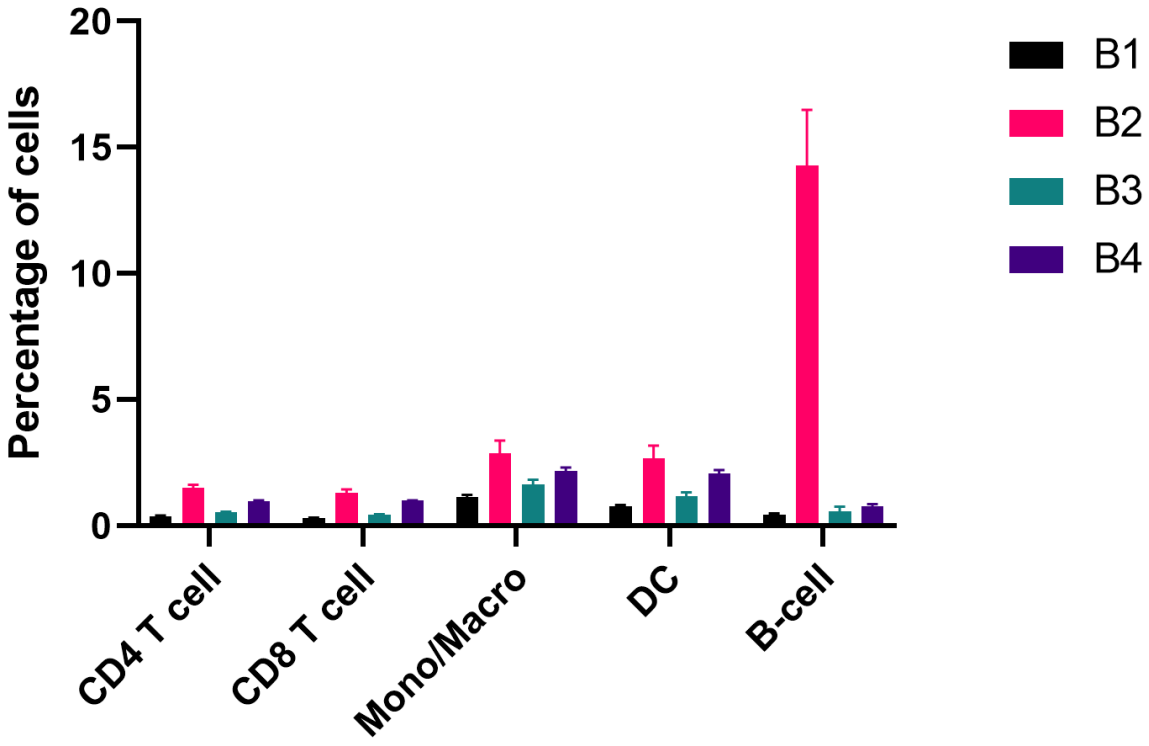


Figure 2: Nanoparticle design used for generating B cell specific nanoparticles for DNA delivery.

B cell specific peptides were linked to the cationic copolymers generated by Dr. Sarah Gong's lab. These peptides were then mixed with plasmid DNA at a calculated N to P ratio (2µg DNA with 0.2µg peptide) for 20 minutes at room temperature to form nanoparticles. The nanoparticles were designed to be redox-sensitive and carried an endosomal escape group. This allowed the nanoparticle to remain stable in the endosome, but on escape to the cytoplasm, nanoparticles were dissociated and released the encapsulated DNA.

Figure 2: Nanoparticle design used for generating B cell specific nanoparticles for DNA delivery.

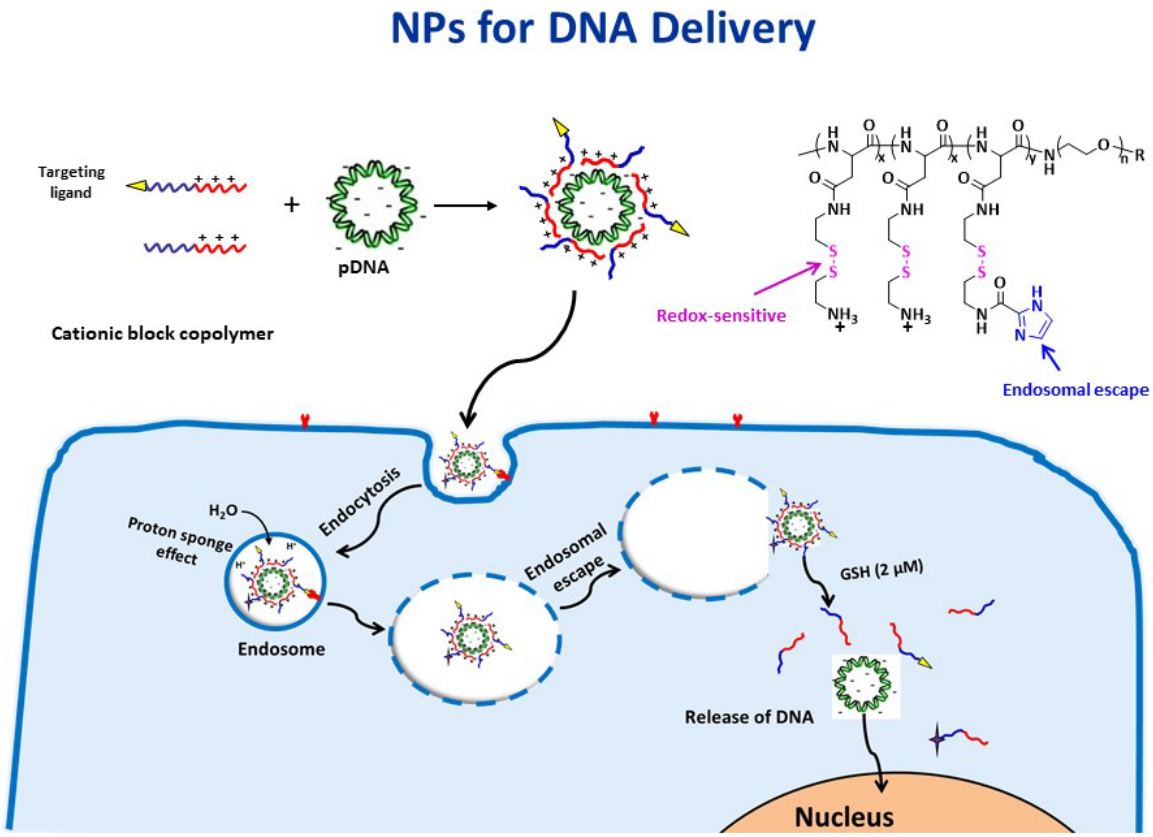


Figure 3: Nanoparticles generated using B cells specific peptide were successfully loaded with plasmid DNA.

Plasmid DNA and B cells specific peptide linked polymers were complexed together as described in Fig 2 to form nanoparticles. Plasmid DNA (Lane 2) and nanoparticle encapsulated DNA (lane 4) were run on a 1% agarose gel by electrophoresis. 1kb DNA ladder (lane 1) was used for band size estimation, and gel was run at 110V. Gel was visualized using UV illuminator. Data presented here is from one experiment.

Figure 3: Nanoparticles generated using B cells specific peptide were successfully loaded with plasmid DNA.

