Strategies to Improve the Efficacy of Vaccines by Selective Manipulation of the Immune System: A Translational Study

by

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For my husbad Joe
I couldn't have done it without you

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ABSTRACT

Novel vaccine technologies are needed to induce protective immunity towards sophisticated diseases for which classical whole-cell vaccines have been inadequate. Modern vaccine development builds upon intricate understanding of immunology and disease pathology. Subunit vaccines containing highly purified protein or peptide antigens are safer than classical vaccines and can be developed for infectious and non-infectious diseases. Transitioning between *in vitro* and *in vivo* systems during pre-clinical development is daunted by the complexity of the vaccine and multifaceted immune response. In this thesis, translational vaccine research was explored with two projects. In the first project, a novel adjuvant system that can boost antibody responses to vaccines was developed *in vitro*. A combination of TLR agonists, poly I:C (TLR3) and Pam3CSK4 (TLR1/2), resulted in enhanced B cell activation characterized by expression of surface receptors, cytokine production and proliferation. The combination promoted B cell differentiation into antibody-producing plasma cells and increased their capacity to induce $CD4^+$ T cell activation in a mixed lymphocyte reaction. When used as an adjuvant system for vaccines containing anthrax or influenza protein antigens *in vivo*, the combination resulted in significantly higher serum antibody titers than vaccines containing either agonist alone. Therefore, the poly I:C/ Pam3CSK4 combination is a promising adjuvant system for humoral vaccines containing protein antigens. The second project describes the development of a combinatorial immunotherapy for cancer using an *in vivo* model. Anti-tumour immune responses induced by vaccines containing tumour associated antigens are thwarted in advanced cancers by tumour-induced immune suppression. To address this, metronomic cyclophosphamide was evaluated as an immune modulator in combination with an $HPV16E7_{49-57}$ peptide vaccine in mice bearing HPV16-induced tumours. The combination provided significant long-term control of tumour growth. Metronomic cyclophosphamide had a pronounced lymphodepletive effect on the vaccine draining lymph node, yet did not reduce antigen-specific $CD8^+$ T cells induced by the vaccine. This enrichment correlated with increased systemic cytotoxic activity and antigen-specific cytotoxic T cells in the tumour. The results provide important insights into the multiple mechanisms of metronomic cyclophosphamide induced immune modulation in the context of a peptide cancer vaccine that may be translated into more effective clinical trial designs.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α	Alpha
~	Approximately
β	Beta
μMT	B cell deficient mice (lacking IgM transmembrane tail)
ºC	Degrees Celsius
γ	Gamma
κ	Kappa
μg	Micrograms
μL	Microlitres
[^{3}H]-TdR	Tritiated thymidine
ACK	Ammonium chloride potassium buffer
ADCC	Antibody dependent cell-mediated cytotoxicity
Ag	Antigen
AICD	Activation induced cell death
ACK	Ammonium-Chloride-Potassium
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
AP-1	Activating protein 1
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
ATRA	All-trans retinoic acid
B10	IL-10 producing regulatory B cell
BAFF	B-cell activator of the TNF family
BCAP	B-cell adaptor protein
BCMA	B cell maturation antigen
BCR	B cell receptor
BID	*bis in die* (twice a day)
BLNK	B cell linker protein
Btk	Bruton's tyrosine kinase
CARD	Caspase-recruitment domains
CBA	Cytokine bead array

CD	Cluster of differentiation
CDC	Complement mediated cytotoxicity
CFSE	Carboxyfluorescein succinimidyl ester
Ci	Curie
COX-2	Cyclooxygenase type 2
CTL	Cytotoxic T lymphocyte ($CD8^+$)
CTLA-4	Cytotoxic T-lymphocyte antigen-4
CPA	Cyclophosphamide
CPM	Counts per minute
CSC	Cancer stem cell
DAG	Diacyl glycerol
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribose nucleic acid
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DPX	DepoVax
DT	Diphtheria toxin
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunosorbent spot assay
F21E	Tetanus toxin peptide (947-967): FNNFTVSFWLRVPKVSASHLE
Fab	Fragment, antigen binding region
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain
FBS	Fetal bovine serum
Fc	Fragment, crystallizable region (constant region)
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FO	Follicular
FoxP3	Forkhead box protein 3
GM-CSF	Granulocyte-macrophage colony stimulating factor
HBSS	Hank's balanced salt solution

HEL	Hen-egg lysozyme
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HRP	Horseradish peroxidase
hTERT	Telomerase reverse transcriptase
IκB	Inhibitor of kappa B
IDO	Indoleamine 2,3-dioxygenase
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Io	Ionomycin
IP_3	Inositol-1,4,5-trisphosphate
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory transcription factor
IMDM	Iscove's modified Dulbecco's medium
IP-10	Interferon gamma-induced protein 10 (CXCL10)
IRAK	Interleukin-1 receptor-associated kinases
ITAM	Immunoreceptor tyrosine based activation motifs
ITIM	Immune tyrosine inhibitory motifs
KLH	Keyhole limpet hemocyanin
KO	Knockout
LNC	Lymph node cells
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MAL	MyD88-adaptor-like
MAPK	Mitogen activated protein kinase
mCPA	Metronomic cyclophosphamide

MDA5	Melanoma differentiation-associated protein 5
MDSC	Myeloid derived suppressor cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mIg	Membrane surface immunoglobulin
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinases
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88
MZ	Marginal zone
n.d.	Not done
NA/LE	No azide, low endotoxin
NALP3	NACHT, LRR and PYD domains-containing protein 3
ND	Not detected
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NLR	Nod-like receptor
NK	Natural killer cell
NS	Not statistically different
OVA	Albumin protein from chicken egg white
Pam3CSK4	N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PD-1	Programmed cell death protein 1
PDL-1 or -2	Programmed cell death receptor ligand 1 or 2
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll
PFA	Paraformaldehyde
PI-3K	Phosphatidylinositol-3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PLCγ2	Phospholipase C gamma 2
PMA	Phorbol myristate acetate
PO	*per os* (oral administration)
Poly I:C	Polyriboinosinic-polyribocytidylic acid
PRR	Pathogen recognition receptor
PTK	Protein tyrosine kinase
R9F	HPV16 E7 peptide (49-57): RAHYNIVTF
RAG	Recombination activating gene
RBC	Red blood cells
rHA	Recombinant hemagglutinin
RIG-I	Retinoic acid-inducible gene 1
RIP-1	Receptor interacting protein 1
RLR	RIG-I-like receptor
RNA	Ribose nucleic acid
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RT-qPCR	Real-time quantitative polymerase chain reaction
S9L	TRP2 peptide (180-188): SVYDFFVWL
SARM	Sterile - and armadillo-motif-containing protein
sbCPA	Single bolus cyclophosphamide
SARM	Sterile - and armadillo-motif-containing protein
SD	Standard deviation
SEM	Standard error of the mean
SFU	Spot forming units
STAT	Signal transducer and activator of transcription
TAA	Tumor associated antigen
TACI	Transmembrane activator calcium modulator and cyclophilin ligand interactor
TAK1	Transforming growth factor-β-activated protein kinase 1
TBK1	TANK-binding kinase 1
TBP	TATA box binding protein
TBST	Tris buffered saline with Tween 20
TCR	T cell receptor

T_{FH}	Follicular T helper cell
TGF-β	Transforming growth factor beta
Th	T-helper cell ($CD4^+$)
TI	T-independent B cell antigen
TIR	Toll-interleukin 1 receptor
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumor necrosis factor
TNFRSF	TNF receptor superfamily
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cell
TRIF	TIR-domain-containing adaptor protein inducing IFN
VEGF	Vascular endothelial growth factor
VLP	Virus like particle

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CHAPTER 1: INTRODUCTION

1.1 Immunology and vaccine development

It has been well over two centuries since Edward Jenner demonstrated in 1796 that smallpox infection can be prevented in humans by prior exposure to non-pathogenic biological preparation, the cowpox vaccine [1]. One hundred years later, in 1885, Louis Pasteur was the first to treat an infection, rabies, with vaccination; this milestone is considered by many to be the birth of immunology [2]. During the 20th century vaccines were developed that eliminated or significantly reduced several types of diseases that previously plagued the world's populations, such as smallpox, measles and polio [3]. These accomplishments are truly amazing given that the immune system was not yet well understood. Today, there are vaccines for over 20 infectious diseases approved for use in North America [3-5]. The goals of vaccine research and development are to improve existing vaccines, and also create new vaccines for other indications [3, 4]. Current vaccine research draws on advanced knowledge of immunology and microbiology to design vaccines for pathogens resistant to vaccination [4]. Pathogens such as HIV, dengue virus and *Plasmodium falciparum* have proven to be difficult targets for vaccination because they have evolved elegant mechanisms to evade immune detection [6-8]. With modern day developments in immunology, the 21st century could also see the creation of vaccines to treat non-communicable diseases such as cancer, Alzheimer's, and substance addiction [9].

A vaccine is a biological preparation that induces the adaptive immune system to recognize and respond to pathogens without causing infection [10]. Prophylactic vaccination generates a memory response so that upon subsequent exposure to the pathogen the immune system can rapidly respond and clear it before the infection becomes symptomatic [10]. The majority of vaccines that are currently licensed were developed by empirical methods and are effective because of their ability to generate an antibody response [4, 11, 12]. Most of these vaccines are prepared from killed or attenuated whole-organisms, somewhat rudimentary methodology. A significant side effect of these vaccines is that they carry the potential of accidental infection due to incomplete deactivation or potential reversion to virulent forms, which precludes their

use in immunocompromised individuals [13]. For some indications, for example respiratory syncytial virus (RSV) and dengue virus, the type of immune response elicited by these vaccines may facilitate infection by inducing inappropriate immune responses [14, 15]. Furthermore, for some indications, such as cholera, whole-organism vaccines do not provide 100% nor long-lasting protection and alternatives are needed [16]. New vaccines are needed that are safe and elicit tailored immune responses towards antigens.

The development and FDA approval of novel vaccine technologies that can generate different types of immune responses has been slow. This process is hindered, in part, by the fact that novel indications are inherently difficult to treat, and also because we still lack necessary understanding of the characteristics of a protective immune response for many difficult to treat diseases [11]. Therefore, vaccine research is intimately tied with advances in immunology to design new vaccine technologies that can predictively manipulate the immune system to provide an appropriate response [10].

1.1.1 Initiation of vaccine induced immune responses

Immune recognition and response to vaccination parallels natural infection in many ways and requires cooperation between the innate and adaptive immune systems [11]. Human vaccines are typically delivered through intramuscular, subcutaneous or intradermal routes [17]. Vaccines consist of immunogenic antigens and immune stimulatory adjuvants. After immunization, the vaccine is phagocytized by innate antigen presenting cells (APCs) that can process and present antigens to the adaptive immune system. Dendritic cells (DCs) are considered to be the most potent APCs as they can recognize a variety of pathogenic stimulus and activate naïve T cells by presenting antigen within major histocompatibility complexes (MHC) on their surface [18]. Activated DCs upregulate several surface receptors that make them adept at stimulating naïve T cells and influencing the quality and quantity of T cell differentiation [19]. DCs process internalized proteins into short peptide fragments within endocytic vesicles. These exogenous peptides may be returned to the DC surface membrane bound to MHC class II for presentation to $CD4^+$ T cells [20]. Endogenous peptides can also be generated by proteasomal processing of internal proteins in the DC cytosol and are presented on the surface of the DC in the context of MHC class I, which is recognized by $CD8^+$ T cells

[20]. Under some conditions, DCs can present exogenous peptides on MHC class I through a process known as cross-presentation [19, 21]. B cells may encounter free antigen or antigen displayed by a variety of APCs, including DCs, neutrophils and macrophages [22, 23]. B cells recognize intact antigens, therefore, they do not require MHC presentation.

The efficiency of APCs to stimulate an adaptive immune response depends on their level of activation, which can be increased by signaling through one or more pathogen-recognition receptors (PRRs) expressed on their surface [24, 25]. Whole-organism vaccines contain a variety of molecules that can stimulate PRRs [26]. These molecules, collectively referred to as pathogen associated molecular patterns (PAMPs), are conserved signature molecules which are essential to the pathogen life-cycle and to which the immune system has evolved specialized detection receptors [27]. Vaccines can mimic this innate cell stimulation by including defined PAMP molecules. PAMP molecules comprise diverse motifs expressed by bacteria (flagellin, cell-wall components), viruses (dsRNA, unmethylated CpG), fungi (zymosan) and parasites (GPI-linked proteins). Different PRRs recognize specific PAMPs, and upon ligand binding they initiate unique intracellular signaling pathways within the APCs leading to expression of receptors and cell signaling molecules which result in an immune response tailored for the clearance of the pathogen type [25]. In general, activated APCs upregulate surface receptors that are needed to interact with cells of the adaptive immune response, chemokine receptors which guide their migration to the lymph node, and cytokine production to promote inflammation [25, 28].

Activated DCs interact with naïve T cells in the paracortex of lymph nodes [18, 29]. Antigenic peptides presented in MHC by the DCs are recognized by T cells through the T cell receptor (TCR) [30, 31]. The TCR is a heterodimeric transmembrane protein that, through genetic rearrangement events during T cell maturation in the thymus, bestows unique antigen-specificity to every naïve T cell generated [32]. The majority of T cells express a TCR composed of α and β chains which associate with CD3 and other accessory molecules to form the TCR/CD3 complex [33]. The TCR/CD3 complex mediates intracellular signaling cascades when the TCR recognizes antigen presented in the context of MHC by APCs, leading to their activation [34]. This signaling is enhanced

by co-stimulation of the T cells provided through interaction of CD28 on the T cells with CD80 (B7-1) or CD86 (B7-2) on the DC [31, 35]. Additionally, depending on which DC PRRs were activated, DCs will provide cytokines that influence the differentiation of the activated T cells [36, 37]. These three signals provided by DCs result in efficient activation of T cells, promoting proliferation and differentiation, and effectively initiating the adaptive immune response [38, 39].

1.1.1.1 Cells of the adaptive immune response

The adaptive immune response is mediated by T and B lymphocytes [40]. T lymphocytes are further divided based on the expression of CD4 or CD8 surface molecules. Naïve T and B cells circulate the blood vessels and lymphoid system until they encounter cognate antigen and appropriate co-stimulation signals [41, 42]. Activation of naïve T and B cells occurs in the secondary lymphoid tissues. Naive T cells are activated by DCs that present cognate peptide antigen within MHC I or II molecules and also provide co-stimulation signals. Naïve B cells do not require peptide antigen presentation, they can recognize intact antigen, but co-stimulation provided by activated $CD4^+$ T cells enables them to differentiate into antibody producing plasma cells. Activated T and B lymphocytes undergo clonal expansion in the lymph node to progressively differentiate into specialized and highly specific effector cells [43, 44]. Effector $CD4^+$ T cells are called T-helper cells, effector $CD8^+$ T cells are called cytotoxic T lymphocytes, and effector B cells are called plasma cells.

T-helper (Th) cells perform a variety of functions that mediate immune responses at different levels and as such are important for many types of immune responses [44]. Th cells provide help signals to other cells of the adaptive immune response in the form of cytokine production or through surface receptor stimulation [45, 46]. Th cells can also enhance the activity of phagocytes and NK cells. On the other end of the spectrum, some Th cells can suppress the immune response and are important mediators of self-tolerance [47]. There are several known Th cell subsets that can differentially mediate these processes [44], which will be discussed in the next section. Th cells carry out their diverse functions through the secretion of cytokines. Cytotoxic T lymphocytes (CTLs) are direct immune effectors that have the capacity to kill cells presenting their cognate

antigen in MHC I. CTLs are important in viral immunity as well as anti-tumour immune responses [48, 49]. CTL cell activation in response to vaccination is discussed in Section 1.3.

B lymphocytes are the primary mediators of humoral immunity through their production of antibodies. Antibodies can perform several functions, namely they neutralize pathogens, opsonize pathogens, enable antibody-dependent cell mediated cytotoxicity (ADCC) and complement mediated cytotoxicity (CDC), [50]. B cells recognize antigen through their surface membrane immunoglobulin which is part of the B cell receptor (BCR) [51]. Unlike T cells, antigen-recognition by B cells is not dependent on MHC presentation. However, Th express the co-stimulation ligand to the CD40 receptor on B cells which promotes B cell differentiation into antibody-producing plasma cells and memory cells [46]. Besides antibody production, B cells may also act as APCs and have the ability to process and present antigens within MHC class II to $CD4^+$ T cells and secrete cytokines [52]. B cell activation in response to vaccination is discussed in detail in Section 1.2.

One of the key features of the adaptive immune response is memory, the persistence of antigen-specific T and B cells that can confer immediate protection upon re-exposure to the same antigen [10, 53]. The magnitude and kinetics of the memory immune response is greater and faster than the initial immune response, resulting in less severe infection upon secondary exposure to pathogen [54]. Development of memory is the last phase of an immune response, preceded by immunological expansion and contraction. The expansion phase for T cells peaks around 1-3 days after pathogen exposure [55]. During this time, T cells undergo clonal expansion to proliferate and increase specificity for pathogen [56, 57]. B cells initially produce high levels of low-affinity IgM antibody, but then migrate to the lymph node follicles for affinity maturation, clonal expansion and proliferation [40]. Class switched high-affinity antibodies are detectable as early as 2 weeks post exposure and peak at 4 weeks [12, 58]. After the peak response, the immune system enters the contraction phase which can last 2 to 4 weeks. During this time the immune response recedes as the vast majority of activated T and B cells undergo apoptosis [55, 59]. A small subset of cells, approximately 5-10% of the peak response, will survive the contraction phase and

transition into the memory immune response [60]. Memory T cells are maintained over time by cytokines IL-7 and IL-15 [61], but the factors contributing to B memory cell persistence are not well understood [62].

1.1.1.2 T-helper Differentiation

Th cells differentiate into one of several different subsets upon activation that have a direct influence on the type of immune response initiated [44]. For many years the adaptive immune response was described in the confines of the Th1/Th2 paradigm proposed in the 1980's by Mosmann & Coffman [63]. Under this model there are two types of Th cells, Th1 and Th2, which are biased towards a cellular or humoral immune response, respectively. This model has since been expanded to include other Th subsets, however, most of the defining principles still apply [44]. Each Th cell subset is identified by the pattern of cytokines it produces and the expression of certain transcription factors. The function of cytokines are three-fold, first and foremost to mediate the biological function of the Th subset, secondly to provide autocrine growth factors to the Th subset and, finally, to enforce polarization by suppressing differentiation to a different Th subset. Differentiation into either subset is controlled by cytokines produced by DCs during DC:$CD4^+$ T cell interactions [64]. However, there is a growing realization that Th cell differentiation may not be as absolute as previously thought as some Th subsets exhibit plasticity [65].

Th1 cells are critical for immune responses towards intracellular pathogens. Differentiation into Th1 cells is promoted by IL-12 and interferon gamma (IFN-γ) [64]. Th1 gene expression is under control of the transcription factor T-bet. The principle effector cytokine of Th1 is IFN-γ [66]. IFN-γ promotes immune responses towards intracellular pathogens by increasing activation of phagocytes and natural killer (NK) cells [67, 68]. IFN-γ enhances CTL differentiation and activation, as well as increasing MHC class I expression to facilitate CTL activity [69, 70]. IFN-γ is also produced by activated CTLs [71]. Th1 cytokines promote B cells to produce antibodies effective at cytotoxic functions, ADCC and CDC [72, 73]. Th1 immunity may also be important to anti-tumour immunity and a major goal of cancer vaccine research is to stimulate an antigen-specific Th1 immune response [74].

Th2 immune responses are critical for the elimination of extracellular pathogens, such as helminthes [44]. IL-4 is a crucial cytokine for Th2 polarization, and the key cytokines produced by Th2 are IL-4 and IL-5 [66]. Th2 gene expression is driven by the transcription factors GATA-3 and STAT6 [75]. Key effector cells of a Th2 response are B cells, eosinophils and mast cells. B cells activated with Th2 cytokines produce antibodies that can sensitize eosinophils and mast cells [66, 73]. A Th2 response is often considered to be the default response as it occurs when DCs are sub-optimally activated, such as in the case of vaccination with a weak antigen or low antigen dose [76, 77].

While Th1 and Th2 subsets are important lineages of differentiated $CD4^+$ T cells, it has become apparent that not all Th cells can easily be classified into one of these categories [44]. An important subset of Th are the regulatory $CD4^+$ T cells (Tregs) which express the transcription factor Forkhead box P3 (FoxP3), the high-affinity IL-2α receptor protein CD25, and produce IL-10 [78]. Tregs are essential in maintaining peripheral tolerance in healthy individuals by suppressing immune responses towards self-antigens [79]. Natural Tregs (nTregs) are preprogrammed in the thymus during T cell development, but a Treg phenotype may also be induced (iTregs) in the periphery by TGF-β [80].

Another distinct subset are the follicular T helper cells (T_{FH}). T_{FH} differentiation can be marked by the induction of B cell lymphoma 6 (Bcl-6) transcription factor upon $CD4^+$ T cell stimulation by dermal $CD14^+$ DCs and expression of the inducible T-cell co-stimulator (CD278) [81]. T_{FH} are specialized helpers to B cell activation and are found in B cell follicles of secondary lymphoid organs [82, 83]. T_{FH} cells are activated by DCs and migrate to the follicular regions of the lymph node by upregulating CXCR5 and downregulating CCR7 expression [84]. T_{FH} secrete IL-21, an important cytokine regulating the survival of B cells. However, there is some debate on whether these cells are a terminally differentiated subset or merely a transient stage of activation since there exists some plasticity in Bcl-6 expression [85, 86].

Besides these lineages several other subsets have been proposed, such as Th9, Th17, and Th22 cells. These subsets have been identified based on their roles in autoimmunity and transplantation rejection, yet they demonstrate a high degree of plasticity which complicates their study [87]. How these subsets are activated and their

homeostatic function are not well understood, but is an active area of investigation [44, 65].