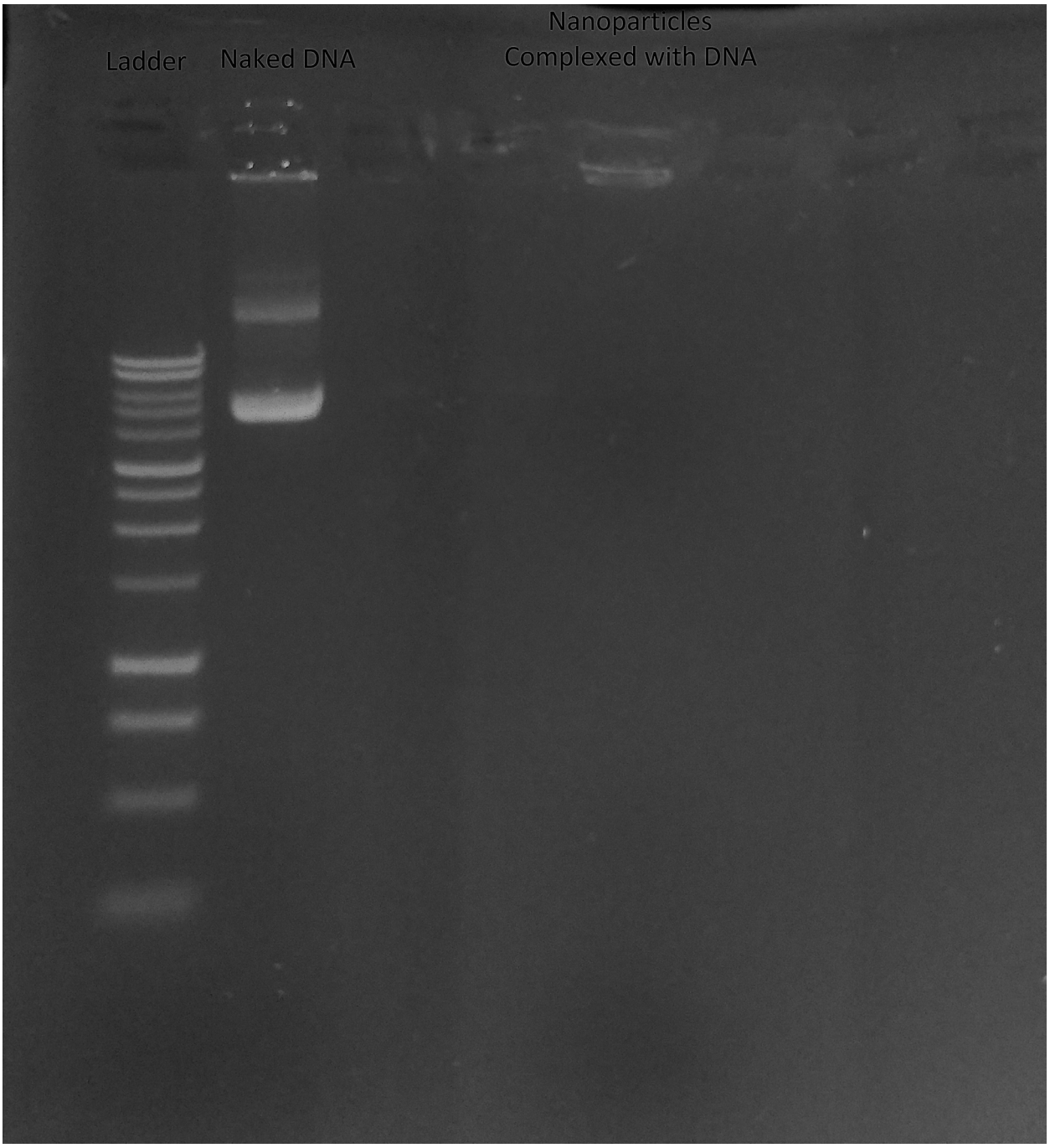


Figure 4: B cell-specific nanoparticles were non-specifically captured by dendritic cells and macrophages.

B cell specific peptide B2 and non-specific peptide B3 were used to generate nanoparticles, loaded with Cy5-labeled plasmid DNA. Nanoparticles were then incubated with PBMCs plated at 0.5 x 106 cells per well. Peptide alone and DNA alone treatment groups were used as negative controls for estimation of background. Cells were analyzed using flow cytometry for detection of (A) B cell specific peptide and (B) DNA in T cells (CD4+, CD8+), macrophages (CD11b+), DCs (CD11c+), and B cells (CD19+). Percentages of cells are presented as bar graphs from three replicates with mean and standard deviation. Data presented here is from one experiment and is representative of three similar experiments.

Figure 4: B cell-specific nanoparticles were non-specifically captured by dendritic cells and macrophages.

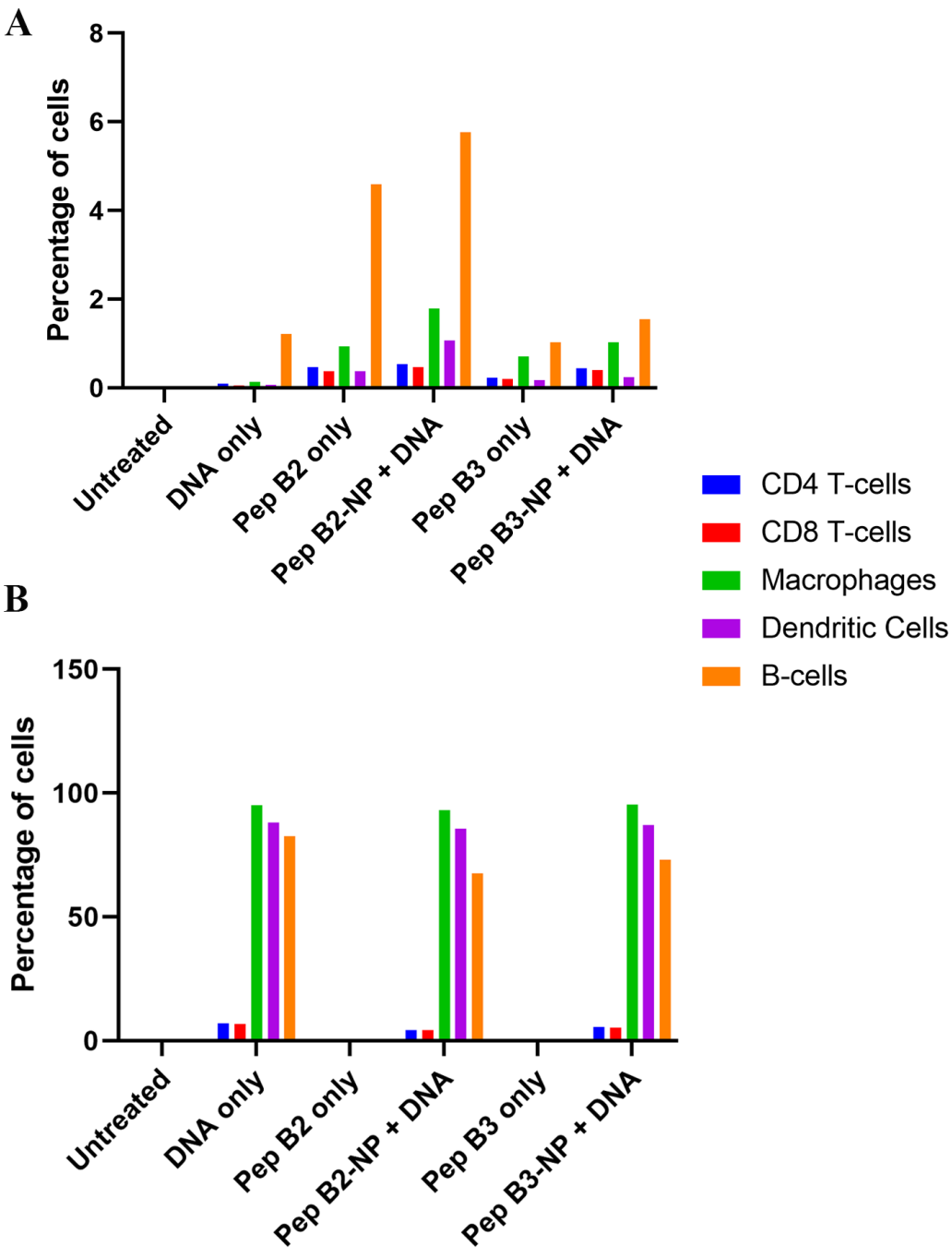
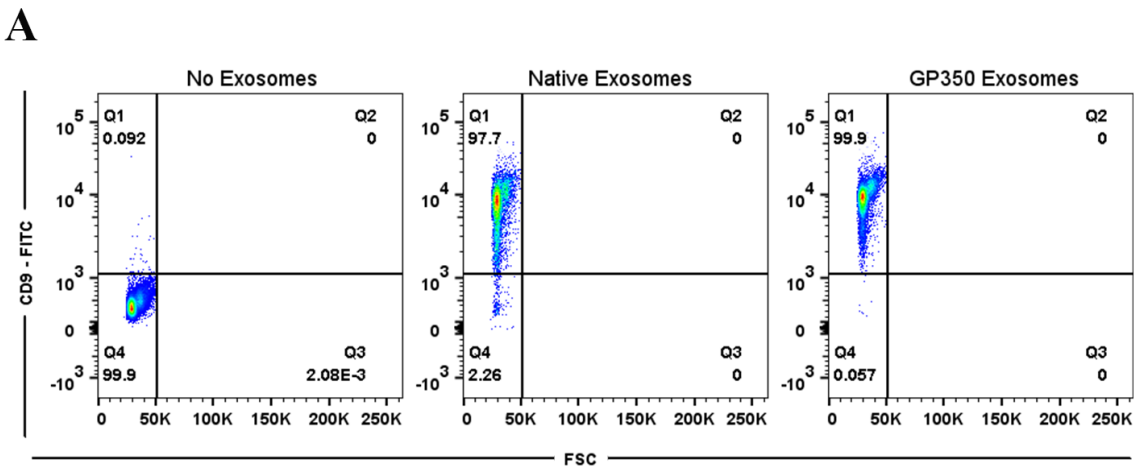


Figure 5: Exosomes generated from iMEF cell line were detected using flow cytometry and quantified for protein content using Bradford assay.

Exosomes were collected from either native or GP350-transduced iMEF (irradiated mouse primary embryonic fibroblast) cells *in vitro*. Collected exosomes were captured using CD9 tagged streptavidin magnetic beads for analysis by flow cytometry. Magnetic beads (CD9 tagged) without exosomes were used as negative control. (A) Flow plots forward scatter (FSC) on X-axis and CD9-FITC on Y-axis. (B) Protein quantification of exosomes using Bradford assay, against a BSA standard curve. Data presented here is from one experiment and is representative of three similar experiments.