1.1.1.3 TLR activation of the innate immune response

The type of immune response induced depends greatly on how innate APCs, and in particular DCs, are first activated [27]. DCs express a variety of PRRs on their surface in order to detect and decipher the type of infection through the recognition of multiple PAMPs. PRRs are also expressed by other innate immune cells, and how they react also plays a role in shaping the immune response [88-90]. Families of PRRs include the Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and receptor for advanced glycation end products (RAGE) [91]. In addition to exogenous PAMP molecules, some PRRs can recognize endogenous molecules that are released upon cell necrosis, these types of immune-stimulatory molecules are called danger associated molecular patterns (DAMPs) [92]. DAMPs include molecules like uric acid, heat shock proteins, cytosolic proteins, and adenosine tri-phosphate [91]. As the earliest discovered PRRs, the TLR family of receptors and ligands are intensely studied in the field of vaccinology for their effects on activating an immune response [93, 94].

TLRs are highly expressed on innate immune cells, but are also expressed on non-hematopoietic cells such as endothelial cells [95], neurons [96] and hepatocytes [97]. TLR signaling in immune cells leads to their activation, but TLR signaling in non-immune cells contributes to development, survival and proliferation [95-97]. There are 9 TLR proteins that share homology between humans and mice, TLRs 1-9 [98]. As well, TLR10 has been identified in humans but not mice, and TLRs 11-13 have been identified in mice only (expression of human vs. mouse TLRs list in Table 1.1). These receptors have structural homology, sharing a horseshoe-like extracellular domain of leucine-rich repeats, a single helical transmembrane domain, and an intracellular Toll-interleukin 1 receptor signaling domain (TIR) motif [99, 100]. Most of the TLRs are found on the cell surface membrane, but a set of intracellular TLRs –3, 7, 8, and 9 – are expressed on internal membranes such as endosomes and recognize various forms of nucleic acids [98]. Each TLR is specialized for detection of a type of PAMP (TLR ligand

examples listed in Table 1.1). TLRs assemble into homodimers upon ligation with their congnate ligand which initiates intrecellular downstream signaling cascades [100]. TLR2 is an exception as it forms a heterodimer with TLR1 or TLR6, and possibly TLR10 in humans [101]. Each TLR2 heterodimer has different ligand specificity [102]. Non-TLR co-receptors may also aid in ligand detection, for example TLR4 recognizes lipopolysaccharide with the help of external accessory proteins MD-2 and CD14 [103].

Intracellular signaling cascades are initiated by receptor dimerization, specifically by the clustering of TIR domains on the intracellular membrane [100]. The TIR domain acts as a scaffold for recruitment of adaptor proteins that initiate the rest of the signaling cascade [99]. A simplified version is depicted in Figure 1.1. Most of the TLRs are associated with the intracellular adaptor protein called myeloid differentiation primary response gene 88 (MyD88) [27]. TLR3 is unique in that it associates with the adaptor protein TIR-domain-containing adaptor protein inducing IFN (TRIF; also known as TICAM1), and TLR4 associates with both MyD88 and TRIF.

In the MyD88-dependent pathway, IL-1 receptor-associated kinases 1 and 4 (IRAK1 and IRAK4) become associated with the MyD88 protein, forming the IRAK1:IRAK4 complex [99]. This complex has a docking site on IRAK1 for tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6). TRAF6 is an ubiquitin E3 ligase and functions with other ubiquitin-conjugating enzymes to ubiquinate several target proteins including TRAF6 itself [99]. Activated TRAF6 in turn activates transforming growth factor-β-activated protein kinase 1 (TAK1). Activated TAK1 activates the IκB kinase complex (IKK), consisting of NEMO and two catalytically active kinases, IKKα and IKKβ. The IKK complex phosphorylates inhibitor of κBα (IκB), which targets it for proteasomal degradation. IκBα is normally found in the cytosol complexed to the transcript factor nuclear factor kappa light chain enhancer of activated B cells (NFκB) [104] to prevent it from translocating into the nucleus. NFκB released from IκBα enters the nucleus where it binds to several promoter or enhancer regions of target genes [104]. TAK1 also initiates the mitogen-activated protein kinase (MAPK) pathway which ultimately results in activation of the dimeric transcription factor called activating protein 1 (AP-1) [105].

In the TRIF-dependent pathway, TRIF associated with TIR activates the kinases TRAF6, TRAF3 and RIP1 [99]. TRAF3 phosphorylates TANK-binding kinase 1 (TBK1), which then activates IRF3 and IRF7, allowing them to translocate into the nucleus and initiate gene transcription. IRF3 and IRF7 provide anti-viral immunity by induction of type I interferons, IFN-α and IFN-β [106, 107]. Activated TRAF6 and RIP1 merge with the MyD88 pathway by also causing polyubiquination of TAK1, which also leads to NFκB activation [99].

Despite redundancies in signaling pathways induced, differential inflammatory responses are initiated by each TLR [108-110]. The signaling cascades can be influenced at several points to fine tune the response. One way this is accomplished is through alternate adaptor proteins recruited to TIR along with MyD88 and/ or TRIF [99]. There are 3 other known adaptor proteins that can influence TIR signaling cascades: MyD88-adaptor-like (MAL, also known as TIRAP), TRIF-related adaptor molecule (TRAM; also known as TICAM2) and sterile-α and armadillo-motif-containing protein (SARM) [111]. MAL is required for signaling by TLR4 and the TLR2 family; it binds to TRAF6 to facilitate its interaction of either of these receptors' TLR domains [112]. TRAM is utilized by TLR4 to bridge MyD88 and TRIF pathways [113]. SARM is a negative regulator of TIR signaling, being able to block TRIF signaling [114].

MyD88 itself may interact differently with each TLR TIR domain [99], for example, Jiang *et al* demonstrated that point mutations in intracellular domains of TLR receptors interfered with MyD88 recruitment and abolished signaling for some receptors and not others [115]. The stimulation of multiple TLR types may result in enhanced or inhibitory effects, indicating that TLRs are involved in cross-regulation [109, 116-118]. Furthermore, the activity of NFκB is regulated by many other signaling pathways and may be influenced by the metabolic state of the cell, growth factors, cytokines or stress signals [104, 119]. The strength and duration of NFκB transcriptional activity is regulated by post-translational modifications of the subunits comprising NFκB [120]. NFκB is not a single transcription factor, but rather a family of factors composed of homo- and hetero-dimers of p105/p50, p100/p52, c-Rel, RelA (p65), and RelB [121, 122]. The level of acetylation and phosphorylation of NFκB is dictated by different

signaling pathways, and is therefore partially dependent on the receptor repertoire of a particular cell.

The strength and duration of the immune response can be determined by the TLR repertoire involved in responding to infection or vaccination [123]. For example, the yellow fever vaccine (YF 17D) is widely regarded as one of the most successful vaccines of all time due to its ability to rapidly generate an immune response after a single dose that lasts up to 10 years [26]. It has been demonstrated that this vaccine, an attenuated whole-organism vaccine, stimulates multiple TLRs expressed on DCs and NK cells that contributes to its potent effects [124, 125].

1.1.2 Manipulating the Immune Response with Subunit Vaccines

While whole-organism vaccines have been effective at reducing and even eliminating the risk of infection for many human pathogens, they are not suitable for all indications [4]. A classic example is shown in the vaccine development history for respiratory syncytial virus (RSV) [126]. RSV is a common pediatric infection, but also a significant cause of hospitalizations of infants and the elderly. Efforts to develop a vaccine in the 1960's led to a clinical trial of an inactivated vaccine in infants with disastrous results. Not only did the vaccine fail to protect, it actually exacerbated disease leading to hospitalization of 80% of vaccinated subjects compared to 5% in the placebo group [14]. The reason was two-fold: the vaccine induced a strong Th2-mediated antibody response but the antibodies that were generated bound only weakly to the virus, being directed against epitopes altered by the inactivation process [127]. Secondly, natural infection is cleared in most individuals through a Th1-type immune response, so by skewing the immunity towards Th2 the vaccine actually inhibited the generation of a protective immune response [127]. Attempts to develop an attenuated version were also unsuccessful since RSV has a particular sensitivity to under or over attenuation. To date there is no vaccine available to prevent RSV infection [126]. Besides RSV, vaccine-enhanced disease has also been documented for measles [128] and dengue [15]. In these diseases, development of antibodies towards these pathogens facilitates viral entry into phagocytic target cells via opsonization.

Second generation vaccines are subunit based, that is, all the components of a whole-cell vaccine are distilled into the most essential units which are packaged into a vaccine composition. Subunit vaccines consist of three basic components: 1) antigen; 2) adjuvant and 3) delivery system (Table 1.2) [129]. Subunit vaccines are considered to be the safest approach to vaccination since the vaccine may be delivered without whole organisms, negating the risk of accidental infection due to virulent strain contamination or reversion [129, 130]. Additionally, each component in the subunit vaccine is produced synthetically and as a result is subjected to tests to confirm identity, purity and reactivity, which provides an additional measure of quality control. Manufacturing of subunit vaccines is inexpensive and reproducible. Subunit vaccines have a huge degree of flexibility since different components may be easily interchanged to produce a tailored immune response to vaccination. However, because a subunit vaccine contains only the essential elements required for induction of immune response, developing effective subunit vaccines requires an understanding of how each component interacts with the immune system [131-133].

1.1.2.1 Subunit vaccine antigens

Immunogenic antigens are molecules that are recognized by a T cell, through the TCR, or a B cell, through the BCR, and are capable of inducing an immune response [134]. Antigens may be proteins, peptides or polysaccharides. An epitope is the portion of the antigen that is recognized by the immune system; a multivalent antigen has multiple epitopes [135]. Antigens for T cells must be presented in MHC class I (for $CD8^+$ T cells responses) or II (for $CD4^+$ T cell responses), and therefore the peptide epitopes are linear amino acid sequences derived from a larger protein. B cell antigens do not have to be presented by MHC for BCR recognition, therefore the epitope may be dictated by the tertiary structure of the antigen and are most efficient when they are multivalent [136]. Selection of an appropriate antigen for a vaccine depends upon many factors; whole proteins are typically used for humoral immune responses since they will induce T and B cell responses, but peptide antigens are preferred for cellular immune responses since they can be presented in MHC class I for efficient presentation to $CD8^+$ T cells [12, 133]. Polysaccharide-antigens tend to induce a weaker antibody-mediated

immune response without activation of T-help or generation of B cell memory [12, 137]. The immunogenicity of some polysaccharide vaccines has been improved by conjugation to a protein carrier, for example, the *Haemophilus influenzae* type b and meningococcal vaccines [137, 138].

MHC molecules are able to present a variety of different peptide sequences, but unlike the TCR or BCR they do not undergo genetic recombination [139]. In humans, MHC protein sequences are encoded by a set of highly polymorphic genes of the human leukocyte antigen (HLA) on chromosome 6. Humans have three loci for classical MHC class I: designated A, B and C. The MHC class II proteins are encoded by the DR, DP and DQ gene families [140]. Inheritance of HLA genes is co-dominant, that is paternal and maternal alleles are both expressed to give two sets of each gene within an individual, and there are thousands of alleles that have been identified in the human population. The polymorphisms of MHC are clustered in the DNA coding the antigen-binding groove, which accounts diversity in antigen recognition [139, 141]. Every individual inherits a maternal and paternal allele for each loci which are expressed co-dominantly [139]. The MHC I protein is composed of a single protein chain stabilized by the β2-microglobin, therefore there are 6 distinct MHC I alleles in heterozygous individuals. The MHC II protein is a dimeric protein consisting of separate α and β chains. Each locus encodes these chains separately, allowing the possibility of many paternal-maternal combinations. Although there are thousands of known alleles for each MHC loci, some are very common while others are rare within a given ethnic population [141, 142].

Identification of peptide epitopes suitable for induction of $CD4^+$ Th and $CD8^+$ CTL responses is an ongoing process for the development of vaccines intended to induce a cellular immune response [135]. Cellular vaccines typically contain a mixture of short MHC I (8-10 amino acids) and longer MHC II (11-30 amino acids) peptides containing one or several epitopes. These types of antigens vary in their capacity to bind different MHC alleles and thus may not be recognized in all individuals [20]. The sequence of antigens that bind to MHC I is strictly restricted by the MHC allele of the individual [143]. Antigens that bind to MHC II, also called T-helper peptides, are more promiscuous in their binding across different alleles [144]. T-helper antigens are

essential to inducing and maintaining a longer lasting, more robust $CD8^+$ T immune response to MHC I antigens [132, 145].

Since MHC proteins are germline encoded, potential antigenic peptides can be predicted by identifying the optimal anchor residues that are the contact points between the HLA and peptide [146]. For example, peptides that bind to HLA-A*02:01, a common allele in North America, are ideally 9 amino acids in length anchored at position 2 by leucine or methionine and position 9 by valine or leucine [147]. This "reverse immunology" approach is frequently used to identify potential MHC class I restricted antigenic peptides for use in vaccines [146, 148]. Knowing the preferred sequence of peptides that bind to a given HLA allele can also be used to modify weakly antigenic peptides in order to increase binding to MHC [149].

1.1.2.2 Subunit vaccine adjuvants

Subunit vaccines, particularly peptide containing vaccines, are not very immunogenic [131, 150]. Without adequate innate cell stimulation at the time of antigen encounter, the immune system may develop tolerance towards the vaccine antigens rather than active immunity [151]. Adjuvants, described as "the immunologists' dirty little secret" by Charles Janeway, are a diverse group of compounds united in their common effect which is to boost the immune response towards an antigen [152]. There are several mechanisms through which a given adjuvant may enhance immune responses [153]. These mechanisms may include: 1) increase activation and antigen uptake by APCs; 2) act as a depot to protect antigen from dilution and degradation; 3) increase local inflammation; 4) increase non-specific proliferation of lymphocytes. Many have attempted to categorize types of adjuvants but so far there is no uniform classification system [94, 152, 154]. In this thesis adjuvants will be divided into two broad classes: delivery systems and immune-stimulants. A delivery system is a specific class of adjuvant with the inherent ability to package antigen(s) to provide stability to the components of the vaccine *in vivo* and facilitate immune recognition. Delivery systems are usually complex preparations, such as water-in-oil emulsions or liposomes [93]. Immune-stimulant adjuvants are defined molecular structures that can stimulate the

innate immune cells via PRRs, for example PAMPs and DAMPs [155, 156] (see Table 1.1 for examples of TLR agonists used as adjuvants).

Adjuvant research is an active area of study in vaccine research, considered essential for the future of vaccines [152, 157]. Not only do new adjuvants need to be discovered, but a detailed understanding of how the known ones work is somewhat lacking at the present time. Most of the current vaccines were developed empirically, through a trial and error process [10, 152]. Until recently, alum was the only adjuvant approved for use in human and veterinary vaccines [158, 159]. Alum-based vaccines, first used in the 1920's, are prepared by adsorbing the antigen to the aluminum hydroxide or aluminum phosphate gels [159, 160]. Upon injection, alum forms a short-lived depot for antigen. Alum preparations induce Th2 humoral responses characterized by IL-4 production, IgG1 and IgE antibodies and eosinophil activation [158]. Alum does not efficiently stimulate Th1 responses. Use of alum pre-dates the modern FDA approval process for new drugs, but has an extensive safety record and is very efficient at stimulating antigen-specific antibody production. The principle mechanism of alum-based adjuvant has been long attributed to formation of a particulate depot [159] that prolongs immune exposure and enables macrophage phagocytosis [161]. Recent studies have shown that alum may also enhance the immune system through stimulation of PRRs [162]. This immune-stimulation by alum is an indirect effect caused by necrosis upon intramuscular injection [163]. Necrotic cell death caused by alum results in the release of endogenous DAMPs, in particular uric acid and HSP70, which can trigger DAMP PRRs on the surrounding DCs [162, 164, 165]. Intracellular signaling associated with alum-adjuvant is linked to activation of the NLRP3-containing inflammasome [166]. The inflammasome is an intracellular complex assembled in response to DAMP stimulation. The molecular events of inflammasome signaling are not yet defined, but lead to the expression of IL-1β, IL-18 and IL-33 which promote Th2-type immunity [167]. TLR agoinsts may or may not be involved as conflicting data on the ability of alum vaccines to generate antibodies in TLR knockout mice has been reported [162, 168].

Besides its inability to stimulate Th1 responses, alum presents other limitations that necessitate development of alternatives. Alum is not effective at boosting immune

responses to all types of antigens; for example, alum does not boost the immunogenicity of polysaccharide vaccines [169] or to small peptide vaccines [158]. Alum vaccine preparations cannot be lyophilized for long term storage and as such have a short shelf life requiring storage at 4°C. Finally, although generally considered safe, alum based vaccines can sometimes cause severe tissue reactions, such as erythema and granulomas, which may increase in severity after booster immunization [158, 159]. Furthermore, due to the promotion of IgE, alum vaccination may exacerbate allergic reactions in some individuals [170].

1.1.2.3 DepoVax™ Vaccine Delivery System

DepoVax™ is a proprietary vaccine delivery system developed by Immunovaccine Inc. (Halifax, NS, Canada). It is an oil-based liposomal preparation that can be formulated with various types of antigens and can be combined with other immune-stimulating adjuvants. Briefly, DepoVax is prepared by mixing antigens and adjuvants in aqueous liposomes, which are then lyophilized and resuspended in metabolizable oil, such as Montanide ISA51 VG [171, 172]. The use of liposomes to encapsulate the antigens and adjuvants allows for mixing of a diverse set of molecules, and the oil diluent creates a strong depot at the injection site. DepoVax has been shown to provide robust humoral and cellular immune responses for a longer period of time than commonly used emulsion formulations, with and without alum (Figure 1.2). Formulation of antigens and adjuvants in DepoVax faciliates their active uptake by phagocytic APCs, enabling more efficient antigen presentation within the lymph node [173]. Several TLR agonists have been formulated with DepoVax to enhance the immune response and skew it towards antibody production or cellular immunity (personal observations). This thesis project was performed in collaboration with Immunovaccine and the vaccines used herein were prepared in DepoVax.

1.1.3 Translational Development of Vaccines

Producing safe and effective vaccines requires about 10-15 years of research, and is estimated to cost over one billion dollars [174]. The current system for testing and validation of vaccines was adopted from methods used to develop small molecule drugs. However, this is an evolving process which is developing to meet the specific

requirements imposed by unique considerations of vaccines. For example, many vaccines are developed with the intention of protecting the host from infection. Demonstrating efficacy in these cases would require exposure to the pathogen, which cannot always be ethically performed in human subjects. For this reason, the FDA has released several guidance documents to help vaccine developers navigate through the regulatory process [175].

The general stages of vaccine development are: exploratory, pre-clinical, and clinical [176, 177]. During the initial exploratory stage, antigens and adjuvants are discovered and tested primarily using *in vitro* techniques. The pre-clinical stage is where these vaccine components are tested *in vivo* using animal models. This is a key stage in vaccine development since it is critical to demonstrate that a vaccine can stimulate the complex immune system in order to generate the desired immune response [4]. During this stage, safety of the vaccine is also evaluated to ensure no adverse reactions or autoimmunity are elicited [178]. Clinical testing in humans is a 3 phase process [176]; for phase I trials, <100 patients are typically enrolled and the vaccine is evaluated primarily for safety. This type of study often involves dose-response testing to optimize the response in human subjects. Phase II trials involve several hundred patients and are designed to evaluate safety, immunogenicity and efficacy in a patient population at risk of acquiring the disease targeted by the vaccine. Finally, phase III trials involve several thousand patients and compare the new treatment to standard of care or placebo to provide significant data demonstrating the efficacy of the vaccine. Once the novel vaccine has completed these developmental stages, the data is presented to the FDA who grant approval and licensure of the vaccine for specific indications [176]. Before the vaccine is available commercially, the manufacturing process must also be inspected, licensed and quality control approved.

During the development stages, research undergoes two important transitions, first from *in vitro* experiments to *in vivo* animal models, and second from *in vivo* animal models to human clinical trials. Successful translation of results between these systems is highly dependent on understanding the genetic and physiological differences between animal models and humans [179, 180]. It has been estimated that only about one-third of

treatments with demonstrated efficacy in animal models will enter phase I clinical testing, and of these only 8% will complete phase I successfully [177].

The amount of data that can be gleaned from *in vitro* testing to support vaccine development is very limited. Two assays commonly used are binding affinity of novel antigens to different MHC alleles [133], and adjuvant activity on cells of the immune system [108]. Particularly for novel adjuvants, these types of assays can be used to uncover the molecular mechanisms involved and therefore identify *in vivo* models where the observed effect would be most beneficial. If an assay can be validated for reliable and predictive results, *in vitro* testing is often much less expensive and time consuming to perform, allowing large scale screening of novel compounds. However, graduating to *in vivo* testing is often a significant hurdle since the complexity of *in vivo* testing cannot be recapitulated using *in vitro* systems.

Animal testing is necessary to vaccine development in order to demonstrate safety, immunogenicity and efficacy [178]. Mice are the most common species for *in vivo* testing; using mouse models, various aspects of the vaccine can be evaluated within the context of a fully functioning immune system. Definitive proof of efficacy is the ultimate validation for any vaccine [12], but herein represents a significant shortfall in the mouse model: mice are not always susceptible to the same diseases as humans. For this reason challenge models exist in a diverse number of species, such as ferrets for influenza [181], cotton rats for RSV infection [182], and armadillos for leprosy [183]. If no model exists, efficacy may be predicted if immune correlates of protection are known, but is not always the case. For example, it is thought that a Th1-type immune response should be induced by an RSV vaccine because this is the type of immune response that develops naturally in individuals that can overcome infection [126]. Therefore, a mouse model can be used to demonstrate that a vaccine can induce a Th1-biased immune response to vaccine even though mice are not a natural host for RSV. But for some diseases, such as HIV, the immune correlate of protection is not known because infection is not naturally overcome [4, 184].

Inherent genetic differences between animal models and humans pose additional challenges to development of both antigens and adjuvants [180, 185]. Peptide antigens are restricted by MHC class I and II alleles, yet the allele restrictions vary between

species. This problem is partially mitigated by the development of humanized transgenic mice expressing HLA molecules [186]. Adjuvants may also work differently in mice and humans owing to differential expression of PRRs on different cell types. TLR4, for example, is expressed constitutively mouse B cells, but only at low levels on human B cells [187]. Additionally, the TLR repertoire varies between mice and humans, there as are 12 TLRs identified in mice but only 10 in humans (Table 1.1) [27].