Figure 5: Exosomes generated from iMEF cell line were detected using flow cytometry and quantified for protein content using Bradford assay.



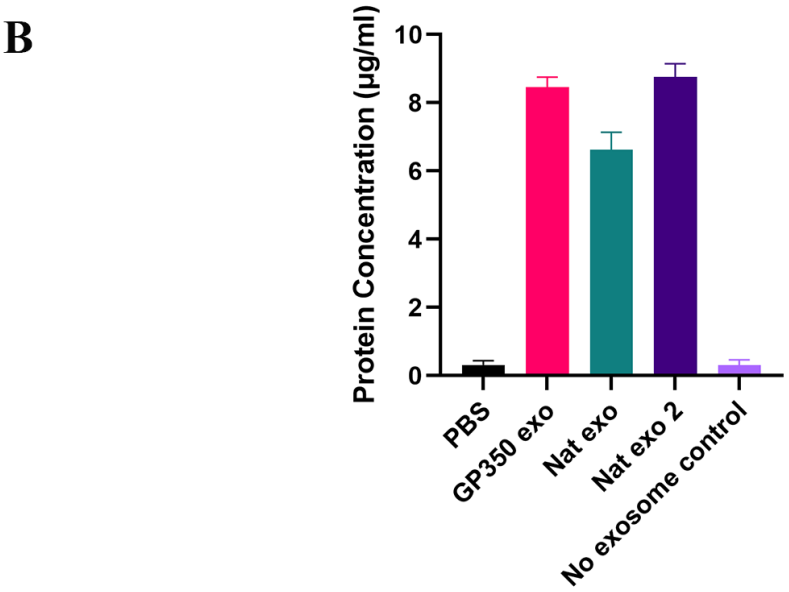


Figure 6: Exosomes were loaded with plasmid DNA either by using electroporation or by transfection

(A) Native and GP350 expressing exosomes were diluted in electroporation buffer. Exosomes were mixed with 20µg plasmid DNA and then electroporated in 0.4mm cuvettes at 400mV voltage and 125µF capacitance, with pulse time of 10-15 milliseconds. No DNA electroporation controls were included to determine the background noise. Exosomes were re-isolated and prepared for analysis with flow cytometry as in Fig 5. Data presented here is from one experiment and is representative of two similar experiments. (B) Native and GP350 expressing exosomes were mixed with 20µg of plasmid DNA in presence of transfection reagent Exo-Fect. No DNA transfection controls were included to determine the background noise. Exosomes were washed, re-isolated and prepared for flow cytometry as in Fig 2. Data presented here is from one experiment and is representative of two similar experiments.

Figure 6: Exosomes were loaded with plasmid DNA either by using electroporation or by transfection

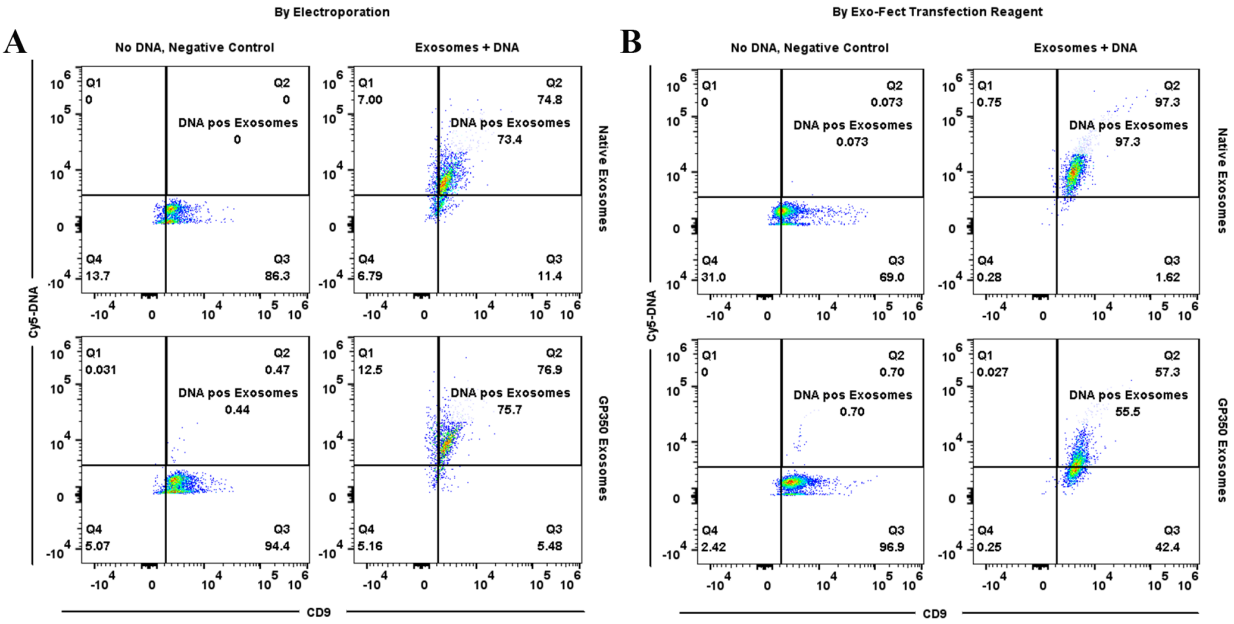
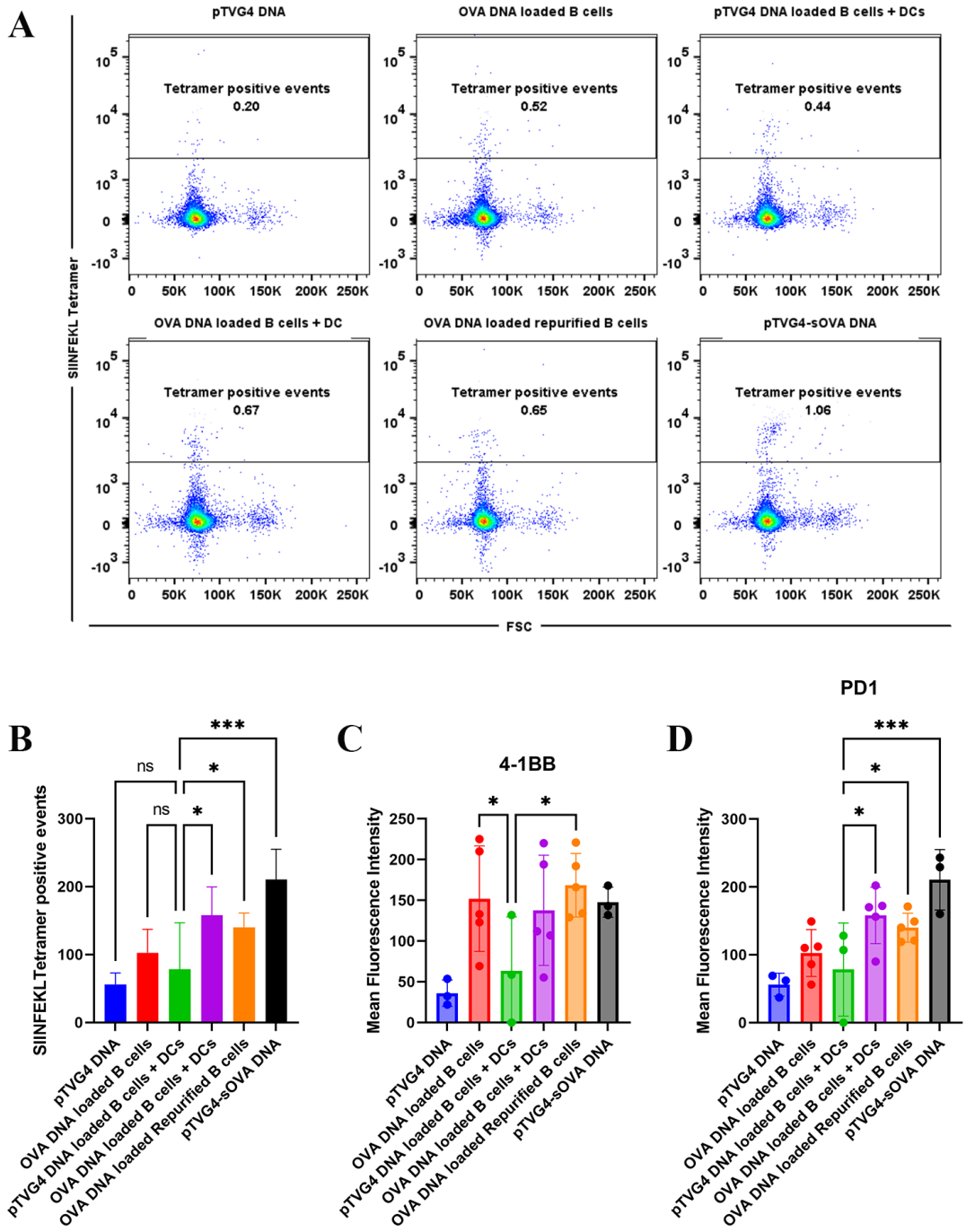


Figure 7: Antigen-specific CD8 T cells were identified *in vivo* after adoptive transfer of DNA-loaded B cells.

DCs isolated from C57Bl/6 mice were cultured in the presence of GM-CSF and IL4 for five days and then DNA loaded B cells were added to the culture along with anti-CD40 mAb. After three days of co-culture, cells were collected and adoptively transferred in to C57Bl/6 mice intradermally. Two similar adoptive transfers were performed seven days apart for each treatment group. Mice were also immunized with naked plasmid DNA, either pTVG4 or pTVG4-sOVA as control groups. Seven days after the second adoptive transfer, mice were sacrificed, and spleens were collected to analyze for presence of antigen-specific CD8 T cells. Re-purified B cells are the cells that have been co-cultured with DCs and then separated using magnetic bead based negative selection. (A) Flow plots show SIINFEKL tetramer positive events recorded from one representative animal from each group. (B) Mean tetramer counts from all animals are plotted as bar graph with standard deviations. (C) 4-1BB and (D) PD1 expression on tetramer positive CD8 T cells are also plotted as bar graphs with mean and standard deviation. Asterisk * indicate $p<0.05$ and *** indicate $p<0.001$. Data presented here is from one experiment with five mice per treatment group and three mice in negative control groups.

Figure 7 – Antigen-specific CD8 T cells were identified *in vivo* after adoptive transfer of DC licensed DNA loaded B cells.



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Acknowledgements

I would like to thank Dr. Sarah Gong and her lab member Guojon Chen for providing guidance on generating B cell specific nanoparticles and materials for their preparation.

Chapter 5

Summary and Future Directions

Summary

DNA vaccines, relative to other vaccine strategies, are an attractive approach for cancer treatment given their safety, easy manipulation, scalability, stability, and economical manufacturing. While a DNA vaccine has been approved for canine melanoma, early phase clinical studies in humans have been generally disappointing. Further studies to understand their mechanism of action, in order to improve their immunogenicity, are therefore needed.

Current delivery approaches use intradermal or intramuscular injections, where the vaccine is primarily taken up and processed by non-professional antigen presenting cells (APC). We believe that part of the reason for limited success of DNA vaccine is relatively weaker immune response generated by low antigen production by non-professional APCs, which leads to poor cross-priming by professional APCs. Based on our preliminary data and literature study, we hypothesized that direct presentation through professional APCs could improve the immunogenicity of DNA vaccines and APC activation via adjuvants such as CD40L or anti-CD40 agonistic antibody can further augment and amplify the immune response against the tumor.

To prove our hypothesis, we formed three major aims: 1) To develop an *in vitro* system for investigating antigen presentation of DNA vaccines and elucidating the mechanism of antigen presentation of DNA vaccines through professional antigen presenting cells, 2) to evaluate and compare the phenotype of CD8 T cells resulting from priming through antigen loaded B cells or DCs, and 3) to develop APC-centered therapeutic strategies focused on improving immunogenicity of DNA vaccines

For the purpose of *in vitro* experiments in Aim 1, we used splenic B cells from wild type C57/Bl6 mice, DCs from Flt3L-treated splenocytes from C57/Bl6 mice, CD8 T-cells from OT-1 mice (T-cell receptor specific for the SIINFEKL peptide epitope of ovalbumin), and plasmid DNA encoding ovalbumin. Antigen-presenting cells (APCs) were loaded with plasmid DNA and tested under variable conditions to evaluate antigen presentation. Activation and proliferation of antigen-specific CD8+ T cells was analyzed by flow cytometry and IFN-γ ELISA.

Previously, we had reported that passive uptake of plasmid DNA by dendritic cells (DC) or macrophages led to its degradation. On the contrary, we observed that passive uptake of DNA by B cells led to transcription of the encoded genes. From the studies reported here we found that B cells, after passive uptake of DNA were able to activate and proliferate antigen-specific CD8+ T cells, but only in the presence of DCs along with GM-CSF and IL4.

Although antigen presentation occurred when both B cells and DCs were in co-culture, we demonstrated by use of MHCI KO APCs and by use of re-purified B cells and DCs from the co-culture following passive uptake of DNA, that only B cells were able to activate and proliferate antigen-specific CD8 T cells. We also demonstrated that B cells required priority access to DNA plasmid, as DCs outcompete B cells for DNA uptake and degrade it, when in co-culture. We further demonstrated that DCs licensed B cells through cell-cell interaction(s) and replacing live DCs with either DC lysate or DC supernatant or their combination was not sufficient for licensing of B cells.

We next demonstrated by cytokine array that co-culture of B cells and DCs resulted in secretion of cytokine related to inflammatory immune response and chemokines related to chemotaxis of several myeloid and lymphoid cell populations. This was performed with a goal to identify cytokines that may be specific to the presence of DCs and may be the key players involved in

transforming B cells into antigen presenting cells. Unfortunately, we could not determine any unique signatures that would distinguish the cultures comparing presence and absence of dendritic cells. Furthermore, for identification of gene expression changes that occurred in B cells after co-culture with DCs, we performed RNA-seq studies. Results of which demonstrated that there were dramatic differences in the gene expression signatures between DNA loaded B cells that have been cultured alone compared to DC-licensed DNA-loaded B cells. GSEA analysis indicated that resulting B cells phenotype is similar to that of B cells activated through TLR7/8 agonist and/or stimulated through BCR. Although gene expression data demonstrated B cell functionality and vast differences in B cell phenotype, our goal to identify signaling pathway(s) specifically activated by DCs remained unanswered.

We also performed a literature search for known protein-protein interactions between B cells and DCs. Among several candidates we shortlisted a few relevant protein-protein interactions such as CD40-CD40L, CD23-IgE and CD27-CD70 that could have a key role in antigen presentation function of B cells. We tested each of these interactions individually by either using blocking antibodies or by using APCs from specific knockout mouse models. The resulting data indicated that both CD40-CD40L and CD23-IgE interactions play crucial roles as blocking these interactions negatively affected antigen presentation, whereas blocking CD27-CD70 interaction did not result in any significant changes.

Alongside our studies that elucidated mechanisms involved in antigen presentation of plasmid DNA through B cells, we investigated the phenotype of CD8 T cells that may result in response to priming through B cells and compared that to CD8 T cells primed through DCs. This was a direct approach to study CD8 T cells and if there were any intrinsic differences in the way that B cells

and DCs activated CD8 T cells. These studies were performed as part of Aim 2, for which we peptide pulsed either immature or LPS-matured B cells or DCs, which were then used to prime antigen-specific CD8 T cells. We then performed an in-depth analysis of activation markers, checkpoint markers, exhaustion markers, memory phenotypes, cytotoxicity related markers and Th2-related cytokine production on the resulting CD8 T cells. Our data demonstrated that B cells and DCs both were similarly capable of activating CD8 T cells, they both generated similar memory phenotypes and also generated similar cytotoxic and proliferation profiles. However, LPS-matured B cells behaved differently and resulted in poor anti-tumor response, they also demonstrated increased expression of IL10 and increased generation of central memory phenotype upon CD8 T cell priming. Taken together this suggested that B cells are equally capable as DCs in eliciting T cell mediated immune response.

For the purpose of developing APC centered therapeutic strategies from Aim 3, our approach had been to selectively target DNA vaccine to B cells. As based on our preliminary data we believed that B cells may be self-sufficient for antigen presentation. Our strategies included use of CD21-targeting nanoparticles, or CD21-targeting exosomes, which would specifically interact with B cells and deliver DNA vaccine. We were successful in developing both B cell targeting nanoparticles and exosomes for targeting DNA vaccine to B cells. However, there were limitations to both approaches which were ultimately not feasible to address. Moreover, from our *in vitro* studies, we gained deeper understanding of B cells as APCs when processing plasmid DNA. Given that DCs were required to "license" B cells, we significantly modified our DNA vaccine delivery approach for *in vivo* investigations.

Our new strategy included *ex vivo* generation of DC-licensed DNA-loaded autologous B cells. We tested *ex vivo* generated B cells against naked plasmid DNA for their ability to generate immune response *in vivo*. We found that B cells loaded with DNA plasmid that had been co-cultured with DCs *in vitro* (when delivered with DCs or after re-purification from DCs) generated a similar number of antigen-specific CD8 T cells as compared to naked plasmid DNA.

Overall, we demonstrated that B cells serve as primary antigen-presenting cells for DNA vaccine, but their ability to subsequently activate and proliferate CD8+ T cells is licensed by DCs. These findings are novel and are important to plan a viable vaccination strategy that utilizes DNA vaccines for treatment of cancer patients. Further evaluation of this mechanism is currently underway to completely understand the role of DCs. We also demonstrated that CD8 T cells primed through antigen pulsed immature B cells and immature DCs were similarly capable of activating CD8 T cells and generated similar anti-tumor responses. Lastly, our *in vivo* immunization studies indicated that DC-licensed B cells loaded with DNA can be a viable approach for delivery of plasmid DNA but required further optimizations.