1.2 Humoral vaccines for infectious diseases

All currently licensed vaccines exert their effect by inducing production of protective antibodies [158, 160]. However, the types of vaccine formulations that are used have limitations in their immune stimulatory mechanisms and applicability. Current research in the development of humoral vaccines is focused on identifying adjuvant alternatives to alum that can efficiently stimulate an antibody mediated response. Understanding how antibody-producing B cells are activated and regulated can contribute to design-based approach for novel humoral vaccines.

1.2.1 B cell biology

1.2.1.1 Development

In adult humans and mice, B cells develop in the bone marrow from hematopoietic progenitors [51]. Developing B cells progress in the bone marrow from pro-B cells, to pre-B cells to immature B cells. During this time, they first express B cell surface receptors CD19 and CD45R (B220) [188, 189]. Each B cell also develops a unique immunoglobulin protein expressed on its membrane surface (mIg) comprised of two heavy and two light chains. The mIg is part of the B cell receptor (BCR) complex and is responsible for antigen recognition. The mIg interacts with epitopes via its complementarity determining regions, which are formed by variable domains of the heavy and light chains [190]. The BCR complex also comprises transmembrane proteins Ig-α and Ig-β, which mediate intracellular signaling upon antigen binding [191]. Before exiting the bone marrow, the BCR has undergone negative selection to remove B cells that recognize self-antigens [51]. Immature B cells enter circulation and migrate to the spleen for maturation into naïve B cells.

B cell development, differentiation and survival are regulated at several stages through the cytokines B-cell activator of the TNF family (BAFF, also known as BLyS) and A proliferation inducing ligand (APRIL). Both are members of the TNF family of cytokines and are recognized by three TNF superfamily receptors found only on B cells: BAFF-Receptor (BAFF-R), transmembrane activator calcium modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA). The expression of these receptors on B cells is regulated by their maturation and activation state [192]. BAFF-R binds only BAFF, while the TACI and BCMA can bind both BAFF and APRIL. BAFF and APRIL are primarily produced by myeloid cells and therefore have no autocrine effect [193]. B cell maturation is completed in the spleen where they receive BAFF survival signals through BAFF-R [194].

1.2.1.2 B cell antigen recognition and activation

Naïve B cells circulate through the blood and lymphoid system seeking antigen recognized by their BCR [42]. The migration of naïve B cells into secondary lymph node follicles is mediated by CXCL13 which binds to the CXCR5 receptor on naïve B cells [195]. B cell encounter of antigen can be facilitated by phagocytic cells which display intact antigens transported from the periphery to the lymph node by engulfing them in specialized non-degradative endosomes [22, 196]. It has also been recently demonstrated that antigens smaller that 70 kDa are able to passively travel to the lymph node where they be recognized by naïve B cells [197]. $CD169^{+}$ macrophages residing in the subcapsular sinus facilitate presentation of intact antigen to B cells within the lymph node [198]. Some B cell subsets may also be involved in transporting antigens to the follicular zone for B cell recognition [199].

BCR clustering is the initial event leading to B cell activation; clustering is the result of binding to multivalent antigens [200]. The chemokine receptor CCR7 is rapidly unregulated on the surface of the B cell, which directs B cell migration to the T cell zone (paracortex) of the lymph node [42]. B cells internalize the protein antigen through BCR and present peptides in MHC class II. In the T cell zone, B cells present peptides within MHC class II to Th cells [46]. Th cells must be previously activated by DCs, and if they recognize peptide antigen presented by B cells they will provide co-stimulation signals,

such as CD40 engagement and cytokines, to B cells to complete activation. Fully activated B cells initially produce a burst of low-affinity germline antibodies of IgM isotype which are detectable in the plasma days after exposure to antigen [201]. Depending on the strength of activation signals provided by the Th, activated B cells can upregulate CXCR5 to reenter the follicle and establish germinal centres [202]. The T_{FH} subset, also expressing CXCR5, co-localizes to the germinal centres and provides signals to B cells which are crucial to the development and regulation of the germinal centre. Within a germinal centre, B cells refine their response to antigen by class switch recombination to change antibody isotype [40]. They also increase antibody affinity for antigen through affinity maturation, a process involving somatic hypermutation of mIg variable regions (VDJ gene segments) followed by positive selection. Class switch recombination and somatic hypermutation are both initiated by the AID enzyme which is expressed in activated B cells [203]. Germinal centre events cumulate in the development of plasma cells producing high affinity, class-switched antibodies and memory B cells [204].

1.2.1.3 Molecular signaling pathways in B cells

An overview of important signaling events that occur after BCR engagement are depicted in Figure 1.3, but the complete picture is much more complex and reviewed in [191]. Clustering of BCR by multivalent antigen activates the *src*-family kinase Lyn, which then phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) found on the Ig-α/β cytoplasmic tails [200]. This creates a docking site for Syk, a protein tyrosine kinase, which in turn activates B-cell linker (BLNK). Activated BLNK is a scaffolding protein essential for the activation of several transcription factors, including Bruton's tyrosine kinase (Btk). Lyn, Syk and Btk are essential protein kinases required for B cell signaling, deficiencies in any one of them results in defective B cell function and development [191]. Inhibitors of Btk are actively being investigated for treatment of various B cell malignancies [205]. Signaling events induced from Btk activation result in activation of the transcription factors NFκB, NFAT and MAP kinases (p38, ERK, and JNK). The CD19 co-receptor, composed of CD19, CD21 and CD81, promotes effective BCR signaling by activating phosphatidylinositol-3-kinase (PI3K) [206]. PI3K promotes

efficient recruitment and activation of Btk and initiates signaling events leading to the activation of Akt [207]. Akt is a serine/ threonine kinase that promotes B cell survival and enhances activation of NFκB.

To become fully activated, B cells require costimulation provided through the interaction of CD40 on the B cell with CD154 (CD40 ligand) on Th (Figure 1.4). CD40 signaling synergizes with BCR signaling to induce vigorous B cell proliferation and differentiation [203]. The importance of CD40 signaling is evidenced by the fact that B cells in $CD40^{-/-}$ mice fail to proliferate or undergo class switch recombination [208, 209]. CD40 is a member of the TNF receptor superfamily and mediates downstream signaling by activating TRAF2, TRAF3 and TRAF6 [203, 210]. CD40 signaling ultimately results in activation of the non-canonical p52-RelB NFκB subunits [211]. B cells may also receive direction in the form of cytokines secreted by Th to skew antibody class switch recombination; for example, IFN-γ promotes IgG2a/c in a Th1 response and IL-4 promotes IgG1 and IgE in a Th2 response in mice [73].

Activated B cells express high affinity isotype switched antibody. B cell activation results in the upregulation of surface receptors such as CD40, CD80, CD86, MHC class II, and CXCR5 [212]. Activated B cells can also secrete a variety of cytokines including IL-6, IL-10, TNF-α, IL-12, IFN-γ and IL-4, which have both autocrine and paracrine effects [213, 214]. The maturational stage of the B cell, the microenvironment, the availability of T-help and the type of antigen can all influence B cell activation [191, 202, 215].

1.2.1.4 T-Independent B cell activation

Some antigens may activate B cells without the need for Th cells. There are two classes of T-independent (TI) antigens, TI-1 and TI-2 [40]. TI-1 antigens are mitogens that can activate B cells irrespective of BCR specificity. A classic TI-1 example is LPS which can bind to murine BCR and will trigger activation by simultaneously clustering BCR and TLR4 [216, 217]. LPS does not have the same effect in naïve human B cells, which do not express TLR4 [187]. LPS stimulates polyclonal expansion and antibody production by B cells. TI-2 antigens are typically large, repetitive multivalent structures capable of binding several BCR at once. Polysaccharides used in vaccines are examples

of TI-2 antigens [138]. TI-2 antigens differ from TI-1 antigens in that they still require a second signal to induce antibody secretion [218]. This second signal for TI-2 antigens may be provided by cytokine released from activated DCs or Th cells nearby, or through TLR signaling on B cells [40]. Polysaccharide antigens are not recognized by MHC and therefore B cells recognizing these type of antigens will not receive Th cell co-stimulation. Without Th cell help, TI-2 antigens do not stimulate affinity maturation to generate high affinity antibodies, nor do they induce memory B cell differentiation [138].

1.2.1.5 B cell subsets

Naïve B cells in the mouse are grouped into two distinct lineages, referred to as B-1 and B-2, based on their origins, phenotype, tissue homing patterns and function [219, 220]. B-1 cells are generated during fetal development, and possess innate-like characteristics. The B-1 subset is capable of self-renewal independent of the bone marrow. They reside in the peritoneal and plural cavities, and may circulate in the blood stream, but are rare in secondary lymph node tissues [219]. However, B-1 cells do not undergo class switch recombination or somatic hypermutation, and their antibodies are encoded by germline immunoglobulin genes. B-1 produced antibodies are low-affinity IgM or IgA that generally recognize TI antigen [221]. B-1 cells produce "natural antibodies" which constitute resting IgM and IgA levels found in normal serum [220]. Although they only constitute about 5% of the total B cell population in an adult, B-1 cells are an important first line of defense against some bacterial infections [222]. In mice, B-1 cells are identified as $B220^{+}CD19^{+}CD11b^{+}CD23^{-}IgD^{-}$ cells isolated from the peritoneum [223]. They can be further subdivided based on CD5 expression into B-1a ($CD5^{+}$) and B-1b ($CD5^{-}$), each participate in first response to different pathogens [224].

B-2 cells are produced in the bone marrow of adults and are involved in recognition and response to T-dependent antigens [220]. B-2 generally reside in secondary lymphoid compartments and can be further subdivided by where they home to in these tissues as follicular (FO) and marginal zone (MZ) B cells. FO B-2 cells are responsible for T-cell dependent antibody responses and constitute the majority of the B cell population [225]. FO B cells create germinal centres to generate high affinity antibodies through affinity maturation, and subsequently persist as memory B cells [226].

In mice, FO B cells may be identified phenotypically as $B220^{+}IgM^{lo+hi}IgD^{hi}CD23^{+}CD21^{-}CD1d^{lo}$ cells [199, 219, 223]. MZ B-2 cells have an innate-like role in the immune response. MZ B-2 cells are restricted to the splenic marginal zone and are involved in the first response to blood-borne infections to rapidly produce IgM, IgG and some IgA [226]. MZ B cells have a limited capacity for affinity maturation, but assist the T-dependent immune response by transporting antigens to FO B cells in the spleen [199]. MZ B cells also express CD1d, a receptor involved in the presentation of lipid antigens to natural killer T (NKT) cells [219]. In mice, MZ B cells can be identified by phenotype $B220^{+}IgM^{hi}IgD^{lo}CD23^{-}CD21^{+}CD1d^{hi}$ [223, 226]. Both FO and MZ express TLR and respond to stimulation, but their repertoire differs as does their capacity to produce different cytokines [223, 227].

Regulatory B cells have also been most recently described [228]. These cells express high amounts of IL-10 and are hence called B10 cells. A high frequency of IL-10 producing B10 cells were found to be $CD5^{+}$ and $CD1d^{high}$, but these markers are not unique to B10 [228]. B10 have an important role in preventing autoimmune diseases since their loss exacerbates experimental autoimmune encephalomyelitis, a mouse multiple sclerosis model [229, 230].

Although most of these B cell subsets are well defined in rodents, evidence of their existence in humans has been controversial. The phenotype of B-1 cells in humans has been defined as $CD20^{+}CD27^{+}CD43^{+}CD70^{-}$ and thus bear little resemblance to murine counterpart [231]. Likewise, human FO and MZ cells are differentiated based on expression of CD21/ CD24 and human MZ cells can be found in other secondary lymph node tissue, including lymph nodes and tonsils [232]. Furthermore, there seems to be significant differences in FO and MZ differentiation and homeostasis between human and mouse [233]. Although not fully understood, these differences indicate that studies of B cell function in mice must be interpreted cautiously when translating to humans.

1.2.1.6 Antibody functions

Antibodies are secreted immunoglobulins similar in structure to the B cell mIg and with identical antigen specificity to their parent B cell [234]. Biologic functional activity is conferred by the isotype of the C-terminal domain of the heavy chains, the Fc

region. The heavy chain gene contains several gene segments that encode different antibody isotypes, which encode the protein isotypes IgD, IgM, IgG, IgA and IgE [235]. Naïve B cells express IgD and IgM, but upon activation the B cells undergo class switch recombination to change the isotype, mediated by the enzyme AID. Isotype switch is dictated by cytokines produced by T_{FH} within germinal centres [235]. Each isotype has unique characteristics that make them suited for particular applications [236]. IgA can mediate mucosal immunity as it is the only isotype capable of traversing the mucosal barrier. IgE mediates mast cell degranulation. There are four subclasses of IgG in humans: IgG1, IgG2, IgG3 and IgG4. There are also four IgG subclasses in mice: IgG1, IgG2a (or IgG2c depending on the strain), IgG2b and IgG3, although the nomenclature differs and their functions are not strictly analogous [180]. Based on *in vitro* studies of human and mouse B cell response to cytokines, human IgG1 is thought to correspond to murine IgG2a/c, and human IgG2, IgG3 and IgG4 correspond to murine IgG3, IgG2b and IgG1, respectively. Each IgG subclass has varying capacity to activate complement and bind Fc receptors to induce phagocytosis, ADCC or CDC [236].

1.2.1.7 B cells as antigen presenting cells

Besides producing antibody, B cells are also efficient APCs with the capacity to activate or tolerize T cells [237]. B cells activated by Th cells in the lymph node upregulate several surface receptors, such as MHC class II, CD80 and CD86, that would allow them to effectively present antigen to naïve $CD4^+$ T cells. The key difference between B cell antigen presentation and presentation by classical APCs, such as DCs, is that B cells internalize specific antigen through a high affinity BCR. This feature allows them to concentrate MHC presentation for epitopes of a particular antigen, thereby focusing the immune response and strengthening the T cell response [237].

Whether or not B cells participate in the priming response to vaccination, without activation by Th, is not well understood. The frequency of naïve B cells that can recognize a new antigen is low, only about 1 in 10^4-10^5 cells, making it less likely that a circulating naïve B cell will encounter cognate antigen in the periphery [237]. Naïve B cells circulate the blood and secondary lymph nodes, but do not home to sites of inflammation [23]. However, some studies indicate that B cells may be more effective at

priming the response to vaccine protein antigens than DCs, which are more effective for peptide antigens. Levin *et al* and Constant *et al* constructed a transgenic MHC class II specific to tobacco hornworm moth cytochrome c (residues 81-103) [238, 239]. They developed mice that preferentially express high levels of the transgenic MHC class II on either B cells or DCs. Immune responses were induced by vaccinating the mice with either whole protein or peptide antigen. They discovered that DCs were essential to priming naive $CD4^+$ T cell responses against peptide antigens, but B cells were critical for naïve $CD4^+$ T cell activation when protein antigens were used for vaccination. Mice deficient for B cells (μMT) were also shown to elicit a weaker $CD4^+$ T cell response after vaccination with various protein antigens, such as ovalbumin, pigeon cytochrome c, conalbumin, and human collagen IV [240]. However, μMT mice could still generate a $CD4^+$ T cell response to peptide vaccination. Other groups have demonstrated that reconstitution of μMT mice with B cells by adoptive transfer restored the $CD4^+$ T cell response to vaccination with ovalbumin [241, 242]. Rodriguez-Pinto *et al* demonstrated that B cells can act as the main APC for $CD4^+$ T cells for protein immunization by transferring B cells expressing a transgenic BCR for hen-egg lysozyme (HEL) antigen into $RAG2^{-/-}$ lymphocyte-deficient mice along with $CD4^+$ T cells bearing TCR specific for HEL peptide [243]. Donor cells were $H\text{-}2^k$ haplotype while recipient mice were $H\text{-}2^b$, ensuring that HEL-specific T cells could only recognize antigen presented by the B cells. Upon immunization with HEL protein, HEL specific B and T cells rapidly proliferated and expressed activation markers. However, these effects were abrogated when CD154 expression was knocked out from HEL-specific T cells. These studies suggested that B cells can act as APCs to prime naïve $CD4^+$ T cells in response to protein vaccination. However, for productive interactions, CD40 co-stimulation must be provided to the B cells, which may be provided by low constituitive expression of CD154 on $CD4^+$ T cells. In none of these studies did they use an immune-stimulatory adjuvant with vaccination, so it remains unclear what effect this could have on B cell APC function.

Although B cells may not be necessary for peptide immunization, the $CD4^+$ T cell response is enhanced by the combined antigen presentation by both DC and B cells [244]. This was demonstrated using mice that expressed transgenic antigen-specific MHC class II (I-E) under control of the CD11c-promoter (DC cells only) or CD19-promoter (B cells

only) or a hybrid containing both DC and B transgenic cells. Adoptively transferred T cells bearing TCR specific for the antigen presented by MHC II most efficiently proliferated in the hybrid mice after vaccination with peptide antigen.

1.2.2 Control of B cell responses through TLRs

The role of TLR signaling on B cell activation, particularly *in vivo*, is not well understood [245]. In general, TLRs 1, 2, 6, 7 and 9 are most commonly found on B cells of both species [223, 227, 246, 247]. Mice tend to have broader TLR expression on B cells, particularly TLR4, which is constitutive on both naïve and memory B cells. On human B cells, TLR expression is increased upon BCR stimulation [247]. TLR4 and TLR9 are the most studied TLRs on B cells due to their constitutive and high expression on murine (TLR4 only) and human B cells [248].

Studies done *in vitro* with mouse B cells suggest TLR engagement can influence B cell activation at several steps. Even without BCR cross-linking, Pam3CSK4 (TLR1/2), lipid A (TLR4), R848 (TLR7) and CpG (TLR9) act as mitogens *in vitro* by inducing B cell proliferation, but do not evoke antibody secretion or class switch recombination, and are not considered TI-1 antigens [217]. However, in the presence of BCR crosslinking and cytokine (IL-4 or IFN-γ), TLR signaling augments proliferation as well as class switch recombination and antibody production by enhancing NFκB activation [217]. TLR stimulation may also enhance B cell capacity to act as APCs to T cells by increasing expression of several co-receptors required for Th activation, such as MHC class II, CD80 and CD86 [249, 250]. Even in naïve human B cells, which express low levels of TLRs, TLR stimulation has been shown to synergize with BCR cross-linking and CD40 engagement to enhance proliferation and differentiation *in vitro* [251]. In human B cells, TLR stimulation has more potent effects on memory B cells since their TLR repertoire is larger than naïve counterparts [252]. TLR stimulation of memory B cells may be one factor contributing to the maintenance of memory B cells [253].

The synergistic effect of TLR and BCR stimulation may be due, in part, to the different signaling pathways both leading to NFκB expression [254]. CD40 signaling in B cells stimulates NFκB activation through the non-canonical pathway which can amplify pro-inflammatory signals (Figure 1.4) [217]. TLR signaling intersects BCR

signaling at several points (Figures 1.1, 1.3). Recently it was shown that TLR signaling can augment BCR induced PI-3K signaling through activation of BCAP [255]. TLR signaling also stabilizes and enhances Syk activation, providing another point of contact between TLR and BCR signaling pathways [256].

1.2.3 TLR Adjuvants to mediate B cell antibody production by vaccination

The role of TLR stimulation on DC to influence Th cell differentiation and subsequent immune response is well documented [110, 257]. Activated B cells promote the development of a robust immune response by providing feedback to Th cells, and B cells may play an important role in the priming of immune responses to some types of vaccine antigens [239, 243]. TLR signaling on B cells *in vitro* can activate B cells and synergize with CD40 signaling [217, 250, 258]. The influence of direct TLR stimulation on activating B cells *in vivo* is not well understood.

The first study to implicate intrinsic TLR signaling of B cells in response to vaccination was done by Pasare and Medzhitov in 2005 [259]. In this study, they reconstituted μMT mice with B cells from wild type, MyD88$^{-/-}$, TLR4$^{-/-}$ or CD40$^{-/-}$ mice. Mice were then immunized with human serum albumin antigen (HSA) adjuvanted with LPS and alum and bled on day 12 to check for antigen-specific antibody levels. They detected antigen-specific IgM and IgG1 in μMT mice reconstituted with wild type B cells, but antibody production was severely diminished in mice reconstituted with MyD88$^{-/-}$ B cells, implicating a role for TLR in antibody production by B cells. They confirmed their hypothesis that MyD88-signaling on B cells was important to antibody production by immunizing transgenic mice in which MyD88 expression was under control of the CD11c promoter and thus only expressed by DCs. The mice were vaccinated with HSA and LPS emulsified in incomplete Freund's adjuvant and bled after 10 days. Total antibody production in the transgenic mice was impaired relative to the wild type mice, demonstrating that MyD88 expression on another cell type besides DCs was an important factor contributing to antibody production. Although this was the first study to indicate that intrinsic TLR signaling may be important for B cell response to vaccination, it was flawed in some respects. The vaccine used contained alum, which is now known to stimulate non-TLR PRRs and thus may influence the B cell response, as

well as LPS that can signal through MyD88-independent pathways, although they only assessed MyD88 knockout mice [260]. Furthermore, they measured antibody production at 12 days, a little early to detect significant differences due to class switch and peak B cell antibody production, which can take up to 4 weeks post-immunization [261].

Two subsequent studies using different models were able to strengthen the hypothesis that TLR adjuvants directly influence B cell antibody production. In 2009, Barr *et al* created chimeric mice by reconstituting irradiated wild type mice with a mixture of bone marrow from MyD88$^{-/-}$ and μMT mice [263]. The chimeric mice, therefore, had a full complement of hematopoietic cells, but the only source of B cells was from MyD88$^{-/-}$ bone marrow. Chimeric mice were vaccinated with ovalbumin protein antigen and LPS emulsified in incomplete Freund's adjuvant and antibody production was monitored by regular bleeding for two months. They found that compared to wild type mice, the chimeric mice were impaired in producing the Th1-type antibody IgG2c in response to vaccination. This correlated with a reduction in thr size of germinal centres formed in chimeric mice. They found that IgG2c impairment may be the result of inefficient IFN-γ production by Th cells; therefore, TLR signaling may be an important factor influencing B cell priming by Th cells.