Future Directions

Within the last decade, immunotherapy as a treatment approach for cancer patients have evolved rapidly. In 2010, the first therapeutic cancer vaccine, Sipuleucel-T was approved by FDA for treatment of castration resistant prostate cancer patients (1). Shortly after, the first checkpoint inhibitor based treatment that blocked CTLA4 was approved by FDA to treat metastatic melanoma patients (2), which was followed by approval of PD1 blockade therapeutics for use in melanoma patients (3). Moreover, identification of tumor neoantigens was demonstrated using next generation sequencing approach (4), which led to development of peptide-based vaccines (5). Treatment approaches involving oncolytic viruses, CAR-T cells and other cancer cell targeted immunotherapy approaches are in development and are currently being investigated in pre-clinical and early-stage clinical trials (6-9).

Nucleic acid vaccines are ones such approach that have been shown to elicit antigen-specific T cell responses in pre-clinical and early-stage clinical trials (10). These included DNA and mRNA based vaccines, a most recent example of these are mRNA vaccines developed for COVID-19 disease, which has seen tremendous success in achieving immunity against SARS-CoV2 (corona virus) (11). Similarly, DNA vaccines are currently being tested, as they provide several advantages over other vaccination approaches, such as ease of manufacturing, storage, modification and most importantly, no side effects (12). Other APC based therapies include generation of autologous DCs, macrophages and B cells and loading them with antigen material such as tumor lysates, tumor-associated proteins, tumor-associated RNA, and peptide antigens (13-17).

Immunizations with DNA vaccines have been shown to efficiently activate CD8 T cell mediated responses by us and others. However, to date only one DNA vaccine has been approved by USDA for treatment of canine melanoma (18). In human clinical trials DNA vaccines have failed to replicate the effects demonstrated in pre-clinical studies (19, 20). Modified approaches for better efficiency of DNA vaccines in clinical are underway, these include 1) modification to plasmid constructs to improve presentation of tumor associated antigen(s), 2) variations in method of administration, such as direct injections to lymph nodes for increased uptake by professional APCs (21, 22), and 3) and other approaches testing delivery vehicles such as nanoparticles and liposomes to enhance antigen presentation (23-26).

We adopted the approach of understanding the mechanism of antigen presentation of plasmid DNA vaccines, specifically through professional antigen presenting cells. Our findings identified B cells as a potential target for DNA vaccines, but further analysis demonstrated their dependency on DCs to gain the APC characteristic. More specifically B cells were licensed by DCs through one or more currently unknown cell surface interactions. Identification of these cell surface interactions is one of the primary future directions of this study, as that will lead to a clear understanding of signals required by B cells to attain APC function for presenting plasmid DNA upon passive uptake.

One known natural interaction between B cells and DCs occur at the germinal centers, where B cells interact with follicular DCs to capture exogenous antigen. During this interaction, B cells undergo BCR stimulation by antigen interaction and get activated by DCs through BAFF, ICAM-1 and VCAM-1 signaling (27). Similar to this we also report increased expression of CD54 (soluble ICAM1) when B cells and DCs are in co-culture. This suggests ICAM1-LFA-1 interaction

between B cells and DCs may be playing a similar role in our *in vitro* antigen presentation system. We further demonstrated a role of CD23-IgE signaling in mediating antigen presentation through B cells. It has been shown that CD23 is expressed on the surface of activated B cells and enhances B cell proliferation and antibody response (28, 29). Moreover, expression of CD23 induces production of IgE, which is a ligand for CD23. This signaling works in a feedback loop to maintain B cell activation and stimulation (30). It has also been reported that the expression of CD23 on B cells can define different immature B cells subsets (31). Results from our studies and known literature indicated that blocking CD23 may have interrupted in B cell activation and therefore resulted in reduced immunogenicity. However, this does not exclude the possibility of DCs being involved in promoting CD23-IgE signaling in B cells. As CD23 has also been shown to interact with DC surface markers CD11b and CD11c, which can act as a ligand for CD23 on B cells and promote similar downstream signaling pathways (32). However, our preliminary studies that tested inducing signaling through ICAM-1-LFA1 and/or CD23-IgE interaction on DNA loaded B cells failed to demonstrate the effects of DC licensing. This suggested that DCs are interacting with B cells in more ways that we have discovered so far, and further investigation of APC function related protein interactions is required for generating B cell APCs without licensing through DCs.

We performed RNAseq on flow sorted B cells that had been cultured with CD8 T cells and with or without DCs. Addition of CD8 T cells may have resulted in increased expression of inflammation related cytokines and chemokines as demonstrated in our analysis. This could have been one of the reasons why we were unable to short list and identify signaling pathways and molecules that are exclusively overexpressed upon B cells and DC interaction. As an alternative we would perform a similar experiment, but without the inclusion of CD8 T cells. This will restrict

the number of genes that get significantly altered between the two groups and can provide a cleaner analysis and probably improve our chances for identifying the interaction(s) between B cells and DCs.

We also demonstrated that activation of B cells was required for antigen presentation in presence of DCs, either by CD4 T cells or by addition of cytokine IL4. Both of these ultimately functioned similarly to induce B cell activation and promoted B cell survival. However, the exact mechanisms by which CD4 T cells and IL4 did this may be different. Results from our cytokine array demonstrated increased amount of IL2 secretion when CD4 T cells were used, which was absent in the cultures where IL4 was used. This shows different modes of activation exist for induction of APC function in B cells. However, not all activation agents work similarly, as we demonstrated that LPS-matured B cells when loaded with antigenic peptide, resulted in active yet non-responsive CD8 T cells. Others have also shown that LPS maturation of B cells results in anergic T cells (33). So, a clear understanding of how each of these stimulation agents function to activate B cells is necessary. This is another future directive of this study, to evaluate B cell activation agents and their effects on APC function of B cells, not just by the activation profile of B cells but by their ability to generate cytotoxic CD8 T cells. Many groups have demonstrated CD40 stimulated, TLR9 agonist stimulated, and BAFF stimulated B cells as potent APCs. However, this has been mostly done by studying the B cell activation profile, and fewer studies exist that evaluate the function of resulting T cells (34-36).

Moreover, it is still unclear as to which B cells subtype can best perform as APCs following passive uptake of plasmid DNA. B cells can be classified in to several subtypes based on the expression and maturation of BCR, as discussed in chapter 1. We hypothesize that the naïve B cells that have

not been exposed to antigen could be the primary B cell subset that processes the plasmid DNA. Furthermore, our preliminary analysis of B cells after they have been exposed to plasmid DNA demonstrated that DNA loaded B cells take on an early mature B cell phenotype (IgM+ IgD+). As a future direction, we would like to track the fluorescently labeled plasmid DNA in B cell sub-populations in a time-course study, following the passive uptake. This could reveal the B cell subtype that can be potentially converted to APCs.

We also demonstrated successful generation of plasmid DNA loaded nanoparticles and exosomes. However, these approaches were developed with B cell targeting in mind and failed to serve our purposes. There is potential in both of these approaches even when DCs capture the plasmid DNA through these delivery vehicles. A major concern for plasmid DNA processing by DCs is endosomal degradation before it could be translocated to nucleus for transcription and further processing. Use of nanoparticles or exosome based encapsulation approaches to protect the DNA against endosomal degradation in DCs and can promote antigen processing by them. The cationic copolymers used by us were linked with an endosomal escape group, which would keep the nanoparticle intact when in endosomes, and therefore protect the DNA against degradation. However, we have not extensively tested nanoparticle based DNA delivery approach with DCs as our primary focus. Current studies involving DNA loaded nanostructures report challenges such as induction of undesired immune response, poor stability in tumor micro-environment and poor efficacy of transportation (37). Given the complicated and less understood biology of B cell APCs, and advantage of DNA protection by encapsulation, employing nanoparticle or exosome based delivery of DNA vaccines to DCs is worth investigating. These studies are part of our future

directives, although we anticipate that *ex vivo* generated B cells loaded with DNA would result in superior immunogenic responses.

Our *in vivo* immunization study demonstrated that DC-licensed DNA-loaded B cells were able to generate antigen-specific and activated CD8 T cells, similar to the naked plasmid DNA immunizations. As a future direction, we plan to evaluate effects of different routes of immunizations and effects of adjuvants such as CD40L, anti-CD40 mAb, and CpG (TLR9 agonist) in our *in vivo* assay. We wish to compare intramuscular, intradermal, intraperitoneal and intravenous routes, for delivery of DC-licensed DNA-loaded B cells, as different routes of administration may result in varying immune responses. Many groups have demonstrated use of adjuvants such as CD40L and anti-CD40 mAb to induce and promote APC function by B cells. Their use could further augment the antigen presentation function of DC-licensed DNA-loaded B cells. We would also perform similar *in vivo* studies to evaluate the anti-tumor response resulting from DC-licensed DNA-loaded B cells in tumor (E.G7-OVA) bearing C57Bl/6 mice. We hypothesize that intradermal route of administration will result in a superior immunogenic response as these injections will drain the transferred B cells in to the lymph nodes where the B cells can home and generate an antigen-specific inflammatory response. We also suspect that use of CD40L or anto-CD40 mAb will result in increased antigen presentation activity of B cells and ultimately result in increased efficacy when compared to traditional approaches of immunizations.