In 2011, Hou *et al* used a Cre-Lox system to selectively knockout MyD88 in DC or B cells and tested the antibody response to various types of vaccines [264]. They found that antibody production, determined 4 weeks after immunization, was not compromised in B cell-MyD88$^{-/-}$ mice if the mice were vaccinated with protein (ovalbumin) and adjuvant (CpG) in an aqueous or liposomal formulation delivered by intraperitoneal injection. In this case, DCs were the most important APC and could direct B cell production of antibodies. However, when mice were immunized with virus-like particle (VLP) encapsulating adjuvant (CpG), only the DC-MyD88$^{-/-}$ mice exhibited impaired antibody production. They concluded that B cells may be more important than DCs in defense against viruses. Others have also reported that B cells are essential to the primary immune response against aggregate-type antigens [265, 266]

1.2.4 Improved Adjuvant Activity with TLR Combinations

Certain PAMP combinations were reported to provide synergistic activation of DCs, suggesting that two is better than one when it comes to adjuvants [257, 267]. This is theoretically sound since natural infection with pathogens is bound to involve several PAMPs and PRRs, and the yellow fever vaccine is a prime example of a successful vaccine that does just that [26, 125]. GlaxoSmithKline has recently developed two adjuvant systems, AS02b and AS04, which are combinations of two different adjuvants and have been shown to work better than either adjuvant alone [268, 269]. Although many TLR combinations have been tested on DCs, few have been tested on B cells. In this dissertation I describe the stimulation of mouse B cells with poly I:C, a TLR3 agonist, and Pam3CSK4, a TLR1/2 agonist, and the use of this adjuvant system *in vivo* to generate an enhanced antibody response to vaccination.

1.2.4.1 Poly I:C-TLR3 signaling on B cells

TLR agonist combinations involving poly I:C (polyriboinosinic-polyribocytidylic acid) are a logical choice for adjuvant combinations since TLR3 uniquely signals through MyD88-independent pathways to produce type 1 IFNs and NFκB activation. Poly I:C is a synthetic double stranded mimic of viral RNA containing repetitive inosine and cytosine residues. Recently it has become apparent that poly I:C, as well as natural viral RNA sequences, are also detected by intracellular receptors MDA5 and RIG-I [270]. These sensors are not TLRs but are helicases found in the cytosol. They contain two caspase-recruitment domains (CARDs) and a DExD/H-box helicase domain. The helicase domain recognizes the RNA structure, while signaling is mediated through CARD and the adaptor protein adaptor inducing interferon β (CARDIF, also known as IPS-2, MAVS and VISA) [271]. Signaling through MDA or RIG-I results in activation of IRF3, IRF7 and NFκB, similar to TLR3 signaling, and is essential in the production of type 1 IFNs, α and β [252, 256, 272]. MDA5, RIG-I and TLR3 recognize different groups of RNA viruses [273] possibly by recognizing different structures of dsRNA. Each receptor preferentially recognizes different lengths of the synthetic ligand poly I:C; where MDA5 recognizes “long” sequences (>1 kbp) and RIG-I recognizes “short” sequences (<1 kbp) [274]. The restrictions for TLR3 are somewhat more relaxed as it

requires a double stranded sequence of at least 50 bp [275]. The relative contribution of each receptor to immune response is not well understood. It has been shown that activation of IRF3 is complex, requiring multiple kinases for maximal phosphorylation, which suggests that the utilization of several receptors is a means to fine-tune the response to different virus types [276, 277].

The expression and responsiveness of TLR3 in B cells is debatable, for example in one study it was found that although human B cells generally have low TLR3 expression they do respond to poly I:C stimulation [278], but in another study, B cells failed to respond to the same stimuli [251]. This may be partially attributed to B cell subsets present, as one group found that a subset of mucosal B cells found in human tonsil express TLR3 and respond strongly to poly I:C stimulation [279]. In mice, antibody production was not impaired in response to infection with influenza in $TLR3^{-/-}$ or $TRIF^{-/-}$, but was compromised in $MyD88^{-/-}$ mice [280], showing that TLR3 does not influence the humoral response to influenza virus infection. Purified murine B cells were found to have low expression of TLR3 relative to other TLRs [227], but two separate studies have documented that murine B cells respond to poly I:C stimulation by increasing expression of CD86 and MHC class II; one of these studies also found an increase in CD40 [281] although the other did not [227]. Activity of poly I:C was attributed to TLR3 signaling since it was abolished in B cells from $TLR3^{-/-}$ or $TRIF^{-/-}$ mice [281].

Poly I:C has long been recognized as a potent adjuvant for use in vaccines; because TLR3 stimulation directly leads to type I IFN production, thus poly I:C tends to skew immune responses towards Th1 [10, 154]. Poly I:C, or derivatives of it, have been used to enhance subunit vaccines for cellular and humoral responses (personal observations) [282-284]. TLR3 is highly expressed by professional APCs, such as macrophages [285] and dendritic cells [286]. TLR3 is also expressed by a variety of non-hematopoietic cells involved in virus detection, such as dermal fibroblasts [287], vascular epithelial cells [288], and keratinocytes [289, 290]. TLR3 mRNA has been detected in the placenta, pancreas, lung, liver, and kidney [291, 292]. The release of type I IFN by these cells upon TLR3 activation has the potential to activate many more cells in the vicinity, propagating the response to TLR3 to other non-TLR3 expressing cells. Recent

evidence has shown that in some cells, such as epithelial cells and fibroblasts, TLR3 is actually expressed on the surface membrane rather than the endosomal membrane [293]. For this reason, the mode of delivery must be carefully considered to prevent release of systemic poly I:C in order to minimize off-target cell stimulation resulting in systemic inflammation [154]. In an early clinical study in the 1970's, poly I:C was administered systemically to cancer patients to increase type I IFNs, but resulted in toxic manifestations in the majority of the patients, precluding subsequent clinical development of the adjuvant [294]. Systemic application of poly I:C has also been linked to pathogenesis of autoimmune disease and chronic inflammation [295, 296]. Even delivered as an emulsion, poly I:C may enter the circulation since it is part of the aqueous phase so careful consideration must be given to its mode of delivery, in particular for prophylactic vaccines which are typically administered to generally healthy individuals [297]. DepoVax is an ideal delivery platform for poly I:C since it forms a strong depot without an emulsion.

1.2.4.2 Pam3CSK4-TLR1/2 signaling on B cells

Pam3CSK4 (N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine) is a triacylated lipopeptide that mimics the acylated amino terminus of Gram positive bacterial lipopeptides. Pam3CSK4 is recognized by the heterodimer formed by TLR1 and TLR2, and not by TLR2 and TLR6 [102]; TLR2 mediates the same downstream signaling in either heterodimer [298]. It was recently shown that Pam3CSK4 may be recognized by the TLR2/10 homodimer and results in alternate down stream signaling, but TLR10 is not expressed in mice [101]. Pam3CSK4 has been known as a potent activator of B cells since the 1980's [299]. MALP-2, a TLR2/6 ligand, also has been reported to activate both human and murine B cells [249, 250, 300]. A comparison of different TLR2 agonists, including MALP-2, on human B cells indicated that Pam3CSK4 had the most potent effects [301]. Although TLR2 expression is low in naïve cells it was upregulated with TLR2 stimulation, and is highly expressed on memory B cells. Stimulation through TLR2 by Pam3CSK4 seemed to promote germinal centre formation as it increased CXCR5 expression. Another study compared Pam3CSK4 stimulation of human B cells to imiquimod (TLR7 agonist) and

CpG (TLR9 agonist) [252]. Of these, Pam3CSK4 was found to be the most stimulatory, causing the upregulation of surface receptors such as CD80, CD86, MHC class II and CD25, as well as cytokine secretion, IL-1, IL-6, TNF-α, IL-10 and IL-13.

Besides B cells, TLR2 and TLR1 are expressed on dendritic cells, monocytes, neutrophils [286] and mast cells [302]. TLR2 is expressed on human keratinocytes [303] and human airway epithelial cells [304]. TLR2 mRNA can be detected in the ovary, lung, pancreas, trachea, and placenta [291, 292]. TLR1 mRNA can be detected in the lung, kidney, pancreas and placenta [291, 292].

Early vaccination studies performed with antigens mixed with Pam3CSK4 in aqueous formulations demonstrated the potency of Pam3CSK4 as a humoral adjuvant, but it tended to induce a Th2-profile [305]. More recently, Pam3CSK4 incorporation into aggregate type vaccines has shown that it can also induce Th1-profiles. Pam3CSK4 included in a virosome vaccine targeting RSS produced higher Th1 IgG2a antibody titer than non-adjuvanted vaccine coupled with increased antigen-specific IFN-γ response in the spleen [306].

1.2.4.3 Poly I:C & Pam3CSK4 Adjuvant System

Synergy between TLR3 and TLR2 stimulation on DCs has been reported. In 2004 Re & Strominger screened stimulation of each known TLR on human PBMCs and DCs [109]. They found that each TLR resulted in a unique cytokine profile detected by mRNA. Two studies published shortly after investigated the potential synergism between TLR ligands, showing that combined stimulation with purified agonists did synergistically enhance DC function. The first, by Bagchi *et al,* found that mice injected with a combination of poly I:C and Pam3CSK4 had increased plasma levels of IL-6 and TNF-α [307]. *In vitro* studies on purified bone marrow derived macrophages indicated that synergism existed between these two TLR pathways because they used different adaptor proteins. Vanhoutte *et al* demonstrated that the *in vitro* synergistic effect of DC stimulation with Pam3CSK4 and poly I:C resulted in enhanced capacity to activate NK cells and promote T cell proliferation [308]. A 2008 study by Zhu *et al* examined potential synergism between poly I:C and various TLR ligands using IL-12p70 production and CD86 expression as markers of activation [257]. Poly I:C in combination

with Pam3CSK4 did not result in increased IL-12p70 production, but did increase CD86 expression relative to either ligand alone. However, MALP-2 (TLR2/6 agonist) did synergize with poly I:C for IL-12p70 production and this combination was tested for adjuvant ability *in vivo*. MALP-2 and poly I:C increased antigen-specific $CD8^+$ T cells towards an aqueous peptide vaccine or when used to activate DCs for vaccination. Most recently, the combination of poly I:C and Pam3CSK4 was demonstrated to increase activation of DC subsets which were then used for vaccination [309]. *In vitro*, the DCs displayed increased expression of co-stimulatory molecules CD40 and CD80 and increased secretion of IL-12p70 and TNF-α. The DCs stimulated with poly I:C and Pam3CSK4 also demonstrated increased capacity for cross-presentation. *In vivo*, DCs stimulated with poly I:C/ Pam3CSK4 were also effective when used for prophylactic vaccination in a tumour model.

The synergistic activity documented between poly I:C and Pam3CSK4 may be due in part to the complementary intracellular signaling cascades elicited by TLR3 (TRIF mediated) and TLR2 (MyD88 mediated) ligation. Additionally, expression of these receptors may be linked as poly I:C has also been shown to enhance mRNA expression for TLR1/2 in human airway epithelial cells [304].

To date, there has been no evaluation of adjuvant combinations on B cells *in vitro*. Furthermore, the combination of poly I:C and Pam3CSK4 is a potent Th1-adjuvant system, but has only been evaluated for CTL induction and not humoral response. The outlined studies suggest that poly I:C and Pam3CSK4 may modulate immune responses through B cell intrinsic signaling, which might provide an effective adjuvant system for boosting antibody responses to protein vaccines.

1.2.5 Summary and research rationale

Novel vaccine technologies are needed to induce protective immunity towards diseases for which conventional whole-cell vaccines have proved to be inadequate. Understanding the factors that contribute to efficient B cell activation may lead to the discovery of novel approaches to boost antibody responses to subunit vaccines containing B cell epitopes. TLR agonists are often studied as vaccine adjuvants due to their multifaceted interactions with cells of the immune system that bear specific

receptors for given agonists. Certain combinations of TLR agonists have a synergistic effect on dendritic cells and macrophages, indicating that two or more of these molecules may be used together as effective adjuvant systems *in vivo*. B cells express several TLRs and stimulation through these receptors influences B cell activation and function. However, the effect of dual TLR stimulation on B cell activation has not been studied. The TLR agonists poly I:C (TLR3) and Pam3CSK4 (TLR1/2) have been reported to synergistically enhance activation of innate APCs. The combination of these TLR agonists has not been used to adjuvant protein vaccines. Therefore, one goal of this thesis was to determine if poly I:C and Pam3CSK4 could influence B cell activation and function *in vitro*, and if this adjuvant combination could be used *in vivo* to promote antibody responses towards protein-antigen vaccines. The results of this project are presented and discussed in Chapter 3.

1.3 Cellular vaccines for cancer therapy

The idea that a vaccine could be used to treat cancer came with the realization that the immune system plays an essential role in detection and control of aberrantly dividing cells [310, 311]. Unlike current standard treatments, such as surgery or chemotherapy, development of a cancer vaccine offers the possibility of providing life-long protection from tumour formation [312]. However, as a cell progresses from abnormally dividing to cancerous, it develops mechanisms to not only avoid but actively escape immune system detection [313]. The complex interplay between tumour and immune system is an important and not fully understood element that impacts the efficacy of cancer vaccines.

It is generally believed that Th1-biased immune response predominated by $CD8^+$ cytotoxic T cells (CTLs) must be induced by an effective cancer vaccine [74]. CTLs programmed to identify tumour cells will initiate controlled killing of target cells. Several strategies to induce effector CTLs by vaccination have been tested both pre-clinically and in clinical trials [314, 315]. Besides subunit vaccines, other vaccine platforms such as DCs, viral vectors and whole-tumour vaccines have been developed with varying degrees of success. The only vaccine currently approved for use in treating cancer is a dendritic-cell based vaccine called Sipuleucel-T® (trade name Provenge) developed by Dendreon Corporation for the treatment of prostate cancer [316]. To

prepare this vaccine, which is done individually for each patient, peripheral blood mononuclear cells (PBMCs) are withdrawn from a patient by leukapheresis and shipped to a manufacturing facility. Dendritic cells are matured from patient derived PBMCs in the presence of prostatic acid phosphatase, a tumour associated protein present on most prostate cancer cells, and growth factor granulocyte-macrophage colony stimulating factor (GM-CSF) [316]. Three days later, the activated product is infused back into the patient. Based on three phase III trials in which it was demonstrated to improve overall survival on average by 4 months, Provenge was approved by the FDA for treatment of advanced prostate cancer [317-319]. However, this technique is far from perfect. Due to the expense of producing a vaccine on a per-patient basis, the cost for Provenge treatment is over $90,000 [320]. Administration of this product also requires careful planning to ensure timely administration after leukapheresis for each treatment. Furthermore, while Provenge has been effective at delaying prostate cancer, it has not been shown to cure disease. Despite these drawbacks, the approval of Provenge was a milestone for the field of cancer vaccine research as it demonstrated for the first time that the human immune system can be trained by a vaccine to provide clinical benefit to cancer patients in a setting where no other alternatives are available [321].

While each type of cancer vaccine has pros and cons to its use, this thesis is concerned with developing subunit vaccines. Subunit cancer vaccines bring the same advantages to cancer therapy as they do to vaccines in general, that is excellent safety profile, flexibility, low cost and scale-up ready manufacturing [150]. Subunit vaccines for cancer typically consist of one to several CTL epitopes as well as a T-helper peptide and adjuvants. Inclusion of adjuvants capable of inducing a Th1-type immune response is important to cancer vaccines [94, 130]. Despite many developments over the last 20 years, an effective subunit vaccine remains elusive in cancer immunology. The challenge of developing an effective therapeutic cancer vaccine is due to the presence of a tumour which has the ability to alter the immune system. “Active, but dysfunctional” is an apt assessment of the immune system in cancer patients [322]. Novel strategies of combining cancer vaccines with immune modulatory agents are now an active area of study for cancer therapy, and the focus of the second project in this thesis.

1.3.1 $CD8^+$ T cell biology

1.3.1.1 $CD8^+$ T cell development

$CD4^+$ and $CD8^+$ T cells develop from hematopoietic stem cells in the bone marrow. T cell precursors migrate from the bone marrow to the thymus to undergo T cell maturation [323]. During this process, thymocytes transition through several stages of development to generate functional and unique TCRs and expression of CD4 or CD8 co-receptor glycoproteins. T cell precursors start off as $CD4^-CD8^-$ double negative cells. During this stage, they undergo genetic rearrangement of the α and β chains of the TCR which is mediated by RAG-1, RAG-2 and TdT proteins, and then become double positive $CD4^+CD8^+$ cells [324]. The variable regions of the TCR are encoded by V(D)J gene segments, like the mIg in BCR. The complete TCR complex is then put through positive selection to ensure that it is capable of interacting with either MHC class I or II, followed by negative selection to remove TCR that are potentially self-reactive. During this process the T cells also commit to a lineage of $CD4^+$ or $CD8^+$ [325]. Phenotypically mature T cells emerge from the thymus as single positive $CD4^+$ or $CD8^+$ T cells bearing a unique TCR receptor in association with the CD3 signaling complex.

1.3.1.2 Cytotoxic T lymphocyte activation

$CD8^+$ T cells become activated in the T-cell zone of lymph nodes by activated DC presenting cognate antigen MHC class I [130]. Endogenous peptides are generated in the cytosol of DCs by proteasome degradation and are directly loaded into MHC class I. Alternatively, exogenous peptides can be presented through a process known as cross-presentation. Cross-presentation pathways are induced by particulate antigens and can be enhanced through TLR stimulation [21, 326]. In addition to antigen presentation, naïve $CD8^+$ T cells require costimulation to become activated; these two signals can only be provided by professional APCs. CD28 is the prototypical costimulatory receptor on murine and human T cells, although in humans there are several other family members that are important [327]. CD28 is stimulated by CD80 or CD86 expressed on the surface of activated APCs and provides synergism to TCR signaling. CTL cell homing to sites of inflammation can be programmed through upregulation of adhesion proteins and chemokine receptors depending on the lymph node where they were activated. For

example, in mice, activation of $CD8^+$ T cells in skin draining lymph nodes causes increased expression of E-selectin ligand and CCR4 to home CTL to the skin, whereas activation in mesenteric lymph nodes increases expression of integrin $\alpha4\beta7$ and CCR9 to home to the gut [328, 329].

Maximal activation of $CD8^+$ T cells takes 1-3 days in the lymph node, and peak activity can be detected *ex vivo* 7-10 days after vaccination [330, 331]. Unlike B cells, T cells do not undergo affinity maturation, but optimization of CTL immunity is mediated through selective expansion of T cells bearing high affinity TCR [332]. Once $CD8^+$ T cells have been activated they become licensed CTLs with the capacity to kill any cell bearing cognate antigen within MHC class I without the need for co-stimulation [130, 330]. Activated $CD4^+$ Th type 1 cells assist CTL differentiation function by providing cytokines to prolong effector function (IFN-γ and IL-12) and ensure generation of memory T cells [45].

T cell activation through TCR and costimulation ultimately results in activation of transcription factors NFκB, AP-1 and nuclear factor of activated T cells (NFAT) [327]. These transcribe cytokine and surface receptor genes essential to T cell activation as well as promoting T cell proliferation and survival. The cytokine IL-2 and the high affinity α subunit of the IL-2 receptor (CD25) are upregulated to provide autocrine survival signals [333]. Th1 cytokines IL-12 and IFN-γ are produced by DCs, $CD4^+$ or $CD8^+$ T cells and mediate a variety of functions in tumour control. IL-12 can induce NK cell activation [334], promote IL-2 signaling in CTLs [333], reduce suppressor cell activity [335], and promote the collapse of tumour stroma to enhance $CD8^+$ T cell infiltration [336]. IFN-γ can increase immunogenicity of tumours by upregulating MHC class I expression [337, 338] and reducing tumour angiogenesis [339]. $CD8^+$ T cells also express tumour TNF-α which mediates several functions essential to CTL activity such as cell-contact independent killing [340]. IFN-γ and TNF-α are both required to activate macrophages [341] and can directly induce tumour growth arrest [342].

CTL activation is counterbalanced by the differential expression of various co-inhibitory receptors throughout their development. There are a variety of co-inhibitory receptors that can be expressed in response to changes in the microenvironment which act to tightly control the quality and quantity of the CTL

response [327, 343]. Co-inhibitory receptors not only protect the host from autoimmune responses, but also limit collateral damage that results from chronic immune stimulation [343]. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is the classical co-inhibitory receptor; it competes with CD28 to bind to CD80 and CD86 and send inhibitory signals and also physically removes the CD80/ CD86 receptors from the surface of the APC [344]. CTLA-4 expression normally accumulates as effector T cells carry out their function to gradually dampen the immune response. Programmed death receptor 1 (PD-1) is another co-inhibitory receptor that is gaining much interest due to its role in tumour-induced immune suppression. PD-1 binds to two ligands, PD-L1 and PD-L2, which are differentially expressed by a variety of hematopoietic and non-hematopoietic cells to provide negative regulation to TCR signaling to self-antigens [345]. Both CTLA-4 and PD-1 ligands are known to be upregulated in tumour microenvironments to suppress CTL activity [346, 347]. Blockade of either or both of these signaling pathways using targeted antibodies has been shown to increase anti-tumour immune responses [348, 349].

CTLs are also subject to activation induced cell death (AICD), which is a self-destruct mechanism responsible for contracting the T cell population as acute phase ends [350]. Fas is an important receptor induced on activated $CD8^+$ T cells that can mediate AICD [351]. Fas expression increases as CTL mature in response to IL-2 signaling [350]. Fas activation is triggered by binding to the Fas ligand (FasL) which is a surface receptor expressed by other CTLs and self-cells. Fas is a member of the TNF receptor superfamily and induces apoptosis through intracellular signaling events involving Fas-associated death domain (FADD) protein and caspase 8 [352]. Fas induction is antigen-specific as it also requires cross-linking of TCR receptors.