Use of autologous B cells or allogeneic B cells is being currently investigated as immunotherapy (38). Major focus has been on CD40-activated B cells as they have been shown to act as potent APCs and are capable of generating antigen-specific T cell responses *in vitro*, in murine models and in dogs (39-42). Current methods for generating human B cell APCs include treatment with

IL4, IL2, IL21, BAFF, and CpG apart from CD40 stimulation by either CD40L, CD40 antibody or co-culture with CD40L expressing cells (41, 43-46). However only a few of these strategies have been tested in early-stage clinical trials for their safety (38). Most of the clinical trials used CD40L activated B cells which are the most studied and defined B cell APCs and reported that they are generally safe and resulted in minimal toxicity (38). Further investigations into antigenic material loaded B cells as APCs is required similar to that of DCs. We know from a decade of research that DC based vaccines have failed to surpass their limited efficacy tag and newer generation of cell therapies is the need of the hour (47). B cells provide an excellent alternative for the same and also pose several advantages over DCs such as easy manufacturing and expansion compared to DCs (45) and of more clinical relevance B cells can evade immune suppression by tumor microenvironment unlike DCs (48).

Overall compiled results from these studies illuminate the potential use of B cells as APCs, either when processing and presenting plasmid DNA based vaccine or when used as peptide-pulsed APCs. These pre-clinical studies can serve as a foundation to development of B cell-centered therapeutic strategies for treatment of cancer patients. Potentially improving the immunogenicity of plasmid DNA and generating a superior anti-tumor response. While the studies presented here answered some questions on APC function of B cells, it raised many more to be addressed and answered in future.

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Chapter 6

Materials and Methods

Most of this work is currently being prepared as part of research manuscripts to be submitted for published in Journal of Immunotherapy in Cancer.

Material and Methods – Chapter 2

Plasmid DNAs

pCI-neo-sOVA plasmid (Cat.# 25098) was purchased from Addgene (Watertown, MA) and the ovalbumin-encoding gene was subcloned into the pTVG4 vector (1). As a negative control we used either pTVG4 (empty vector) or pTVG4-SSX2 (non-specific antigen-encoding plasmid DNA). For protein expression studies, pcDNA3-EGFP plasmid (Cat.# 13031-DNA.cg) was purchased from Addgene (Watertown, MA) and the EGFP gene was similarly cloned into pTVG4 vector.

Mouse models and Cell lines

C57Bl/6 mice (stock no. 000664), OT1 mice (stock no. 003831), OT2 mice (stock no. 004194), MHCI knockout mice (Stock no. 002087) and CD40 knockout mice (stock no. 002928) were obtained from the Jackson laboratory (Bar Harbor, ME) and were housed and monitored by the Wisconsin Institute of Medical Research vivarium facility. All mice were maintained under aseptic conditions and all experiments were conducted under an IACUC-approved protocol.

B3Z cell line (Lonza, Basel, Switzerland) is a T cell hybridoma expressing a TCR that specifically recognizes SIINFEKL in context of H-2Kb. These cells carry a β-galactosidase construct driven by NF-AT element from the IL2 promoter. Cells were cultured and maintained according to manufacturer recommended guidelines.

Materials:

Flow cytometry antibodies: Anti-mouse CD19-PE-Cy7 (Cat.# 561739), anti-mouse CD80-APC (Cat.# 560016), anti-mouse CD86-BV421 (Cat.# 564198), anti-mouse PD-1-PE-CF594 (Cat.# 562523), anti-mouse CD4-BUV395 (Cat.# 563790), anti-mouse CD8-BV786 (Cat.# 563332), anti-mouse MHCI-BV711 (Cat.# 749707), anti-mouse MHCII-BUV805 (Cat.# 748844), anti-mouse CD45R (B220)-BV786 (Cat.# 563894), anti-mouse CD24-PE-CF594 (Cat.# 562477), anti-mouse CD43-BUV395 (Cat.# 740224), anti-mouse IgD-BV510 (Cat.# 563110), and anti-mouse IgM-BV711 (Cat.# 743327) were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-mouse CD11c-PE (Cat.# 50-0114-U100) and Ghost780 live dead dye (Cat.# 13-0865-T500) were purchased from Tonbo Biosciences (San Diego, CA). Anti-mouse 4-1BB-PerCP-eF710 (Cat.# 46-1371-82) was purchased from LifeTech technologies (Carlsbad, CA).

ELISA antibodies: Purified anti-mouse IFN-γ (Cat.# 551216) and biotinylated anti-mouse IFN-γ (Cat.# 554410) were purchased from Thermo Fisher Scientific (Waltham, MA). Avidin-HRP (Cat.# 170-6528) was purchased from Bio-Rad laboratories (Hercules, CA).

Other antibodies: Purified anti-mouse CD23 (Cat.# 101602) was purchased from BioLegend (San Diego, CA), anti-mouse CD70 (Cat.# BE0022) was purchased from BioXCell (Lebanon, NH) and anti-mouse CD40 (Cat.# 553721) was purchased from BD Biosciences (Franklin Lakes, NJ)

Tetramer: BV421-labeled SIINFEKL tetramer was provided by the NIH tetramer core facility at Emory University (Atlanta, GA).

Reagents: Recombinant mouse GM-CSF (Cat.# 576304) and recombinant mouse BAFF (Cat.# 591202) were purchased from BioLegend (San Diego, CA). Recombinant mouse IL4 (Cat.# 21-8041-U0020) was purchased from Tonbo Biosciences (San Diego, CA). Recombinant mouse CD40 (Cat.# 1215-CD-050), recombinant mouse CD40L (Cat.# 8230-CL-050/CF), and recombinant mouse IL21 (Cat.# 594-ml-025) were purchased from R&D systems (Minneapolis, MN). TLR7/8 agonist, R848 (Cat.# vac-R848) and TLR9 agonist, CpG (Cat.# trlr-2395) were purchased from InvivoGen (San Diego,CA). RPMI-1640 (Cat.# 10-040-cv) and penicillin/streptomycin solution (Cat.# 15140122) were purchased from Thermo Fisher Scientific (Waltham, MA). BenchMark FBS (Cat.# 100-106 500ml) was purchased from Gemini Bio (Sacremento, CA), TMB-substrate (Cat.# 50-76-00) was purchased from Sera Care Life Sciences (Milford, MA)

B cell, DC, and T cell isolations

Mouse spleens were acquired at necropsy and processed to single cell suspension following red blood cell lysis. B cells were isolated using a negative selection kit (Cat.# 12210-110) from Akadeum technologies (Ann Harbor, MI) following the manufacturer's protocol. CD8 and CD4 T cells were isolated using negative selection kits (Cat.# 19853 and Cat.# 19852) from Stemcell technologies (Vancouver, Canada) following the manufacturer's protocol. A B16/Flt3-L cell line

was implanted in C57Bl/6 mice for generation of primary DCs *in vivo*, as described below. DCs were isolated by either CD11c positive selection (Cat.# 17684) or negative selection enrichment (Cat. # 19763) from Stemcell technologies (Vancouver, Canada) following the manufacturer's protocol.

DC Preparation

C57Bl/6 mice were injected subcutaneously in the right flank with 5×10^6 B16-BL6 melanoma tumor cells engineered to secrete FMS like Tyrosine kinase 3 ligand (Flt3-L). Spleens were harvested between day-15 to day-20, injected with 2 mg/ml Collagenase D (Roche, San Francisco, CA) buffer with DNase (1 µg/ml) and incubated at 37°C for 30 minutes. Spleens were then disrupted with a syringe plunger and passed through nylon mesh screen. Splenocytes were washed and treated with ACK buffer to lyse the red blood cells. Single cell suspension of splenocytes were then frozen in liquid nitrogen, to be thawed before usage.

B3Z assay

B cells were isolated using CD19 positive selection using PE positive selection kit Stem Cell technologies (Vancouver, Canada, Cat. # 18554) following manufacturer's protocol. In a 96-wel plate, 1 x 10^6 cells per well were incubated with plasmid DNA for passive uptake, in PBS for 1 hour and then overnight in RPMI media supplemented with 10% FBS. Following day 0.5 x 10^6 B3Z cells were added per well along with the B cell activation agents. After overnight incubation, cells were washed twice with PBS and then 100µl of Lac-Z lysis buffer (PBS + 0.125% NP-40 +

9mM $MgCl_2$ + 100µM β-mercaptoethanol + 0.15 mM Chlorophenol red β-galactopyranoside) was added to each well. After four to five hours of incubation with buffer, optical density (OD) for each well were recorded at 570nm. Data are represented as recorded OD values for each control and treatment group.