1.3.1.3 CTL mediated killing

CTLs mediate cell-contact dependent killing through perforin/ granzyme-mediated or Fas-mediated pathways [330]. Both of these pathways ultimately result in apoptosis by activation of caspases in the target cell [353]. After recognition of a target cell presenting cognate antigen in MHC class I, the perforin/ granzyme pathway is initiated by formation of an immunological synapse between the CTL and target cell

[354]. Perforin released into the synapse polymerizes in the target cell membrane to form pores, thus allowing the transfer of cytotoxic granules containing granzymes from the CTL [355]. Although there are several known granzymes, granzyme B is the most abundant and most efficient of the granzymes [355]. Granzyme B can mediate cell death by caspase activation and also through loss of mitochondrial membrane potential. Caspase-3 is activated by granzyme B by proteolysis and orchestrates a variety of processes to systematically dismantle the cell [353]. Granzyme B initiates mitochondrial permeabilization by proteolysis of the pro-apoptotic protein BID to allow it to insert into the mitochondrial membrane. This triggers oligomerization of other pro-apoptotic enzymes BAX and BAK within the membrane which facilitates release of cytochrome *c* into the cytosol. Cytosolic cytochrome *c* assembles an apoptosome which then activates other members of the caspase family [355].

The second pathway of CTL mediated cell-contact dependent killing is through Fas expressed by target cell interaction with FasL expressed on CTL. CTLs upregulate expression of Fas and FasL upon activation which is an inherent mechanism to self- limit their proliferation. Although efficient, this mechanism is more important in the maintenance of T cell homeostasis, particularly for controlling response to self-antigens, than it is for target cell killing [351]. Fas-FasL killing pathway is not as rapid as the perforin/ granzyme pathway; recycling of FasL expression takes several hours whereas generation of new perforin/ granzyme granules takes only minutes [330].

Apoptotic cell death induced by CTLs is non-immunologic, that is it does not initiate inflammation. The end result of apoptosis is the packaging of cellular components into small apoptotic bodies which are quietly cleared by professional phagocytic cells [353]. In contrast to apoptosis, necrosis is immunologic cell death that is caused by cellular toxicity, stress or damage. After a cell dies by necrosis, its internal contents are released and trigger inflammation through PRRs, these contents are DAMPs [92, 353]. Necrosis may be caused by alum vaccination, cytotoxic chemotherapy or radiation therapy. While necrosis should be avoided due to its non-specific and variable nature, it is sometimes helpful to induce anti-tumour immune responses by simultaneously causing release of DAMPs and tumour antigens [356].

To kill tumour cells, CTLs must be able to differentiate between normal and cancer cells by recognizing peptides presented in MHC class I. Tumor associated antigens (TAA) are peptide-antigens derived from proteins expressed in tumour cells but not normal cells. There are several families of TAAs: growth promoting proteins (e.g. survivin, HER-2/*neu*), mutated genes (e.g. β-catenin, k-ras), cancer-germline (e.g. MAGE, NY-ESO1), tissue-specific differentiation antigens (e.g. tyrosinase, MART-1), proteins with abnormal posttranslational modification (e.g. MUC-1), or viral antigens (e.g. EBV, HPV) [313, 357]. Although technically many these are self antigens, they are not exposed to T cells during thymic selection process due to their limited expression in normal cells. These include mutated antigens, antigens involved in cell proliferation and antigens involved in fetal development [358]. Detection of spontaneously occurring antigen-specific CTLs in cancer patients demonstrates that given adequate stimulation, tumour-reactive CTLs can be generated and participate in anti-tumour immune response [359, 360].

1.3.2 Tumor initiation and immune evasion

In a paper published in 2000, Hanahan and Weinberg proposed 6 hallmarks of tumour cells that enable the progression from a normal to neoplastic state, these are 1) resistance to cell death; 2) sustaining proliferative signaling; 3) evasion of growth suppressors; 4) invasion and metastasis; 5) immortalization and 6) neo-angiogenesis [361]. The list was expanded in 2011 to include four new hallmarks: 7) dysregulated metabolism; 8) genome instability and mutation; 9) avoiding immune destruction; and 10) tumour promoting inflammation [362]. The updated list reflects a new appreciation for the role of the immune system in not only mediating tumour destruction, but also for the role it plays in tumour development.

Tumors typically arise from a few cells that acquire a particular set of mutations that allows them to grow unregulated. These cells have been recently coined “cancer stem cells” (CSCs) since they are elusive to immune detection, and can be the parent cells for tumour expansion [363]. The term “stem cell” is a bit of a misnomer, however, since it can imply that tumours arise from a set of predefined undifferentiated cells, akin to hematopoietic stem cells. However, the nature of the CSC is very different than other

stem cells. As tumours evolve and adapt resistance to immune detection and chemotherapies, new CSCs can develop through negative selection [364].

CTLs play a critical role in prevention of tumour growth. This is evident from increased incidence of tumours in mice deficient for β2 microglobulin, perforin or IFN-γ [365, 366]. Furthermore, in humans, adoptive transfer of highly-activated T cells specific to TAA can cause tumour regression [367]. Ironically, it is immune pressure that sculpts the tumour during development, by negatively selecting those cells with the right set of mutations to escape immune detection. To combat CTL recognition, tumour cells downregulate, or completely eliminate, MHC class I expression [368, 369]. Many tumours have defects in antigen processing pathway, such as TAP deficiencies or immunoproteasome subunits LMP2 and LMP7, thereby preventing presentation of antigens on MHC [370, 371]. Even if tumours express an antigen that is recognized by the immune system, they can adapt to lose expression of that protein under immune pressure [372, 373]. Furthermore, tumours can become resistant to some forms of CTL killing, such as by developing mutations in IFN-γ receptor pathway [374] or TNF-TRAIL induced apoptosis [375].

1.3.3 Tumor induced immune suppression

1.3.3.1 Tumor induced cytokines

Besides evading the immune system, tumours can actively suppress immune responses by tapping into natural mechanisms of immune suppression. The tumour microenvironment contains a network of cytokines that not only support the growth and invasiveness of the tumour, but also promote development of immune suppressor cells [322]. The pleiotropic nature and redundancy of these cytokines has made it difficult to delineate their precise contributions to tumour growth. Transforming growth factor β (TGF-β), TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-18 are some of the most common cytokines found in the microenvironment of different cancer types [322, 376, 377]. These cytokines are all pleiotropic in effect and generally are involved in promoting inflammation. In the context of the tumour microenvironment, these cytokines contribute to the development, growth and spread of tumours. For example, TGF-β inhibits immune responses by suppressing IL-2 production and signaling, inducing tolerogenic DC

through expression of indoleamine 2,3-dioxygenase (IDO), inhibiting macrophage and NK function, and inhibiting T cell differentiation into Th1 or Th2, yet supporting iTreg and Th17 [378]. IL-1β cytokine is known as a potent driver of inflammation. Signaling through the IL-1 receptor (IL-1R) induces a cascade of pro-inflammatory genes as well as more IL-1 to propagate the response. IL-1R induces cyclooxygenase type 2 (COX-2), inducible nitric oxide synthase (iNOS), other inflammatory cytokines and chemokines and matrix metalloproteinases (MMPs) [379]. IL-10 is an important homeostatic immune regulatory cytokine that is involved in contracting immune responses. IL-10 downregulates the expression of Th1 cytokines (IL-2, TNF-α, IFN-γ), MHC class II and co-stimulatory molecules [380]. IL-10 can also suppress activated T cells by inducing PD-L1 expression on macrophages [381].

1.3.3.2 Tumor induced immune suppressor cells

The inflammatory environment created by tumour induced cytokines not only promotes tumour growth and metastatic potential, but also induces development of immune suppressor cells [382, 383]. Elevated levels of $CD4^+CD25^{hi}FoxP3^+$ Tregs are frequently detected in tumours and peripheral blood of cancer patients and animal models [80, 372]. Increased Treg frequency is often a negative prognostic marker in cancer [384, 385]. Tregs have a critical role in immune homeostasis by curtailing overactive immune responses and preventing autoimmunity [47, 79]. The origin of Tregs in cancer remains controversial. Specifically, there is debate on whether tumour-associated Tregs are natural Tregs recruited to the tumour, or whether the tumour microenvironment promotes differentiation of inducible Tregs [386]. In any case, Tregs accumulate in response to tumour growth and exert suppressive function through secretion of IL-10 and TGF-β. The high expression of the high-affinity IL-2 receptor α chain (CD25) also allows them to remove excess IL-2, which is essential for proliferation of activated T cells [387].

Myeloid derived suppressor cells (MDSCs) are a heterogeneous group of early myeloid progenitors identified in mice as granulocytic-MDSCs ($CD11b^+Ly6G^+Ly6C^{lo}$) or monocytic-MDSCs ($CD11b^+Ly6G^-Ly6C^{hi}$) [388]. These two populations have different morphologies which is a reflection of their origin, but both are immune suppressive. The Gr-1 antibody, which can detect both Ly6G and Ly6C, is often used as

a marker with CD11b in mice [389]. MDSCs may also share phenotypic markers with granulocyte, DCs and neutrophils, reflecting their hematopoietic origin. Identification of MDSCs by surface marker expression in humans is somewhat more challenging, being characterized as $CD16^{low}CD11b^{+}HLA\text{-}DR^{-}CD15^{+}CD33^{+}$ [390]. The markers used for mice and humans are not specific to MDSC population, so absolute identification also requires functional assay to test the suppressive ability of the MDSC population [388]. MDSCs are frequently induced in many cancer types, but the levels and phenotype can differ depending on the cancer type or model being studied [391, 392]. MDSCs are released prematurely from the bone marrow in response to IL-1β induced inflammation [393, 394]. They accumulate in the inflammatory environment of the tumour where they can exert several suppressive activities [395]. Tumor derived COX-2 can trigger overexpression of arginase I in MDSCs which depletes arginine necessary for T cell activity [396]. MDSCs can suppress antigen-specific CTLs by nitrating the TCR receptor to prevent interaction with MHC [397, 398]. MDSC may also induce T cell tolerance through release of IDO, which depletes tryptophan, an essential amino acid to T cell proliferation [399].

Tumor derived TGF-β and prostaglandin E2 (PGE2) can also promote the development of tolerogenic DCs [400]. Tolerogenic DCs have downregulated receptors required for antigen presentation, such as MHC and CD40, but upregulated co-inhibitory molecules such as PD-L1 and PD-L2 [401]. Like MDSC, tolerogenic DCs also express IDO and arginase I. However, infiltration of tumours by DCs in general is limited and the majority of DCs within the tumour are rendered inactive.

Other suppressive cell types such as M2 macrophages [402], B10 cells [403] and suppressive $CD8^{+}$ T cells [404] have also been identified in tumour models and/ or clinical samples. The common theme is that the immunosuppressive and inflammatory environment created by the tumour alters the immune response, converting immune effectors into immune suppressors.

1.3.4 Strategies to improve cancer vaccines with immune modulation

Cancer vaccines attempt to rekindle effective tumour control by induction of tumour-specific CTLs; however, to be effective they must be able to overcome tumour

immune evasion and suppressor mechanisms [130, 314]. Due to immune-avoidance mechanisms, cancers are able to grow silently without triggering an immune response. Cancer vaccines alone have little direct effect on the tumour to increase tumour immunogenicity or reverse tumour-induced suppression. Certainly, careful consideration must be given to the selection of antigen. Tumors have a remarkable ability to escape immune recognition by shedding recognized antigens [405]. However, not all antigens are easily replaced and their loss compromises the tumours' ability to thrive [406]. For example, the TAAs survivin and telomerase reverse transcriptase (hTERT) are both highly overexpressed in a variety of cancers [407, 408]. Therefore, from an expression standpoint, these are excellent TAAs based on their high frequency of overexpression in tumour cells compared to normal cells. Additionally, both proteins are involved in maintaining immortality of the tumour; survivin is an inhibitor of apoptosis and hTERT prevents shortening of telomeres during replication. Therefore, loss of these antigens would compromise tumour pro-survival pathways and render it susceptible to death by necrosis or apoptosis. Another strategy to improve generation of high-affinity T cells is to use peptide mimotopes; these are peptides derived from TAAs and modified to improve binding to HLA and recognition by TCR [409, 410]. To increase binding to HLA, peptides can be altered at their anchor residues to an amino acid preferred for binding in the HLA-peptide binding groove [149, 411]. Finally, the use of adjuvants that promote Th1-biased immune responses aid in providing the maximal immunogenicity to peptide vaccines [130].

Another way to improve the efficacy of a cancer vaccine is to combine it with an agent that can directly counteract tumour immune evasion and suppression. There are three main classes of agents that can modulate immune responses to benefit cancer vaccines: 1) cytokines, 2) monoclonal antibodies and 3) low dose chemotherapy. Co-administration of Th1-type cytokines, such as IL-2 and IL-12, with vaccination has been used as a strategy to boost responses towards the vaccine. Unfortunately, administration of cytokines can be associated with systemic toxicity and the effect may be hampered by short half life *in vivo* [412, 413]. Cytokine use is typically relegated to live cell vaccines, such as modified tumour cells or DCs, which can be programmed to secrete desired cytokines [414, 415]. Monoclonal antibodies and low dose

chemotherapies are perhaps the best options for combination with subunit cancer vaccines [416].

There are several mechanisms through which monoclonal antibodies (mAbs) can contribute to an anti-tumour immune response [417]. First, some mAbs target tumour cells directly to induce CDC or ADCC. For example, rituximab is used to deplete malignant B cells in chronic lymphocytic leukemia. Rituximab binds to CD20, which is intensely expressed on malignant B cells relative to normal B cells, and results in their elimination through CDC and ADCC [418]. Monoclonal antibodies can also be used to block receptors on tumour to prevent survival signaling. For example, trastuzumab binds to HER2/ *neu*, which is a growth factor over-expressed in some cancers. Not only does it prevent binding of the growth receptor ligand, but it can also trigger endocytic destruction of the receptor [419]. Alternatively, mAb can bind to soluble factors to prevent signal transmission, for example bevacizumab binds and neutralizes vascular-endothelial growth factor A (VEGF-A) to prevent signaling through VEGF receptor, thereby reducing angiogenesis [420, 421]. Finally, mAb are also being used as immune modulators by binding to targets on immune cells to reverse suppression and promote immune response. Ipilimumab (anti-CTLA-4) was recently approved for used in advanced metastatic melanoma [422]. Ipilimumab targets CLTA-4 on T cells to prevent co-inhibition. Blockade of PD-1 has been shown to work in a similar way and two mAb targeting PD-1 on T cells have shown promise in clinical studies [423], lambrolizumab [424] and nivolumab [425]. Immune modulatory mAb are also being designed to reverse tumour induced immune suppression by targeting suppressor cells [426]; for example, anti-GITR mAb targets Treg cells and impairs their ability to accumulate within tumours [427]. Immune modulatory mAb may also be used in combination with vaccine therapy, where the development of an active immune response is enabled by preventing tumour-induced immune suppression.

Development and production of mAb for human therapeutics has advanced considerably over the past 20 years. Initially, mAb were developed by grafting antigen-recognition domains from antigen-specific murine antibodies onto human Fc domains [428]. Depending on the production technique, these were called chimeric or humanized mAb. Many of these types of mAb were approved for human use, and are still being

used today, for example rituximab (chimera) and trastuzumab (humanized). However, they are limited by a complex discovery and production process, and also retain immunogenicity due to their murine content [428]. In the 1990's, new molecular biology techniques were pioneered that not only allowed high throughput screening of antibodies, but also the production of fully human mAb was made possible by creating a mouse that only expressed human IgG. These fully human mAb have little immunogenicity and are relatively easier to produce.

However, mAb, even if fully human, still have limitations on their applicability. Despite advances, discovery and pre-clinical development of mAb takes many years. Transition from pre-clinical research to clinical testing also presents its own unique challenges. The only relevant species for pre-clinical development are non-human primates, which can be very costly and not always predictive [429]. Although similar, there are irreconcilable differences between human and non-human primate immune systems that can obscure potentially dangerous complications of interfering with the immune response in humans. An unfortunate example of this is the clinical trial for TGN1412, a humanized IgG4 mAb targeting CD28 which was designed to promote proliferation of activated T cells [430]. In a small phase I trial, all 6 patients injected with a small dose (1/500 of a dose tested safely in non-human primates) rapidly developed multi-organ failure resulting in hospitalization as a result of cytokine storm. Most mAb cause some degree of immune-related adverse events that can have a range of severity, but do not prevent their use [431]. For example ipilimumab (fully human IgG1) is associated with adverse events affecting the gastrointestinal tract (diarrhea or colitis) or skin (rash, pruritus) [422], and nivolumab (fully human IgG4) causes pneumonitis, vitiligo, colitis, hepatitis, hypophysitis, and thyroiditis [432].

Direct mAb toxicity towards solid tumours is also limited by their inability to efficiently enter the tumour microenvironment. Only ~20% of all antibodies injected will interact with the tumour, and of this very little actually penetrates the tumour [433]. Steric hindrance, disorganized vasculature structure and high interstitial pressure within the tumour also oppose antibody diffusion [433].

Use of low dose chemotherapy for immune modulation may be a more suitable option than mAb. Many chemotherapeutic drugs, already approved for use in humans at

high cytotoxic doses, have been demonstrated to have immune-modulatory effects when used at low, non-toxic doses. There are multiple immune modulatory mechanisms attributed to chemotherapies. They have been known to selectively reduce suppressor cells such as Tregs (cyclophosphamide [434]) , MDSCs (gemcitabine [435]) and tumour associated macrophages (doxorubicin [436]), increase activity and proliferation of T cells (cyclophosphamide, docetaxel [48]), increase DC activity (cyclophosphamide [437]), cause immunologic cell death (docetaxel [48], oxaliplatin [438]), increase NK activity (decitabine [439]), and increase tumour immunogenicity (carboplatin [440], taxol [441]). Low dose chemotherapy probably targets the immune system because hematopoietic cells are rapidly dividing, but how chemotherapeutic drugs target specific cells of the immune system is not understood. Cyclophosphamide (CPA) was the first chemotherapy to demonstrate immune-modulatory activity when used at low doses and has since been extensively studied in both mice and humans [442]. CPA is already the one of the most common types of chemotherapy used to treat wide variety of tumours. Although CPA immune modulation was initially attributed to its selective depletion of suppressor cells [434, 443, 444], it has since been shown to have a plethora of immune modulatory activities which make it particularly well suited for combination therapy with cancer vaccines [445].

1.3.5 Low dose cyclophosphamide: administration and pharmacokinetics

Cyclophosphamide (N,N-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide) is a nitrogen mustard alkylating agent traditionally used to impart cytotoxic effects on tumours as a chemotherapy drug for cancer patients. It is also known and referred to under the trade names Endoxan®, Cytoxan®, Neosar®, Procytox® and Revimmune®.

CPA is a pro drug which is typically administered via intravenous infusion, but also can be administered orally as a pill [446] with little difference in bioavailability [447]. CPA is converted to its active metabolites, 4-hydroxy-CPA and aldophosphamide, by oxidation by P450 enzymes in the liver [152, 448]. The active metabolites of CPA are lipid soluble and enter cells through passive diffusion. Intracellular 4-OH-CPA spontaneously decomposes into phosphoramide mustard which is the ultimate active

metabolite. Phosphoramide mustard catalyzes intra- and interstrand DNA cross-links as well as DNA-protein cross-links that inhibit DNA replication leading to cell death [446]. Phosphoramide mustard is eliminated by enzymatic conversion to carboxyphoshphamide by cytoplasmic aldehyde dehydrogenase (ALDH) [152]. Cells with low levels of ALDH tend to accumulate CPA metabolites and are more sensitive to its effects, and indeed tumour upregulation of ALDH is one mechanism of CPA resistance [449]. Besides ALDH, low intracellular ATP levels have also been associated with CPA sensitivity of particular cells types [450]. At high doses, typically in the range of 1-5 g/m^2, the effects of CPA are most cytotoxic to rapidly dividing cells regardless of cell type, and CPA is myelosuppressive since most hematopoietic cells are rapidly dividing [451, 452].

Total systemic clearance of CPA and its metabolites in humans varies between 5-9 hours, and peak plasma levels of the parent drug also vary considerably between patients (3-11 hours) reflecting individual genetic differences in metabolism [453, 454]. Repeated administration of CPA is reported to shorten elimination half-life by increasing activity of enzymes involved in metabolism [455], but whether this leads to increased metabolism of the active metabolite is still controversial [446], particularly at low doses [448].

In the last two decades low dose CPA has been appreciated for its immune modulatory and anti-angiogenic effects. In contrast to high dose CPA, low doses of CPA, typically 100-300 mg/m^2, lack widespread cytotoxic activity but do appear to enhance immune-mediated tumour elimination by selectively modulating cells of the immune system and also by reducing angiogenesis within the tumour microenvironment [442]. Alone, low dose CPA therapy delays tumour growth in animal models, but is ineffective at complete tumour eradication. Pioneering studies by Robert North in the 1980's [443, 444] were the first to indicate that low dose CPA selectively depletes immune suppressor cells and could be used to augment tumour immunotherapy. Using mice with established Meth A fibrosarcoma, North *et al* administered sbCPA (100 mg/kg) 1 hour before intravenous infusion with spleen cells isolated from Meth A-tumour vaccinated mice. They demonstrated complete regression of the tumours and speculated that sbCPA treatment temporarily depleted "suppressor" cells induced by the tumour. Since then, low dose CPA has also been reported to selectively reduce and

impair functionality of $CD4^+CD25^{hi}FoxP3^+$ regulatory T cells [434], inhibit tumour angiogenesis [456], increase activation of DCs [457], skew immune response towards Th1 [458] and restore T and NK effector function [459]. In mice, the effects of a single bolus low dose administration of CPA are transient, typically reaching nadir within 4 days after administration and returning to normal by 7-10 days [434, 460].