In vitro antigen presentation assay

In general, APCs were isolated as described above, re-suspended in PBS at 10^7 cells/ml and incubated with plasmid DNA (25µg/ml) for 60 minutes, with gentle mixing every 15 minutes. RPMI media supplemented with 10% fetal calf serum and 1% penicillin/streptomycin was then added to the culture and the cells were incubated overnight at 37°C. The following day, other cell populations (such as CD4 T cells, CD8 T cells or DCs, each at ratio of 1:2 B cells) were added to the culture, as were GM-CSF (25 ng/ml) and/or IL4 (20 ng/ml). In some studies, CD8 T cells added to culture were labeled with PKH67 (Cat#. PKH67GL-1KT, Sigma Aldrich, St. Louis, MO) following the manufacturer's protocol. Additional treatments, such as activation agents and blocking antibodies, were also added on second day of the culture where indicated. After three to five days of further incubation, the cells and media supernatant from the culture were collected separately for analysis. Cells were analyzed by flow cytometry and media supernatants were analyzed for secreted IFNγ via ELISA as described below. Flow cytometry was performed using a BD-Fortessa instrument. Data obtained were analyzed using FlowJo software (version 10.8). For GFP expression analysis, cell images were recorded using Amnis ImageStream imaging flow cytometer and analyzed using IDEAS software (version 6.2). Expression of cytokines and

chemokines in media supernatant was analyzed using the Proteome Profiler Mouse Cytokine Array (Cat.# ARY006, R&D Systems, Minneapolis, MN), following the manufacturer's protocol.

Enzyme-linked immunosorbent assay (ELISA)

The presence of IFNγ in the supernatants was determined by a quantitative capture ELISA. Specifically, murine monoclonal antibody specific for rat IFNγ were diluted to 2.5 μg/ml in 50 mM sodium carbonate buffer (pH 9.6) and adsorbed to wells of Immulon-4 polystyrene plates (Dynex Technologies Inc., Chantilly, VA) overnight at 4 °C. Wells were then blocked with PBS + 1% bovine serum albumin (BSA) for 2 hours and washed with PBS + 0.1% Tween-20. Supernatants from the *in vitro* cultures were added in replicate to experimental wells. A standard curve of purified IFNγ (0–20,000 pg/ml) was included on each plate. Plates were then incubated overnight at 4 °C, washed, then incubated at room temperature with a biotinylated IFNγ detection antibody at 1 μg/ml. After washing, the plates were incubated for 1 h at room temperature with peroxidase-conjugated streptavidin, washed again, and developed with a tetramethylbenzidine (TMB) colorimetric substrate and reactions were stopped with 1 N HCl. The optical density (OD) of individual wells was measured at 450 nm. The concentrations of IFNγ were calculated by comparison of the obtained OD with the standard curve.

RNA Seq

B cells isolated from C57Bl/6 mice splenocytes were incubated with OVA plasmid DNA overnight as described above, and then cultured with or without DCs (2:1 ratio). DCs had been pre-cultured

in the presence of GM-CSF (25 ng/ml) and IL4 (20 ng/ml) for five days before addition of B cells to the co-culture. After three days of co-culture, B cells were then sorted by flow cytometry using CD19 surface expression and total RNA was isolated. cDNA was prepared, amplified, and indexed using SMART-seq v4 ultra low RNA whole transcriptome kit (Cat.# 634890, Takara Bio USA, San Jose, CA). cDNA was then sequenced using NovaSeq6000 for 30 million reads per sample (DNA sequencing facility, University of Wisconsin-Madison Biotech Center). Raw files were processed using Galaxy analysis interface (usegalaxy.org) (2), gene ontology search and functional profiling were performed using gProfiler (https://biit.cs.ut.ee/gprofiler/gost), and gene set enrichment analysis (GSEA) was performed (3, 4).

Statistical Analysis

All data presented are representative of at least three or more replicates of each experiment/assay. Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to calculate statistical significance for all data presented that had more than two groups for comparison. Two-way ANOVA was used for experiments that had only two experimental groups. $p<0.05$ was considered statistically significant.

Materials and methods – Chapter 3

Cell lines and Mouse models

E.G7-OVA (derivative of EL4, with constitutive expression of ovalbumin) cell line was purchased from ATCC (Manassas, VA, Cat. # CRL-2113) and maintained according ATCC recommended guidelines.

C57Bl/6 mice (stock no. 000664) and OT1 mice (stock no. 003831) were obtained from the Jackson laboratory (Bar Harbor, ME) and were housed and monitored by the Wisconsin Institute of Medical Research vivarium facility. All mice were maintained under aseptic conditions and all experiments were conducted under an IACUC-approved protocol.

Materials:

Flow cytometry antibodies: Anti-mouse CD25-PE (Cat. #553866), CD28-PE-CF594 (Cat. #562765), CD62L-BV650 (Cat. #564108), CD27-BUV805 (Cat. #BDB741959), CD8-BV786 (Cat. #563332), CD19-FITC (Cat. #553785), CD11c-FITC (Cat. #553801), PD1-PE-CF594 (Cat. #562523), CD272-BV480 (Cat. #746417), LAG3-BV711 (Cat. #563179), CD244-BUV395 (Cat. #744290), KLRG1-PE-CF594 (Cat. #565393), CD160-PerCp-Cy5.5 (Cat. #562218), CD107a-BV421 (Cat. #564347), CD278 (ICOS)-BV480 (Cat. #746539), TNF-α-PE-Cy7 (Cat. #557644), IL10-BV510 (Cat. #563277), and EOMES-BUV395 (Cat. #567171) were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-mouse CD40L-PE-Cy7 (Cat. #157008), OX40-APC (Cat.

#119414), CD96-PE (Cat. #131705), CTLA4-PE-Cy7 (Cat. #106314), VISTA-PerCp-Cy5.5 (Cat. #150210), and TIGIT-PE-Cy7 (Cat. #142108) were purchased from BioLegend (San Diego, CA).Anti-mouse CD44-PerCp-Cy5.5 (Cat. #560570), CD69-BV510 (Cat. #563030), FAS-L-PE (Cat. #555293), FAS-R-BV711 (Cat. #740716), TRAIL-APC (Cat. #17-5951-82), Perforin-FITC (Cat. #11-9392-82), IL4-BV421 (Cat. #566288), Ki67-BV711 (Cat. #563755), CD19-BUV805 (Cat. #749027), and CD11c-BUV805 (Cat. #749038) were purchased from Fisher Scientific (Waltham, MA). Anti-mouse TIM3-APC (Cat. # 17587182) and Granzyme-B-PE-eF610 (Cat. #61-8898-82) were purchased from Invitrogen (Waltham, MA). Anti-mouse CD137 (4-1BB)-PerCp-eF710 (Cat. # 46-1371-82) was purchased from Life Technologies (Carlsbad, CA). Ghost dye 780 (Live/dead stain) (Cat. # 13-0865-T500) was purchased from Tonbo Biosciences (San Diego, CA).

ELISA antibodies: Purified anti-mouse IFN-γ (Cat.# 551216) and biotinylated anti-mouse IFN-γ (Cat.# 554410) were purchased from Thermo Fisher Scientific (Waltham, MA). Avidin-HRP (Cat.# 170-6528) was purchased from Bio-Rad laboratories (Hercules, CA).

Reagents: Recombinant mouse GM-CSF (Cat.# 576304) was purchased from BioLegend (San Diego, CA). Recombinant mouse IL4 (Cat.# 21-8041-U0020) was purchased from Tonbo Biosciences (San Diego, CA). RPMI-1640 (Cat.# 10-040-cv) and penicillin/streptomycin solution (Cat.# 15140122) were purchased from Thermo Fisher Scientific (Waltham, MA). BenchMark FBS (Cat.# 100-106 500ml) was purchased from Gemini Bio (Sacremento, CA), TMB-substrate (Cat.# 50-76-00) was purchased from Sera Care Life Sciences (Milford, MA)

Peptide:

Peptide for the H-2b-restricted epitope from chicken ovalbumin (SIINFEKL), was synthesized, and the purity and identity were confirmed by mass spectrometry and gas chromatography (LifeTein, LLC., Hillsborough, NJ). Peptides were reconstituted in DMSO (2 mg/ml) and stored at −80°C until use.

B cell, DC, and T cell isolations

Mouse spleens were acquired at necropsy and processed to single cell suspension following red blood cell lysis. B cells were isolated using a negative selection kit (Cat.# 12210-110) from Akadeum technologies (Ann Harbor, MI) following the manufacturer's protocol. CD8 T cells were isolated using a negative selection kit (Cat.# 19853) from Stemcell technologies (Vancouver, Canada) following the manufacturer's protocol. A B16/Flt3-L cell line was implanted in C57Bl/6 mice for generation of primary DCs *in vivo*, as previously described in chapter 2. DCs were isolated by negative selection enrichment (Cat. # 19763) from Stemcell technologies (Vancouver, Canada) following the manufacturer's protocol.

***In vitro* assay**

Purified B cells and DCs were plated at 2.5 x 10^5 cells per well in a 96 well plate. For maturation B cells and DCs were treated with LPS (Sigma Aldrich, St. Louis, MO, Cat. # L4516-1mg) at 1µg/ml and 10µg/ml respectively for 24 hours. Non-LPS treated cells were used as immature B

cells and DCs. Following LPS treatment, cells were washed twice with PBS and then treated with SIINFEKL peptide (SIIN). After four hours of peptide treatment, cells were washed twice with PBS and CD8 T cells were added to the culture. After 24 or 48 hours of culture, CD8 T cells were analyzed by cell surface marker and intracellular protein expression using flow cytometry, ELISA or used for adoptive transfer study. For proliferation studies, CD8 T cells were labeled with CFSE (BioLegend, San Diego, CA, Cat. #423801) before they were added to the culture and loss of CFSE was quantified using flow cytometry.

Tumor study

C57Bl/6 mice were inoculated with E.G7-OVA cells (melanoma cell line that expresses ovalbumin), 1 x 10^6 cells were injected per mouse subcutaneously in the flank region. After eight days, tumor volumes were measured, and mice were randomized to different treatment groups. Antigen-primed CD8 T cells from LPS-matured and immature B cells or DCs treated with GM-CSF and IL4 were collected after 48 hours of *in vitro* culture, and 1 x 10^6 cells per mice were adoptively transferred intra-peritoneally on day 9. Tumor volumes were measured and recorded every second or third day. Mice were sacrificed when tumor volumes reached 2 cm^3.

Statistical Analysis

All data presented are representative of at least two or more similar experiments/assays. Tumor study was performed once, with four to six mice per treatment group. Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to calculate statistical

significance for all data presented that had more than two groups for comparison. Two-way ANOVA was used for experiments that had only two experimental groups. Survival analysis was conducted using a Mantel-Cox log-rank test. $p<0.05$ was considered statistically significant.

Materials and Methods – Chapter 4

Plasmid DNAs

pCI-neo-sOVA plasmid (Cat.# 25098) was purchased from Addgene (Watertown, MA) and the ovalbumin-encoding gene was subcloned into the pTVG4 vector (1). As a negative control we used pTVG4 (empty vector).

Mouse models

C57Bl/6 mice (stock no. 000664) were obtained from the Jackson laboratory (Bar Harbor, ME) and were housed and monitored by the Wisconsin Institute of Medical Research vivarium facility. All mice were maintained under aseptic conditions and all experiments were conducted under an IACUC-approved protocol.

Materials:

Flow cytometry antibodies: Anti-mouse CD19-PE-Cy7 (Cat.# 561739), anti-mouse PD-1-PE-CF594 (Cat.# 562523), anti-mouse CD4-BUV395 (Cat.# 563790), and anti-mouse CD8-BV786 (Cat.# 563332) were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-mouse CD11c-PE (Cat.# 50-0114-U100) and Ghost780 live dead dye (Cat.# 13-0865-T500) were purchased from Tonbo Biosciences (San Diego, CA). Anti-mouse 4-1BB-PerCP-eF710 (Cat.# 46-1371-82) was purchased from LifeTech technologies (Carlsbad, CA).

Other antibodies: Anti-mouse CD40 (Cat.# 553721) was purchased from BD Biosciences (Franklin Lakes, NJ)

Tetramer: BV421-labeled SIINFEKL tetramer was provided by the NIH tetramer core facility at Emory University (Atlanta, GA).

Peptide: FITC-labeled peptides specific for B cells surface marker CD21, FITC-CGGGGEDPGFFNVE (B1) and FITC-CGGGGKKKKKKKEDPGFFNVE (B2) were synthesized, and the purity and identity were confirmed by mass spectrometry and gas chromatography (LifeTein, LLC., Hillsborough, NJ). Peptides were reconstituted in DMSO (2 mg/ml) and stored at −20°C until use.

Reagents: Recombinant mouse GM-CSF (Cat.# 576304) was purchased from BioLegend (San Diego, CA). Recombinant mouse IL4 (Cat.# 21-8041-U0020) was purchased from Tonbo Biosciences (San Diego, CA). RPMI-1640 (Cat.# 10-040-cv) and penicillin/streptomycin solution (Cat.# 15140122) were purchased from Thermo Fisher Scientific (Waltham, MA). BenchMark FBS (Cat.# 100-106 500ml) was purchased from Gemini Bio (Sacremento, CA), TMB-substrate (Cat.# 50-76-00) was purchased from Sera Care Life Sciences (Milford, MA). Opti-prep solution (Cat. # D1556-250ML) was purchased from Sigma Aldrich (St Louis, MO).

Peptide and nanoparticle targeting assay

PBMCs were plated at 0.5 x 106 cells per well and each peptide (B1, B2, B3 and B4) at 4µg/ml was added to the individual wells. Cells were incubated at 4°C for 30 minutes. After which cells were washed twice with PBS and then processed for analysis by flow cytometry. For nanoparticle

targeting studies, similar method was used, however number of nanoparticles added to each well was decided based on the amount of DNA complexed with nanoparticles (25µg/ml – final DNA concentration). DNA enclosed in nanoparticles was labeled with Cy5 dye using Label-IT® Tracker™ Intracellular Nucleic Acid Localization Kit (Cat. # MIR 7021) from Mirus Bio (Madison, WI) following manufacturer's protocol.

Nanoparticle generation

B2 or B3 linked cationic copolymers were mixed with plasmid DNA at a calculated N to P ratio, for every 2µg of plasmid DNA, 0.2µg of peptide linked copolymer was used. Components were mixed well and incubated at room temperature for 20 minutes. Following incubation, nanoparticles were used at the same concentration as naked plasmid DNA (25µg/ml) for *in vitro* testing.

Exosome detection by flow cytometry

Exosomes were isolated using Exo-Quick TC (System BioSciences, Palo Alto, CA), and detected by flow cytometry by using Exo-Flow exosome capture kit (System BioSciences, Palo Alto, CA) following manufacturer's protocol. After capturing and staining the exosomes were visualized by flow cytometry using BD Fortessa and analyzed by FlowJo (version 10.7).

DNA loading in exosome

Electroporation: Exosomes were diluted in electroporation buffer (1.15mM potassium phosphate, 25mM potassium chloride and 21% opti-prep in ddH2O). Briefly, 100µl of exosome containing media was mixed with 20µg of plasmid DNA, and final volume of 400µl was made up with electroporation buffer. Using BioRad electroporator and 0.4 mm cuvettes, exosomes were electroporated at voltage of 400mV, capacitance of 125µF and pulse time of 10 to 15 ms. Following electroporation, Exo-Quick TC was used to re-isolate exosomes from the electroporation buffer.

Transfection: Exosomes were transfected using Exo-Fect (Cat. # EXFT20a-1, System Biosciences, Palo Alto, CA) following manufacturer's protocol. Briefly, 100µl of exosome containing solution was mixed with 20µg of plasmid DNA with transfection reagent.

B cell and DC isolations for *in vivo* study

Mouse spleens were acquired at necropsy and processed to single cell suspension following red blood cell lysis. B cells were isolated using a negative selection kit (Cat.# 12210-110) from Akadeum technologies (Ann Harbor, MI) following the manufacturer's protocol. A B16/Flt3-L cell line was implanted in C57Bl/6 mice for generation of primary DCs *in vivo*, as previously described in Chapter 2. DCs were isolated by either CD11c positive selection (Cat.# 17684) or negative selection enrichment (Cat. # 19763) from Stemcell technologies (Vancouver, Canada) following the manufacturer's protocol.

In vivo adoptive transfer study

DCs were isolated using negative selection and cultured with GM-CSF (25ng/ml) and IL4 (20 ng/ml) for five days. On fourth day of DC culture, B cells were isolated using negative selection and incubated with plasmid DNA (pTVG4 or pTVG4-sOVA) as previously describes in chapter 2. On fifth day of DC culture, B cells were collected, washed with PBS, and added to DC culture with antii-CD40 mAb (10µg/ml). After three additional days of culture B cells were either re-purified or not for adoptive transfer in C57Bl/6 mice. Two similar immunizations were performed one week apart. As a control, B cells that have not been cultured with DCs, but have been incubated with DNA for three days in presence of GM-CSF, IL4 and anti-CD40 mAb (as above) were also collected for adoptive transfer. Naked DNA immunizations using either pTVG4 or pTVG40sOVA plasmid DNA were also performed for negative and positive controls. All immunizations were performed intradermally in the ear of the mice. Mice were sacrificed seven days after the second immunization and spleens were collected. Spleens were processed into single cell suspension following red blood cell lysis using ACK buffer. Splenocytes were analyzed using flow cytometry.

Flow Cytometry

Splenocytes were plated at 0.5 x 106 cells per well and were stained with cell surface markers for identification of SIINFEKL tetramer positive CD8 T cells and for evaluating their activation status by using 4-1BB and PD1 surface markers. For staining 1:100 dilution of each antibody was used, diluted in FACS wash, and incubated at 4°C for 30 minutes. After that, cells were washed twice

with FACS wash buffer and then finally re-suspended in 100µl of FACS wash buffer for analysis using BD Fortessa. Data was analysis using FlowJo software version 10.7.

Statistical Analysis

All data presented are representative of at least three or more replicates of each experiment/assay. Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to calculate statistical significance for all data presented that had more than two groups for comparison. Two-way ANOVA was used for experiments that had only two experimental groups. $p<0.05$ was considered statistically significant.

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