Dose translation from human to murine studies is calculated using the following equation:

$$\frac{\text{Human dose (mg/kg)}}{\text{Animal dose (mg/kg)}} = \frac{\text{Animal } Km}{\text{Human } Km}$$

The constant mouse *Km* value is 3 and human *Km* value is 37 [461]. Using this calculation, a treatment of 50 mg twice a day in humans is equivalent to 20.56 mg/kg in mouse. The dose of 20 mg/kg PO has been evaluated in pre-clinical models and determined to be biologically equivalent to the 50 mg human dose [462, 463].

1.3.5.1 Single bolus cyclophosphamide in combination with cancer vaccines

Initial studies combining low dose CPA with vaccination administered CPA as a single bolus injection before vaccination; in this document, sbCPA refers to this method of administration. Treatment with sbCPA has been combined with various types of cancer vaccines in pre-clinical models and found to increase their efficacy and immunogenicity as measured by increased antigen-specific $CD8^+$ T cells [464, 465]. However, the enhancement of vaccine immunogenicity after sbCPA is very sensitive to timing in mouse models. If vaccine is administered too early after sbCPA (i.e. within 6 hours) it can result in increased systemic toxicity [464], yet delivered too late (i.e. sbCPA 7 days after vaccination) results in abrogation of synergistic effects [466]. The therapeutic window appears to be vaccination 1 to 3 days after sbCPA administration [467]. This timing has been applied to human studies, but no clinical study has directly evaluated different time intervals between sbCPA and vaccination in humans.

Several small phase I clinical studies were conducted to evaluate the optimal dose of sbCPA and timing relative to vaccination. In general, it was concluded that doses less than 300 mg/m^2 administered 1-3 days before vaccination provided significant reduction in Tregs without abrogating immune responses to vaccination. However, not all studies

have reported consistent results. Treatment with sbCPA has failed to consistently reduce circulating Tregs [468-471]. Although two studies have reported that there is a tendency for sbCPA treated patients to have better overall survival, they have not been correlated with increased antigen-specific immune responses to vaccine [469, 472]. Some of these differences may be attributed to differences in sbCPA administration, vaccine type, small patient enrollment (<20) or advanced disease stage.

Two large phase II studies evaluating sbCPA in combination with vaccine have been reported in the last two years. In the first study, sbCPA (300 mg/m^2) was combined with a peptide vaccine (containing 10 HLA-A2 restricted peptide antigens and 1 HLA-DR T-helper peptide antigen) delivered in an aqueous buffer [472]. A total of 68 renal cell carcinoma patients were randomized 1:1 to receive vaccine alone or in combination with sbCPA. Patients were treated with 17 immunizations over 9 months; the sbCPA arm received a single treatment 3 days before the first vaccination only. In this study, circulating Tregs ($CD45^+CD3^+CD4^+CD8^-FOXP3^+CD25^{hi}CD127^{low}$) were significantly reduced at 3 days post sbCPA administration. The Treg population also had reduced expression of Ki-67, a surface marker associated with proliferating cells. They did not detect any differences in antigen-specific T cells as detected by multimer flow cytometry between the two arms, nor was there a significant difference in overall survival. However, retrospective analysis indicated that patients who developed strong immune responses to vaccine and were treated with sbCPA tended to have better overall survival.

The second study evaluated sbCPA (300 mg/m^2) in combination with a peptide vaccine (containing 4 peptide antigens modified to increase binding to HLA-A2) emulsified in oil [473]. A total of 43 patients with stage II/ III melanoma were randomized 1:1 into an untreated control arm or to receive sbCPA in combination with vaccine. Patients received 6 vaccinations over a 16 week period with sbCPA given one week before the first vaccination, and again on weeks 7 and 11. In addition, patients received IL-2 on weeks 13 and 15. In this study, a lymph node biopsy was taken from all patients after the first two vaccinations. Patients in the treated group had fewer Tregs ($CD4^+CD25^{hi}FoxP3^+$) in the lymph node corresponding to lower inflammatory cytokines such as IL-10, IL-6 and VEGF. Analysis of PBMC populations throughout the study indicated a slight and transient decrease in Tregs. After the IL-2 treatment, the number of

Tregs spiked for one week. However, this increase in Tregs was followed by an increase in Th1-type $CD4^+$ T cells, indicating that perhaps the "Treg" population amplified by IL-2 was merely a transient differentiation stage of $CD4^+$ T cell development.

Due to the inconsistencies observed with sbCPA treatment in combination with vaccine in clinical trials, it has been suggested that providing low continuous dose (i.e. metronomic) would result in more effective and prolonged immune modulation [442].

1.3.5.2 Metronomic cyclophosphamide in combination with vaccines

Metronomic CPA (mCPA) refers to the repeated administration of very low doses of CPA. This can be done with intravenous or intraperitoneal injection, and also orally. In mice, CPA can be added to drinking water and in humans given as a pill. Oral administration is an advantage to mCPA treatment over sbCPA in terms of convenience for both animal studies and human clinical trials. Metronomic CPA has been shown to exert many of the same effects on the immune system as sbCPA, and may also provide additional benefits. Early pre-clinical studies of mCPA demonstrated that administration of 170 mg/kg every 6 days resulted in reduced tumour angiogenesis, resulting in retarded tumour growth [456]. These results could be recapitulated in mice with even less stringent regimen consisting of 20 mg/kg/day provided in drinking water [448, 463].

Recently, mCPA has been evaluated with cancer vaccines in a variety of pre-clinical models. Peng *et al* compared sbCPA to mCPA using TC-1 (expressing human papilloma virus 16 E7 oncoprotein; HPV16 E7) transplanted tumours [474]. Mice bearing 8 day old tumours were treated with mCPA (10 mg/kg/day for 14 days continuously) or sbCPA (50 mg/kg on days 8, 15 and 22). Mice were vaccinated every week starting on day 9 with a DNA vaccine encoding the HPV16 E7 protein. They found both sbCPA and mCPA combined with vaccine to provide increased tumour protection, and this was correlated with increases in antigen-specific $CD8^+$ T cells. They also detected increases in splenic MDSC, but only for sbCPA treatment. Mice treated with sbCPA and vaccine had same MDSC levels as untreated mice.

Treatment with mCPA has also provided enhancement to two other types of DNA-based cancer vaccines in pre-clinical models. Hermans *et al* examined the efficacy

of low dose CPA treatment in tumour bearing mice that had been prophylactically immunized with a DNA/ MVA prime/boost strategy [475]. Briefly, mice were immunized with plasmid DNA encoding the tumour antigen mel3, then boosted with MVA encoding the same antigen 14 days later. Seven days after the MVA boost, mice were challenged with B16-F10 tumours and then treated every 6 days with low dose CPA (175 mg/kg, IP). They found that mice previously immunized and then treated with low dose CPA had a significant delay in tumour growth, but either treatment alone had no effect. They did not detect an increase in number of antigen-specific $CD8^+$ T cells, but they did find that mCPA treatment did not reduce the number of memory $CD8^+CD43^+$ T cells. A study by Barbon *et al* in non-tumour bearing mice demonstrated that 3-5 daily injections of low dose CPA (20 mg/kg/day IP) given prior to a DNA vaccine encoding the CYP1B1 antigen provided better immune responses than single administration of low or high dose (200 mg/kg) CPA [476]. They found that CPA treatment was more effective at reducing total numbers of Tregs ($CD4^+CD25^+FoxP3^+$) without altering effector $CD8^+$ T cells.

There are two reported clinical studies evaluating mCPA in cancer patients. Ghiringhelli *et al* evaluated metronomic dosing of CPA monotherapy in 9 patients with various types of advanced solid tumours, without concurrent vaccine treatment [459]. Patients were given 50 mg oral CPA twice daily (BID) for one month and they assessed peripheral blood immune profile before and at the end of the study (day 0 and day 30). They found that treatment resulted in significant decrease in circulating Tregs ($CD4^+CD25^+$), but total circulating levels of $CD4^+$, $CD8^+$ and NK ($CD3^-CD56^+$) cells were not affected. In addition, they reported that T cells and NK cells in treated patients had increased proliferation capacity, indicating that mCPA treatment may combine well with vaccine.

A single arm phase II study in melanoma patients (n=28) tested the combination of DC vaccine (loaded with KLH, survivin, hTERT, and p53 antigens) and mCPA (50 mg BID, one week on-one week off) [477] also did not observe any decrease in circulating Tregs. Although there was no vaccine only group within this study, the authors stated that the patients treated with vaccine and mCPA generated stronger responses than a historical control. Additionally, in this study, the number of patients

achieving stable disease doubled compared to a previous trial testing vaccine alone. The authors also noted strange kinetics of circulating MDSCs – an initial decline followed by rise, which could not be explained.

1.3.6 Summary and research rationale

Cancer vaccines are often able to generate anti-tumour immune responses, but are ineffective at overcoming tumour induced immune suppression. Immune suppression may be reversed by administering other types of immune therapies. Low dose CPA has demonstrated considerable immune enhancing effects when tested in pre-clinical models, but has not provided consistent benefit in human clinical trials. An alternative form of low dose CPA treatment is repeated low dose administration (metronomic). mCPA may offer more effective and long-term benefit and translate better into human clinical trials. Treatment with mCPA has shown immune modulatory effects similar to sbCPA in some pre-clinical and clinical trials, but has not been extensively studied as sbCPA. Furthermore, providing mCPA for continual periods presents unique scheduling obstacles in designing clinical trials involving both mCPA and vaccination. In order to design effective clinical trials to test mCPA in combination with peptide-based cancer vaccines it will be important to understand the kinetics of immune modulation.

In this project, the immune modulatory activity of mCPA will be evaluated in combination with a vaccine containing peptide antigens in a murine C3 tumour model. This tumour expresses the oncogenic protein HPV16 E7 and can be treated by efficient induction of $CD8^+$ T cells specific to the peptide HPV16 $E7_{49\text{-}57}$ (RAHNIVTF, R9F). Mice bearing established C3 tumours will be treated with mCPA and a DepoVax vaccine containing the peptide R9F (DPX-R9F) to determine if mCPA can provide enhanced efficacy and immunogenicity. The results of this project are presented and discussed in Chapter 4.

1.4 Hypothesis & Objectives

1.4.1 *In vitro* to *in vivo* translational study of a novel vaccine adjuvant system

Hypothesis

Stimulation of B cells through TLR3 and TLR1/2, using the specific agonists poly I:C and Pam3CSK4 respectively, will result in more efficient activation of B cells *in vitro*. The combination of these two agonists when used to adjuvant a protein vaccine may help to boost antibody responses *in vivo*.

Objectives

1) Identify the doses of poly I:C and Pam3CSK4 that can promote B cell activation *in vitro* and determine if they can provide enhanced activation signals to B cells when used in combination.
2) Confirm that poly I:C and Pam3CSK4 induced B cell activation is mediated through their interaction with TLR3 and TLR1/2 receptors expressed by B cells.
3) Investigate intracellular signaling events leading to NFκB activation triggered by stimulation of B cells with poly I:C, Pam3CSK4 or the combination.
4) Determine if the poly I:C/ Pam3CSK4 combination can enhance T-dependent B cell activation, differentiation and functions *in vitro.*
5) Test the efficacy of poly I:C and Pam3CSK4 adjuvant system *in vivo* using protein-based vaccines against anthrax and influenza.

1.4.2 *In vivo* development of a combinatorial Immunotherpay for cancer

Hypothesis

Metronomic cyclophosphamide will enhance anti-tumour immune responses induced by a peptide vaccine. This enhancement will lead to better control of tumour growth. This project will be performed using HPV16E7-induced C3 tumours with a DepoVax vaccine containing the HPV16E7$_{49\text{-}57}$ CD8^{+} peptide epitope (DPX-R9F).

Objectives

1) Compare the efficacy of mCPA or sbCPA treatment in combination with DPX-R9F to treat C3 tumours in mice.

2) Explore scheduling of mCPA relative to vaccination and the impacts on efficacy and immunogenicity.
3) Characterize the antigen-specific immune response in tumour bearing mice induced by DPX-R9F with and without mCPA treatment.
4) Explore the mechanisms through which mCPA can potentially enhance the effect of vaccination by examining immune cell profiles in the lymph node, spleen and tumour following treatment.

1.5 Tables & Figures

Table 1.1: Toll-like receptors and their ligands. Thirteen TLR receptors have been identified in humans (H) and mice (M). TLRs can be classified depending on the intracellular adaptor molecule they associate with, their cellular localization or agonist type. TLRs recognize a diverse selection of bacterial, viral, fungal and parasitic PAMPs. TLRs are found primarily on cells of the innate immune system, but also other immune cells like B cells and non-hematopoietic cells. Signaling through TLRs generally leads to inflammation. [155, 180, 478]

Receptor	Adaptor Molecule	Cellular Location	Type of Agonist	Agonist Examples
TLR1/2 (H, M)	MyD88	Surface	Bacterial triacyl lipopeptides	Pam3CSK4
TLR2/6 (H, M)	MyD88	Surface	Bacterial diacyl lipopeptides	Pam2Cys MALP-2 FSL-1
TLR3 (H, M)	TRIF	Endosomal	dsRNA	Poly I:C
TLR4 (H, M)	MyD88/TRIF	Surface	Lipopolysaccharide	LPS MPL A
TLR5 (H, M)	MyD88	Surface	Protein	Flagellin
TLR7 (H, M)	MyD88	Endosomal	ssRNA, purine analogs	Imiquimod Loxoribine Aldara®
TLR8 (H. M)	MyD88	Endosomal	ssRNA, small synthetic compounds	Resiquimod R848
TLR9 (H, M)	MyD88	Endosomal	dsDNA	CpG
TLR10 (H, M*)	MyD88	Surface	Unknown, possibly lipopeptides	Unknown
TLR11 (M)	MyD88	Surface	Flagellin, profillin	Toxoplasma gondii
TLR12 (M)	MyD88	Surface	Flagellin, profillin	Toxoplasma gondii
TLR13 (M)	MyD88	Endosomal	Bacterial RNA,	23S rRNA

*Pseudogene in mouse (non-coding)

Table 1.2: Components of subunit vaccines. A subunit vaccine is comprised of defined antigen(s) and adjuvant(s) assembled into a delivery system. [93, 94, 152]

Component	Definition	Examples
Antigen	Molecule that is unique to the target pathogen that the immune system can recognize and develop an adaptive immune response towards	Protein Peptide Glycopeptide DNA plasmid Virus-like particle Polysaccharide
Adjuvant	Molecule that can non-specifically stimulate the immune system, in particular antigen presenting cells, to develop immune response to the antigen. Also can direct the type of immune response developed.	LPS Poly I:C Pam3CSK4 Muramyl dipeptide
Delivery System	A type of adjuvant with the specific ability of packaging and delivering the antigen to increase stability *in vivo* and ensure presentation to the immune system	Water-in-oil emulsion Oil-in-water emulsion Liposomes Nanoparticle MF59

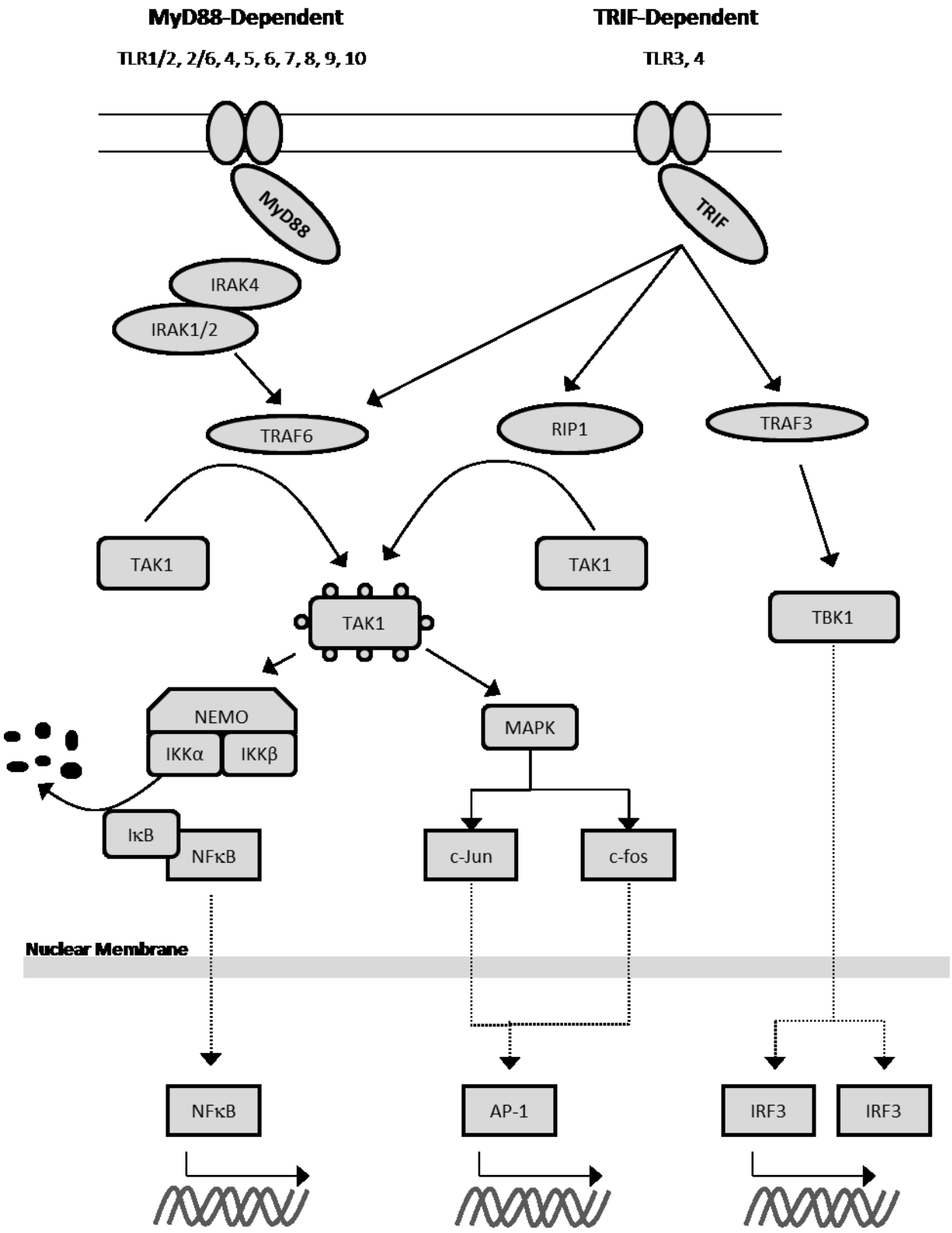
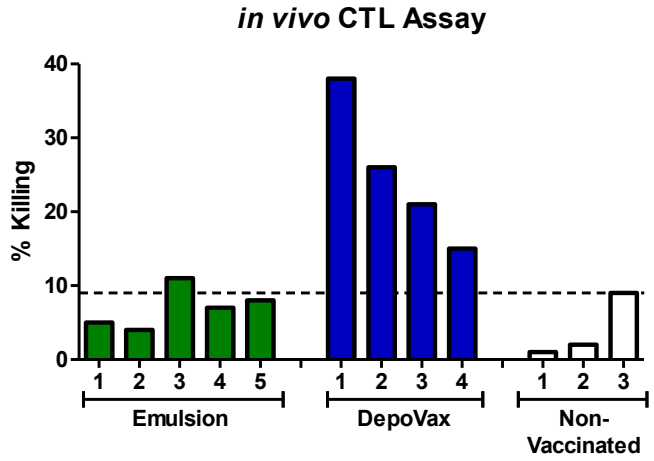
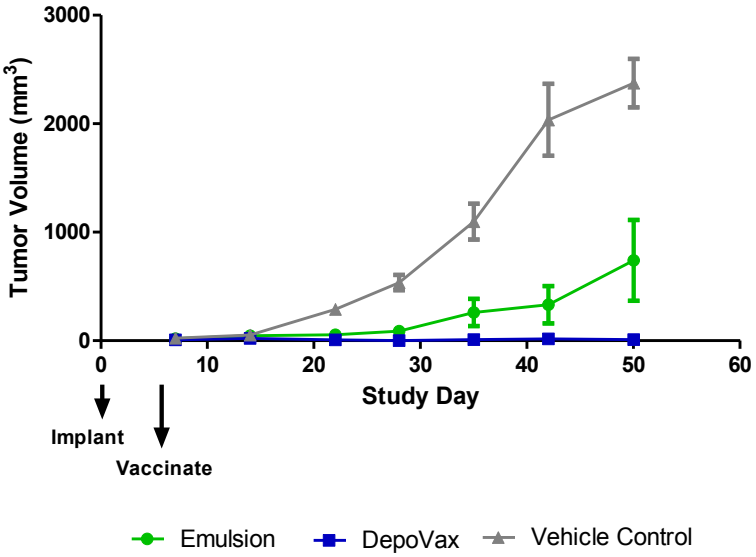

MyD88-Dependent
TLR1/2, 2/6, 4, 5, 6, 7, 8, 9, 10
TRIF-Dependent
TLR3, 4
MyD88
TRIF
IRAK4
IRAK1/2
TRAF6
RIP1
TRAF3
TAK1
TAK1
TAK1
TBK1
NEMO
IKKα
IKKβ
MAPK
IκB
NFκB
c-Jun
c-fos
Nuclear Membrane
NFκB
AP-1
IRF3
IRF3

Figure 1.1: TLR signal transduction. TLRs can be broadly classified based on signaling through the MyD88-dependent (TLR1,2,4,5,6,7,8,9,10) or MyD88-idenpendant pathways using TRIF (TLR3, 4). Only TLR4 can signal through both pathways. TLR3, 7, 8 and 9 are found on endosomal membranes, the rest are found on the surface membrane. The MyD88 pathway leads to NFκB and AP-1 activation, these transcription factors initiate the transcription of several inflammatory genes such as IL-1, IL-6 and IFN-γ. The TRIF pathway leads to activation of IRF3 and IRF7 which initiate the transcription of type I IFNs. These pathways are described in detail in section 1.1.2.

A.


in vivo CTL Assay
% Killing
40
30
20
10
0
1 2 3 4 5
1 2 3 4
1 2 3
Emulsion
DepoVax
Non-
Vaccinated

B.


Tumor Volume (mm3)
3000
2000
1000
0
0 10 20 30 40 50 60
Study Day
Implant
Vaccinate
Emulsion
DepoVax
Vehicle Control

C.

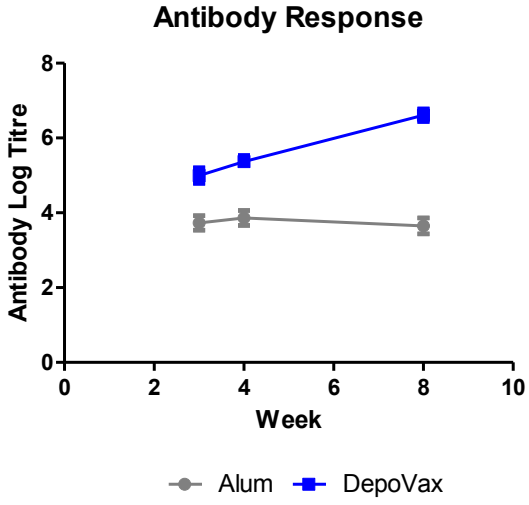

Antibody Response
Antibody Log Titre
8
6
4
2
0
0 2 4 6 8 10
Week
Alum
DepoVax

Figure 1.2: DepoVax enhances peptide and protein vaccine immunogenicity. **(A)** DepoVax formulation generates more effective cytotoxic T lymphocytes against vaccine peptide antigens than a comparable emulsion based vaccine. **(B)** DepoVax provides more effective tumour protection in a therapeutic cancer challenge model than a comparable emulsion based vaccines. **(C)** DepoVax generates higher antibody titers towards a protein vaccine antigen than alum based control. Studies performed by Immunovaccine, Figures used with permission.

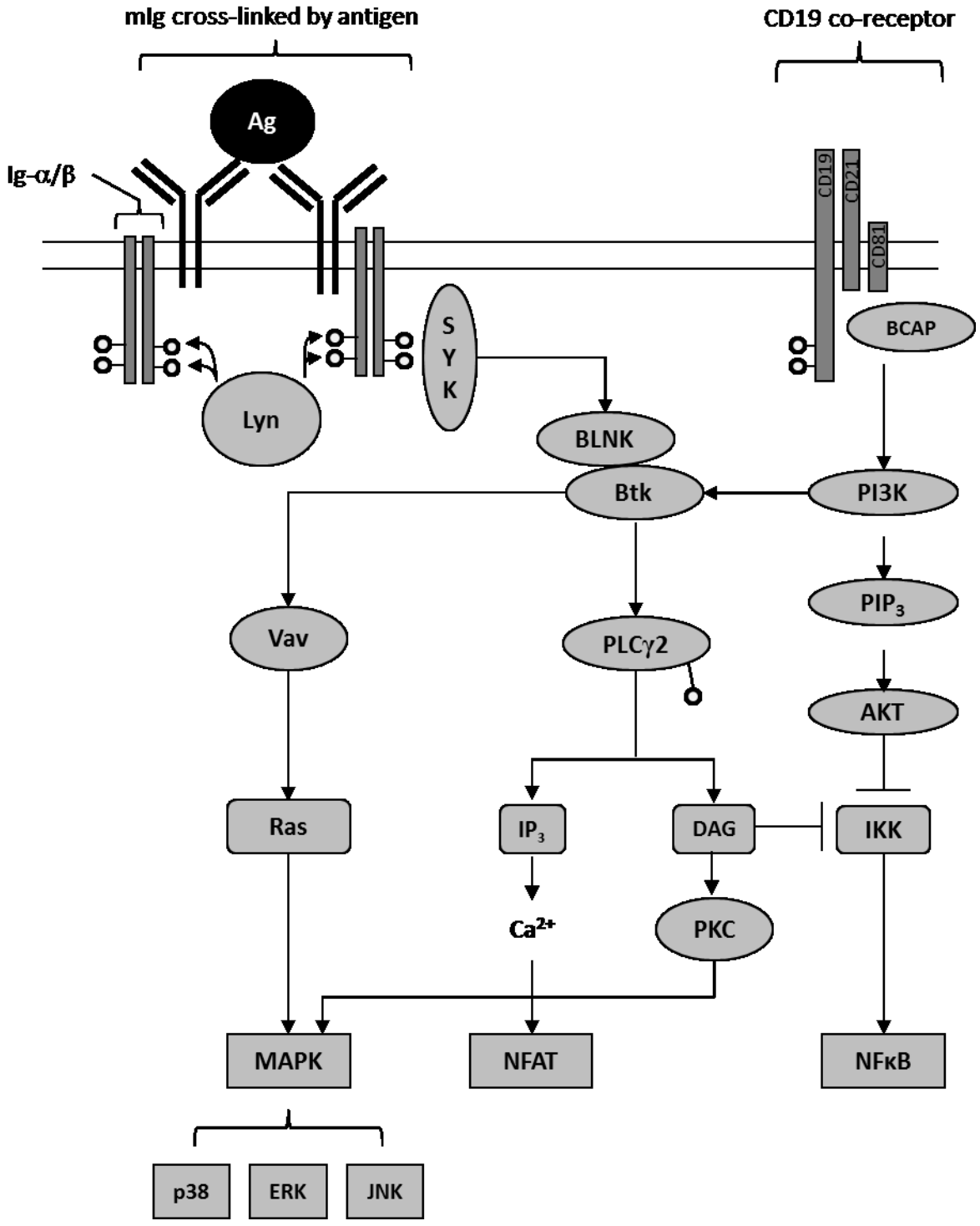

mIg cross-linked by antigen
CD19 co-receptor
Ag
Ig-α/β
CD19
CD21
CD81
BCAP
SYK
Lyn
BLNK
Btk
PI3K
PIP3
Vav
PLCγ2
AKT
Ras
IP3
DAG
IKK
Ca2+
PKC
MAPK
NFAT
NFκB
p38
ERK
JNK

Figure 1.3: Overview of signaling events triggered by BCR engagement. BCR clustering by binding multivalent antigens triggers BCR receptor signal transduction. Clustering of the BCR triggers Lyn to phosphorylate ITAM motifs on the BCR and CD19 co-receptor. Phosphorylated BCR creates a binding site for the tyrosine kinase Syk. Activated Syk phosphorylates BLNK which acts as a molecular scaffold for several signaling pathways. Shown here, phosphorylated BLNK associated with Btk activates Vav leading to induction of Ras-MAPK. BLNK/ Btk can also activate phospholipase C gamma 2 (PLγC2) which cleaves membrane associated phosphatidylinositol diphosphate (PIP_2) into second messengers inositol triphosphate (IP_3) and diacylgylcerol (DAG). IP_3 causes increase of intracellular Ca^{2+} levels which is required for activation of NFAT via calcineurin. DAG activates protein kinase C (PKC) which feeds into the Ras pathway leading to activation of MAPK. DAG also synergizes with CD19 co-receptor signaling enhancing the degradation of IKK and leading to activation of NFκB. CD19 co-receptor signaling activates the Akt serine/ threonine kinase that, besides from enhancing NFκB activation, also promotes activation of other transcription factors promoting B cell survival.

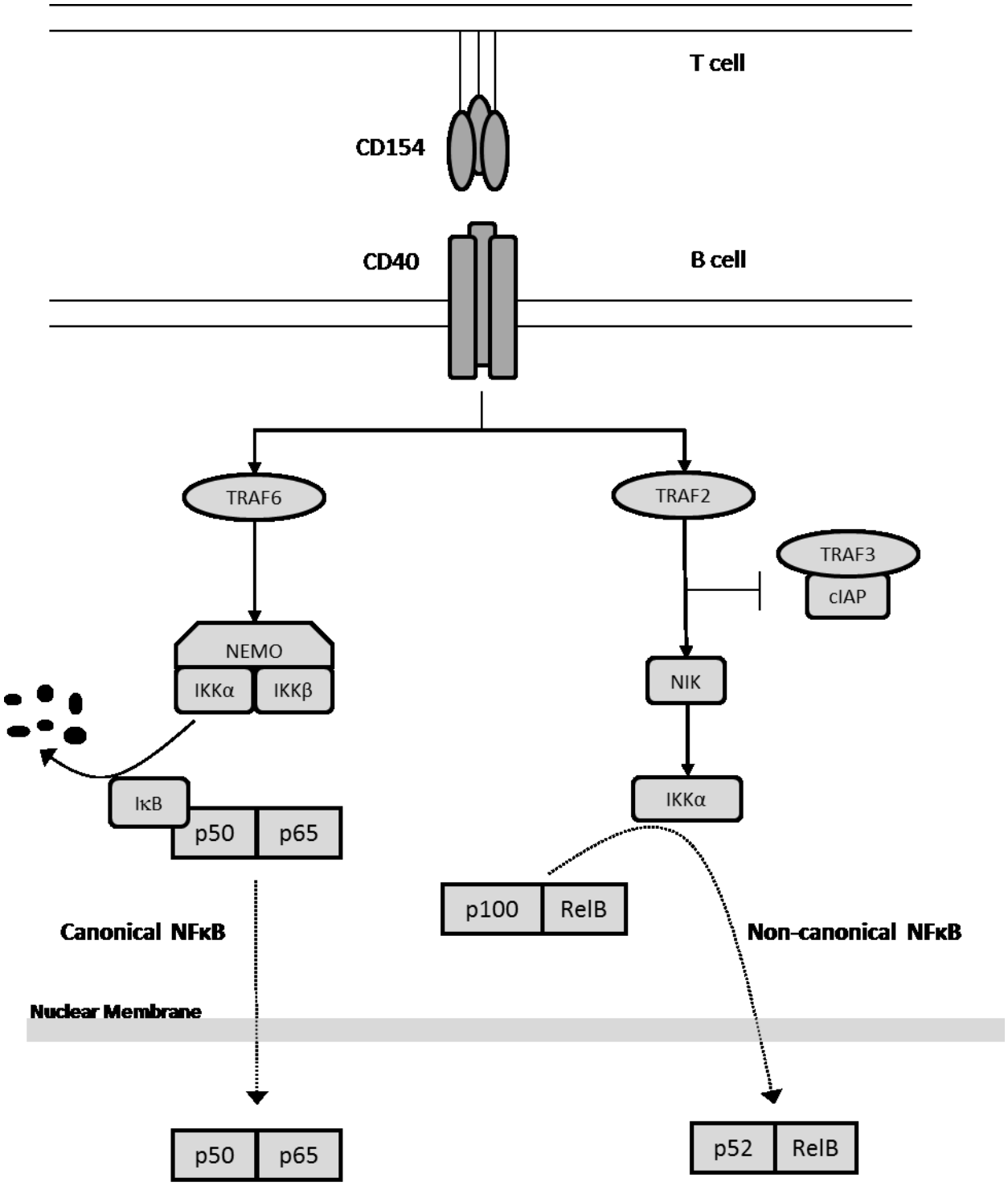

T cell
CD154
CD40
B cell
TRAF6
TRAF2
TRAF3
cIAP
NEMO
IKKα
IKKβ
NIK
IKKα
IκB
p50
p65
p100
RelB
Canonical NFκB
Non-canonical NFκB
Nuclear Membrane
p50
p65
p52
RelB

Figure 1.4: Activation of NFκB pathways by CD40 signaling in B cells. B cell engagement of CD40 by CD154 on T cells results in intracellular signaling mediated through TRAF2, 3 and 6. TRAF6 activates the canonical NFκB transcription factors p50/p65 by activation of the IKK complex (consisting of NEMO, IKKα and IKKβ) and subsequent degradation of the inhibitory IκB proteins, similar to TLR signaling. CD40 also recruits TRAF2 which destabilizes TRAF3, resulting in the inhibition of cIAP and activation of NIK. NIK then activates IKKα which induces the phosphorylation and proteasomal processing of p100 into p52. The NFκB dimer of p52/ RelB translocates into the nucleus to induce transcription.

CHAPTER 2: MATERIALS & METHODS

2.1 Methods

2.1.1 Media and Buffers

Complete RPMI culture medium was prepared by supplementing Roswell Park Memorial Institute (RPMI) 1640 media (Hyclone, Rockford, IL) with 10% heat-inactivated fetal bovine serum (FBS; HyClone) 2% penicillin-streptomycin (Gibco, Burlington, ON), 50 mM mercaptoethanol (Gibco) and 2mM L-glutamine (Gibco). Ammonium-chloride-potassium (ACK) lysing buffer was prepared with 162 mM ammonium chloride, 10 mM sodium bicarbonate and 1 mM EDTA, pH 7.4. Magnetic activated cell sorting (MACS) buffer was prepared with 0.5% FBS (Hyclone) and 2mM EDTA (Sigma) diluted in phosphate buffered saline (PBS) (Hyclone). PBS containing 0.5% tween (PBST) was purchased as a powder from Sigma and reconstituted in ddH_2O prior to use. Tris-buffered saline containing 0.5% tween (TBST) was prepared as 100 mM Tris (Roche, Mississauga, ON), 1.5 M NaCl (EMD, Mississauga, ON), 0.5% Tween 20 (BioBasic, Markham, ON) in double distilled water (ddH_2O). Flow cytometry buffer was prepared as 5% bovine serum albumin (BSA; Roche), 0.1% sodium azide (VWR, Mississauga, ON) in Dulbeco's PBS containing Ca^{2+} and Mg^{2+} (Gibco). Alkaline phosphatase buffer was prepared as 100 mM Tris (Roche), 100 mM sodium chloride (EMD) and 5 mM magnesium chloride (VWR).

2.1.2 TLR Agonists

Pam3CSK4 (MW: 1510 g/ mol) was obtained from EMC Microcollections (Tuebingen, Germany). Poly I:C (MW: 989,486 g/ mol) was obtained from Thermo Fisher Scientific (Milwaukee, WS). Lipopolysaccharide (LPS) and CpG 1826 were purchased from Sigma-Aldrich (St. Louis, MO). R848 was purchased from Invivogen (San Diego, CA). All agonists were reconstituted in water at concentration recommended by manufacturer, for cell stimulation dilutions were prepared in complete RPMI medium.

2.1.3 Peptides and proteins

Recombinant hemagglutinin (rHA) protein H5N1 from influenza strain A/Vietnam/1203/2004 was obtained from Protein Sciences (Meriden, CT). Recombinant protective antigen (rPA) was obtained from List Biologicals (Campbell, CA). All peptides were synthesized by NeoMPS (San Diego, CA) at >95% purity. CD8 epitopes used were: HPV16 $E7_{49\text{-}57}$ (RAHNIVYTIF; R9F) and $TRP2_{180\text{-}188}$ (SVYDFFVWL; S9L). All peptide vaccines contained a universal T helper peptide, either PADRE (AKXVAAWTLKAA) or $TT_{830\text{-}843}$ (FNNFTVSFWLRVPKVSASHLE; F21E).

2.1.4 Animals

Female C57BL/6NCrl (C57BL/6), BALB/c and CD-1 mice were obtained from Charles River Labs (St. Constant, PQ, Canada). TLR2 knockout mice had a C57BL/6 background and were a kind gift from Dr. Jean Marshall (Dalhousie University). TLR3 knockout mice (B6;129S1-*Tlr3*^{tm1Flv}/J) and wild type controls (B6;129SF2/J) were obtained from Jackson (Bar Harbor, ME) [479]. Mice were used at 7 weeks of age with average body weight of 20 g ± 2 g. All mice were maintained in the Carleton Animal Care Facility at Dalhousie University following institutional guidelines for procedures and provided food and water *ad libitum*.

2.1.5 Dendritic cell isolation and culture

Dendritic cells (DCs) were isolated from the femur bone marrow of naive C57BL/6 mice on day 0 and cultured at 1.2×10^6 cells/mL in a 6-well plate in 5 mL of complete RPMI medium supplemented with 30 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ) at 37°C, 5% CO_2. Cells were fed on day 3 with fresh medium and 30 ng/mL murine GM-CSF. On day 6, non-adherent cells were washed and resuspended in fresh medium with 15 ng/mL murine GM-CSF then transferred into into a new 6-well plate. DC purity was assessed on day 8 by flow cytometry using anti-mouse CD11c.

2.1.6 B cell isolation and culture

B cells were isolated from naïve C57BL/6 mouse spleens using negative selection magnetic beads from Miltenyi Biotech (Auburn, CA). Briefly, a single cell suspension

was prepared by crushing the spleens with a 3 mL plunger against a 40 μM nylon mesh screen (Fisher Scientific, Nepean, ON). RBCs were lysed using ACK buffer. Cells were resuspended in MACS buffer and then fractionated by MACS LS immuno-columns according to the manufacturer's protocol. Purity was confirmed to be >95% by flow cytometry using anti-mouse CD19. For stimulation, B cells were set up in triplicate in a 96-well plate at 10^5 cells/ well in complete RPMI medium. Pam3CSK4 and poly I:C dilutions were made in complete RPMI medium and added into the wells containing B cells for a final volume of 200 μL per well. T-dependent activation was simulated by adding purified hamster anti-mouse CD40 (2.5 μg/mL; clone HM40-3, NA/LE, BD Biosciences) and purified rat anti-mouse kappa Ig (1 μg/mL; clone 187.1, BD Biosciences) to the B cell suspensions. Plates were incubated at 37°C/ 5% CO2 for 1 to 4 days, depending on the type of assay.

2.1.7 Flow cytometry

All antibodies were purchased from eBioscience (San Diego, CA), unless stated otherwise; a description of antibodies used for flow cytometry can be found in Table 2.1. R9F-dextramer-PE was obtained from Immudex (Copenhagen, Denmark). Cells (~5-10×10^6 cells/ tube) were washed once in flow cytometry buffer followed by Fc block using 1:50 dilution of anti-mouse CD16/CD32 and incubated for 10 minutes at room temperature. Antibody cocktails were then added and cells incubated for an additional 30 minutes at 4°C. Intracellular staining for FoxP3 was performed after surface staining using a permeabilization kit from eBioscience. Cells were washed in flow cytometry buffer and resuspended in 1% paraformaldehyde/ PBS and kept at 4°C until acquisition on FACSCalibur (BD Biosciences). Flow cytometer data was analysed using WinList 7.0 software (Verity Software, Topsham, ME).

2.1.8 Quantitation of cytokine production by dendritic cells

Supernatants were collected from day 8 DCs after 24 hours of stimulation with the indicated adjuvants. The concentration of TNF-α, IFN-γ, CCL2, IL-10 and IL-6 was measured using the Cytometric Bead Array Mouse Cytokines kit (BD Bioscience) following manufacturer's instructions. Standard curves were prepared from supplied lyophilized proteins for each cytokine at various ranges.

2.1.9 ELISAs

2.1.9.1 Quantitation of cytokine production by B cells

ELISA kits were used to measure the concentration of IL-6 (eBioscience), IL-10 (Southern Biotech), IFN-γ (eBioscience) and CXCL10 (eBioscience) in B cell supernatants. IL-6 and CXCL10 was measured after 24 hours of stimulation; IL-10 and IFN-γ were each measured after 24 hours and after 4 days of culture. Briefly, enzyme-immunoassay (EIA) flat-bottomed 96-well plates (BioRad Laboratories Inc, Mississauga, ON) were coated overnight with a capture antibody diluted in coating buffer supplied with the kit. Next day, plates were washed with PBST and blocked with assay diluent (supplied with kit) for 1 hour at room temperature. Plates were washed 5 times with PBST then supernatants (1:2 dilution) or standard were added and plates were incubated for 2 hours at room temperature. Plates were then washed 5 times with PBST and biotinylated detection antibody was added and plates incubated for 2 hours. Plates were thoroughly washed again and avidin-conjugated horseradish peroxidase (HRP) reagent added for 30 minutes at room temperature. Plates were washed 7 times with PBST and then 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution added for 15 minutes. Colour development was then stopped by adding 1 M H_3PO_4 stop solution. Plates were read on an ELISA plate reader (ASYS Expert Plus Microplate reader, Biochrom, Holliston, MA) at 450nm.

2.1.9.2 Detection of secreted IgG in B cell supernatant

B cell cultures were stimulated for 4 days. Triplicate wells were pooled and supernatants collected and frozen at -80°C. Total IgG was detected using an ELISA kit from eBioscience. Briefly, EIA flat bottomed 96-well plates were coated with purified anti-IgG in PBS overnight at 4°C. Next day, plates were blocked with assay diluent for 2 hours at room temperature. Plates were washed with PBST then standards and supernatants (diluted 1:10 in assay diluent) were added with anti-IgG-HRP detection antibody. Plates were incubated for 3 hours at room temperature, then washed 4 times with PBST. Plates were developed with TMB substrate and stopped with H_3PO_4. Absorbance was read on a microplate reader (ASYS Expert Plus Microplate reader, Biochrom) at 450nm.

2.1.9.3 Serum antibody titer

Mice were bled by facial venupuncture on indicated weeks post vaccination. Antibody titres were determined in the serum by endpoint titration. Briefly, EIA 96-well flat bottomed plates (Biorad) were coated with antigen at 1 μg/mL in bicarbonate coating buffer (pH 9.5) overnight at 4°C. Next day, plates were washed in TBST and blocked with 3% gelatin (Biorad) in TBST at 37°C for 30 minutes. Plates were washed again in TBST and serum pre-diluted in TBST was added to the top row. Serial dilutions of 1:2 were prepared down the plate from row A to row H. On each plate at least two naïve serum samples were included as background control. Plates were incubated overnight at 4°C. The next day, plates were washed with TBST and loaded with alkaline phosphatase -conjugated Protein G to detect total antibody titers (Calbiochem, Gibbstown, NJ) at 1 μg/mL. Plates were incubated for 1 hour at 37°C, washed then loaded with alkaline phosphatase substrate solution and incubated for 1 hour at 37°C. Absorbance was read on a microplate reader (ASYS Expert Plus Microplate reader, Biochrom) at 405nm. Endpoint titers were calculated as described in Frey A. *et. al* [480]. Endpoint titers represent the highest dilution at which a statistically significant increase in absorbance is observed in serum samples of immunized mice versus serum samples from naïve, non-immunized control mice.

2.1.10 Proliferation of B cells

To measure proliferation, B cells were incubated for 3 days and were pulsed with 0.5 μCi of tritiated thymidine ([^{3}H]-TdR; MP Biomedical, Irvine, CA) for the last 18 hours of culture. Cells were harvested onto fiberglass filter mats (Skatron Instruments, Sterling, VA) with Titertek Cell Harvester (Skatron Instruments). [^{3}H]-TdR incorporation was measured using Beckman LS6000IC liquid scintillation counter (Beckman Coulter Inc., Mississauga, ON) and quantified as counts per minute (CPM).

2.1.11 Western blotting

Spleen B cells were resuspended in complete RPMI medium and stimulated as indicated. At the end of stimulation time, B cells were immediately placed on ice and washed once with ice-cold PBS. Cell lysates were prepared using ice-cold lysis buffer

(50mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM Na_2PO_4, 0.25% sodium deoxycholate [w/v], 0.1% NP-40 [v/v], 5 mM EDTA and 5 mM EGTA) containing freshly added protease and phosphatase inhibitors (5 μg/mL leupeptin, 5 μg/mL pepstatin A, 10 mM NaF, 1 mM DTT, 100 μM $NaVO_4$, 10 uM PAO and 10 g/mL aprotinin). Samples were incubated on ice for 15-30 minutes and clarified by centrifugation at 14,000 g for 10 min. Total protein was collected and quantified by colorometric assay using Bio-Rad Assay Dye Reagent (Bio-Rad Laboratories Inc.) and bovine serum albumin (BSA; Sigma-Aldrich) standard of known concentration, which were read at 570 nm on a ELx800 UV universal microplate reader (BioTeck Instruments, Inc.). Protein levels were equalized between samples and then denatured by the addition of SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer (200 mM Tris-HCl [pH 6.8], 30% glycerol [v/v], 6% SDS [w/v], and 0.01% bromophenol blue [w/v]). Each sample was then heated to 95°C for 5 min and frozen at -80°C until use.

Duplicate protein samples were resolved in parallel on two Tris-HCl acrylamide gel (12% resolving gel: 375 mM Tris-HCl [pH 8.8]0.1% SDS [w/v], 0.1% APS [w/v], and 0.15% TEMED [v/v] and 4% stacking gel: 125 mM Tris-HCl [pH 6.8], 0.1% SDS, 0.1% APS, and 0.3% TEMED) and transferred to PVDF membrane using iBlot (Life Technologies). Membranes were blocked with 5% milk in TBST for 1 hour at room temperature washed 5x TBST, and incubated overnight at 4°C with primary antibodies. The following antibodies were used: anti-p65 (clone D14E12, Cell Signaling Technologies, Danvers, MA), anti-pSer539-p65 (clone 93H1, Cell Signaling Technologies), anti-IκBα (polyclonal, Cell Signaling Technologies) anti-pSer32-IκBα (clone MA5-15087, ThermoFisher). Total and phosphorylated proteins were probed separately on duplicate blots. All primary antibodies were reconstituted at dilution recommended by manufacturer in 5% BSA/ TBST. Blots were washed 5x in TBST and incubated with secondary anti-rabbit-HRP antibody (1:1000 5% milk/ TBST; Santa Cruz) for 1 hour at room temperature. Membranes were reacted with chemiluminescence reagents (GE Healthcare, Baie d'Urfe, QC) for 1 min then exposed to x-ray film (Sci-Med Inc., Truro, NS), which was developed in a Kodak X-MAT 1000A automated X-ray developer. To confirm equal protein loading, membranes were re-probed for actin expression. Protein bands were quantified by densitometry using ImageJ software (NIH).

Individual band densities were first normalized to respective actin band, then to unstimulated B cell control which was run on each gel.

2.1.12 Vaccines and immunization

DepoVax vaccines were prepared as previously described [171, 172]. Briefly, proteins and adjuvants were solubilized in appropriate buffer and mixed with lipids and cholesterol. The aqueous mixture containing liposomes was lyophilized to a dry cake and then reconstituted with Montanide ISA51 VG (SEPPIC, France) just prior to injection.

For anthrax and influenza protein vaccines used in Chapter 3, lipids were prepared with S100 lecithin (Lipoid GmBH, Bermany). These vaccines were delivered via intramuscular injections of 25 μL on each the right and left leg. Each 50 μL dose of anthrax vaccine contained 1 μg rPA and adjuvants as indicated in figure legends. Each 50 μL dose of influenza vaccine contained 0.5 μg rHA and adjuvants as indicated in figure legends.

For peptide vaccines used in Chapter 4, lipids were prepared with DOPC lecithin (Lipoid). Mice were vaccinated subcutaneously on the right flank with 50 μL of vaccine. Each dose of vaccine contained 10 μg R9F fused to PADRE (R9F-PADRE) + 20 μg adjuvant or 5 μg R9F + 5 μg F21E + 20 μg adjuvant, where indicated. Irrelevant peptide control vaccine contained 5 μg S9L + 5 μg F21E + 20 μg adjuvant. When multiple vaccinations were administered they were given in the same general area but avoiding previous immunization sites.

2.1.13 Tumor cells and implantation

The C3 tumour line was provided by Dr. Martin Kast (USC, Los Angeles, USA). The C3 cells are derived from C57BL/6 mouse embryo cells transfected to express HPV16 [481]. The C3 tumour line was maintained in IMDM (Gibco) supplemented with 10% FBC (HyClone) 2% penicillin-streptomycin (Gibco), 50 mM mercaptoethanol (Gibco) and 2 mM L-glutamine (Gibco). For tumour implantation, mice were subcutaneously injected in the left flank with 5×10^5 C3 tumour cells. Tumor growth was measured with digital calipers twice weekly and tumour volume calculated using the formula [($width^2 \times length$)/2]. For experiments requiring determinations of survival, endpoint was determined to be when mice had tumour volumes of ≥ 2000 mm^3, or

showed significant signs of ill health, such as wasting, severe dehydration, significant decrease in activity and hunched or prostate posture. When endpoint was determined, mice were humanely euthanized per CCAC guidelines.

2.1.14 Cyclophosphamide treatment

CPA (Sigma-Aldrich, St. Louis, MO) was reconstituted in PBS and given either as a single intravenous injection at 100 mg/kg or provided for consecutive days in drinking water (PO) at 0.133 mg/mL calculated to deliver 20 mg/kg/day based on 3 mL water/ mouse/ day [463]. For PO administration, water was changed every 2-3 days.

2.1.15 IFN-γ ELISPOT

2.1.15.1 Lymph node ELISPOT

DCs were prepared from bone marrow cells as described above. On day 7, they were loaded with peptides at 20 μg/mL. On day 8, they were collected, washed and resuspended in complete RPMI medium to which additional peptide was added at 20 μg/ mL. Right inguinal lymph nodes were collected from mice upon termination. Single cell suspensions were prepared by crushing the tissues against a 40 μM nylon mesh (BD Biosciences). Cells were counted in trypan blue using the Countess® Automated Cell Counter (Life Technologies, Carlsbad, CA) and after washing lymph node cells (LNC) were resuspended at 2×10^6 cells/ mL in complete RPMI medium. IFN-γ ELISPOT kits were obtained from BD Biosciences and used according to the manufacturer's instructions. Briefly, ELISPOT plates were coated overnight with anti-mouse-IFN-γ diluted in PBS and then blocked with complete RPMI medium for two hours at room temperature. LNC (100 μL) were loaded onto the ELISPOT plate and stimulated in duplicate with peptide loaded DCs (100 μL) at a 10:1 ratio. For background control, LNC were also stimulated in duplicate with unloaded DCs. The ELISPOT plate was incubated overnight at 37°C, 5% CO_2 and then developed the next day using AEC kit (Sigma-Aldrich). Spots were counted using ELISPOT Reader (C.T.L. Ltd, Shaker Heights, OH) and results enumerated as number of spot-forming units (SFU).

2.1.15.2 Splenocyte ELISPOT

Spleens were collected from mice upon termination. Single cell suspensions were prepared by crushing spleens in a petri dish with 3 mL syringe plunger. Red blood cells were lysed with ACK buffer. Cells were resuspended in complete RPMI medium and counted with Countess® Automated Cell Counter (Life Technologies) and resuspended at 5×10^6 cells/ mL in complete RPMI medium. IFN-γ ELISPOT plates were prepared as above. Splenocytes (100 µL) were loaded onto the ELISPOT plate and stimulated in duplicate with 100 µL of R9F peptide (20 µg/mL), irrelevant S9L peptide (20 µg/mL), C3 tumour cells (500,000 cells/ mL) or unstimulated (medium). ELISPOT plate was incubated overnight and developed as described above.

2.1.16 *In vivo* cytotoxic lymphocyte assay

In vivo CTL assays were performed as described previously [482]. Briefly, RBC-lysed splenocytes from donor naïve syngeneic mice were resuspended at 10^8 cells/ mL in HBSS + 1% HEPES buffer (Gibco) and divided into two tubes which were pulsed with R9F peptide at 20 µg/mL or unpulsed for 1.5 hours at 37°C. Both fractions were washed and resuspended in 4 mL PBS. Each fraction was labeled with Oregon Green 488 (Life Technologies, Burlington, ON) at two different concentrations (5 µM for peptide-pulsed, 0.5 µM for nonpulsed) for 7 minutes at room temperature. Labeling was quenched with the addition of 5 mL FBS. The fractions were washed, resuspended in HBSS+HEPES at 10^8 cells/ mL and pooled 1:1. Recipient mice received 100 µL of cells via tail vein injection of the cell mixture on day 8 after vaccination. Eighteen hours later, the mice were terminated and spleens collected. A single cell splenocyte suspension was prepared and the relative levels of each target population detected by flow cytometry. Percent specific lysis of fluorescent donor spleen cells in each mouse is calculated as follows: $1-(r_{naive}/r_{vaccinated}) \times 100\%$, where "r" = number of unpulsed target cells/ number of peptide-pulsed target cells.

2.1.17 Dissociation of tumours

Procedure adapted from [483]. Mice were anesthetized and tumours removed and cleaned of external skin as much as possible using scissors. Tumors were placed in

complete RPMI medium and kept on ice until processing. Tumors were cut into small pieces with a scalpel in 5 mL digestion buffer (200 U/mL collagenase type I [Gibco], 0.1 mg/mL DNAse I [Sigma-Aldrich], 5% FBS, RPMI 1640). Pieces and buffer were transferred to a 15 mL tube and incubated on an orbital shaker at 37°C for 30 minutes. Samples were strained through a 40 μM cell strainer and washed in excess complete RPMI medium. Cells were counted and ~10^7 were transferred into FACS tubes for staining by flow cytometry. All tumour samples were stained with anti-mouse CD45 to allow gating on leukocyte population.

2.1.18 MDSC functional assay

MDSCs were isolated from splenocytes using biotinylated anti-mouse Gr-1 antibody followed by strepdavidin-magnetic beads (Miltenyi). Labeled cells were separated using MACs separation columns (Miltenyi) and the Gr-1/ CD11b phenotype confirmed by flow cytometry to detect CD11b. Responder T cells were isolated from naïve mice by magnetic separation using CD8α negative isolation kit (Stem Cell Technologies, Vancouver, BC) and labeled with Oregon Green 488 (Life Technologies). $CD8^+$ T cells were mixed with MDSCs in triplicate wells of a 96-well plate in a 1:2 ratio and anti-CD3/ anti-CD28 beads (Life Technologies) added to stimulate T cell proliferation. Three days later, cells were collected and triplicates pooled. T cell proliferation was assessed by Oregon Green 488 dilution by on the FACSCalibur.

2.1.19 Adoptive transfer of immunity

Total T cells ($CD3^+$) or $CD8^+$ T cells were isolated from donor mice using negative magnetic isolation kits (Stem Cell Technologies). Purity was assessed by flow cytometry (Appendix B.5). Cells were prepared in HBSS and 10×10^6 T cells or 4×10^6 CD8 T cells were injected intravenously into recipient mice that had been challenged with C3 tumours two days previously.

2.1.20 Real time quantitative polymerase chain reaction (RT-qPCR)

2.1.20.1 RNA extractions

Tumor samples were collected in RNAlater RNA Stabilization Reagent (QIAGEN, Gaithersburg, MD) and stored at -80°C. Total RNA was isolated from tumours using RNeasy Plus Mini Kit (QIAGEN) according to the recommended protocol.

2.1.20.2 RT-qPCR

To remove contaminating DNA, 5 µg aliquots of isolated total RNA were treated with 1U of DNAse I (Invitrogen, Carlsbad, CA). The total RNA were reverse transcribed using a SuperScript III reverse transcriptase kit (Invitrogen) and oligo(dT) primer (Invitrogen); cDNA samples were precipitated with 100% ethanol overnight at -20°C and resuspended in 400 µl of water.

Quantitative PCR primers for the amplification of *Gzmb*, *Pdcd1*, *Ifng*, *Il2*, *Il4*, *Ctla4*, *Il10*, and *Tbp* were designed using Primer-BLAST algorithm (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are listed in Table 2.2. Amplifications of these transcripts were performed on a Rotor Gene 3000 real-time PCR machine (Corbett Research, Sydney, Australia) using a QuantiFast SYBR Green PCR kit (QIAGEN) in accordance with the protocol provided. Each cDNA sample was examined in duplicated 20 µl qPCR containing 5 µl of the cDNA template. All data were analyzed based on the standard curve method. To compensate for any variance in the amount or quality of the input RNA, the presented mRNA levels are shown relative to the levels of TBP mRNA that was used as an internal control.

Quantitative amplifications of pre-designed primers for *Cd4*, *Foxp3*, *Klrc1*, *Cd19*, *Cd8a*, and *Tbp* (housekeeping control) were performed using RT² qPCR Primer Assays (QIAGEN) and RT² SYBR Green ROX FAST kit (QIAGEN). Each cDNA sample was analyzed in duplicated 20 µl qPCR containing 5 µl of the cDNA template. Quantifications were performed based on the ΔΔCT method as recommended in the provided protocol.

2.1.21 Statistical analysis

Statistical analysis was conducted with GraphPad Prism 5 software (La Jolla, CA). Data was analysed by one-way ANOVA using Tukey multiple comparisons post-test, two-way ANOVA with Bonferroni multiple comparisons post-test, or Student's t-test as indicated; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

2.2 Tables

Table 2.1: List of anti-Mouse Monoclonal Antibodies Used for Flow Cytometry. All antibodies were purchased from eBioscience, except the PerCP conjugates (*) which were obtained from BD Biosciences.

Target	Clone	Host	Isotype	Conjugate
Isotype	eBRG1	Rat	IgG1, κ	APC
Isotype	eBR2a	Rat	IgG2a, κ	APC
Isotype	eB149/10H5	Rat	IgG2b, κ	FITC, PE
Isotype	eBio299Arm	Armenian Hamster	IgG	FITC, PE
CD3	145-2C11	Armenian Hamster	IgG	PE
CD4	GK1.5	Rat	IgG2b, κ	FITC, PE, APC
CD4	RM4-5	Rat	IgG2a, κ	PerCP*
CD8α	53-6.7	Rat	IgG2b, κ	FITC, PerCP*, APC
CD11c	N418	Armenian Hamster	IgG	FITC, PE, APC
CD11b	M1/70	Rat	IgG2b, κ	FITC
CD16/ CD32	93	Rat	IgG2a, λ	Purified
CD19	1D3	Rat	IgG2a, κ	FITC, PE, APC
CD25	PC61	Rat	IgG1, λ	APC
CD40	1C10	Rat	IgG2a, κ	PE
CD45	30-F11	Rat	IgG2b, κ	PerCP*
CD69	H1.2F3	Armenian Hamster	IgG	FITC
CD80	16-10A1	Armenian Hamster	IgG	FITC, PE
CD86	GL1	Rat	IgG2a, κ	PE
CD138	281-2	Rat	IgG2a, κ	APC
CD197	4B12	Rat	IgG2a, κ	PE
CD267	Ebio8F10-3	Rat	IgG2a, κ	PE
CD279	J43	Armenian Hamster	IgG	FITC
FoxP3	FJK-16s	Rat	IgG2a, κ	PE
Gr-1 (Ly-6G/C)	RB6-8C5	Rat	IgG2b, κ	APC
H-2Db (MHC I)	28-14-8	Mouse	IgG2a, κ	FITC
IA/IE (MHC II)	M5/114.15.2	Rat	IgG2b, κ	FITC, APC
NK1.1	PK136	Mouse	IgG2a, κ	PE

Table 2.2: List of Primers for RT-qPCR.

Gene	Forward primer	Reverse primer
Gzmb	5'AGTCAAGCCCCACTCTCGAC-3'	5'-AGGGATGACTTGCTGGGTCT-3'
Ifng	5'-TCAGCAACAGCAAGGCGAAA-3'	5'-CAATCTCTTCCCCACCCCGA-3'
Foxp3	5'- CACAATATGCGACCCCCTTT -3'	5'-TCCCTTCTCGCTCTCCACTC-3'
Il4	5'-GTGAGCTCGTCTGTAGGGCT-3'	5'-TCAGTGATGTGGACTTGGACTCA-3'
Tbp	5'-CCTGCCACACCAGCTTCTGA-3'	5'-ATCAACGCAGTTGTCCGTGG-3'
Il10	5'-AACTGCACCCACTTCCCAGTC-3'	5'-TGGGGCATCACTTCTACCAGG-3'
Vegf	5'-CGCAGCGACAAGGCAGACTA-3'	5'-GGGAGTGAAGAACCAACCTCCT-3'
Ctla4	5'- CACCGCCATACTTTGTGGGC-3'	5'-GGCTCTGTTGGGGGCATTTT-3'
Pdcd1	5'-AGAAGGCCGGTTTCAAGGCA-3'	5'-GGCCACACTAGGGACAGGTG-3'
Klrc1	5'-AAACCAAGGGGTCCTCGCAG-3'	5'- GACAAAACAGATGAGGCCCAGG-3'
Cd4	5'-GCTGGTTCTGGCAACCTGAC-3'	5'-CTCAGGGGCCACCACTTGAA-3'
Cd8a	5'-TTCTGCCATGAGGGACACGA-3'	5'-GGTGCACAGGTGAGGGAGTT-3'

CHAPTER 3:

IN VITRO TO *IN VIVO* TRANSLATIONAL STUDY OF A NOVEL VACCINE ADJUVANT SYSTEM

Portions of this chapter are being prepared for publication and patent application:

Weir GM, Karkada M, Stanford MM, MacDonald L, Hoskin D, Mansour M, & Liwski RS.

Contributions: GW participated in project conception, designed and executed experiments, analysed and interpreted data, and prepared manuscript. LM participated in experiment design and analysis of *in vivo* studies. MK, and RL contributed to project conception, experiment design, data interpretation and manuscript revision. MS, DH and MM contributed to experiment design, data interpretation and manuscript revision. Technical assistance to perform experiments was provided by R. Rajagopalan.

3.1 Results

3.1.1 Stimulation of dendritic cells with the combination of poly I:C and Pam3CSK4 increases activation

Poly I:C and Pam3CSK4 have been reported to have a synergistic effect on the *in vitro* activation of human DCs [109] as well as mouse DCs [308] and macrophages [307]. To confirm reported observations, we tested the effect of poly I:C and Pam3CSK4 stimulation of DCs. Bone marrow derived DCs were treated overnight with poly I:C (5 μg/mL), Pam3CSK4 (10 μg/mL), or the combination of both. Next day, the expression of surface molecules associated with activation (MHC class II, CD40, CD80, CCR7) were measured by flow cytometry (Figure 3.1A) and cytokines in the supernatant detected by cytokine bead array (TNF-α, IFN-γ, CCL2, IL-10, IL-6; Figure 3.1B).

As shown in Figure 3.1A, stimulation with either agonist alone resulted in increases (non-significant over untreated) in the expression of receptors, but the agonist combination produced the highest and most significant increases in expression of all receptors. Poly I:C did not induce significant production of the cytokines examined (Figure 3.1B), but Pam3CSK4 induced elevated levels of TNF-α and IL-6. Together, poly I:C and Pam3CSK4 induced significantly higher levels of TNF-α, IL-6 and the chemokine CCL2. The combination of poly I:C and Pam3CSK4 increased the secretion of IL-6 and CCL2 greater than the sum of each individual adjuvant, indicating a synergistic effect. No IFN-γ or IL-10 was detected under any stimulation condition.

3.1.2 Poly I:C and Pam3CSK4 stimulate B cells through TLR3 and TLR2 to enhance B cell activation independent of T cell help

3.1.2.1 Dosing poly I:C and Pam3CSK4 on B cells in vitro

We next evaluated the effect of poly I:C and Pam3CSK4 stimulation on B cells. B cells were isolated from naïve C57BL/6 spleens and confirmed to be >95% pure by flow cytometry using CD19 marker with less than 1% $CD11c^+$ DCs (Appendix Figure B.1). To identify the optimal doses of each agonist, we performed a preliminary dose-response test of poly I:C (0.1, 0.5, 1, 5, 10, 25, and 50 μg/mL) and Pam3CSK4 (0.01, 0.1, 0.5, 1, 5, and 10 μg/mL) and measured expression of activation markers by

flow cytometry after 24 hours and proliferation by [^{3}H]-TdR uptake after 3 days. These readouts have been used to measure B cell response to TLR stimulation by others [223, 227].

Figure 3.2 shows the results of the dose-response testing. We found that Pam3CSK4 induced CD40 expression starting at 0.01 μg/mL and CD80 expression starting at 0.5 μg/mL. Pam3CSK4 significantly increased proliferation at all doses tested. Poly I:C did not induce expression of CD80 at any dose, but could induce CD40 expression starting at 10 μg/mL. Poly I:C induced weak proliferation of B cells that was not statistically greater than that of untreated B cells.

We selected a dose of 1 μg/ mL Pam3CSK4 to evaluate in combination with poly I:C. This dose of Pam3CSK4 induced significant expression of CD80 and CD40, but not maximal, and doses of Pam3CSK4 between 0.5-10 μg/mL resulted in similar levels of proliferation. Poly I:C did not have a strong effect on induction of CD80 or proliferation, but could increase CD40 expression modestly at doses ≥ 10 μg/mL. For poly I:C, we selected the dose of 25 μg/mL.

3.1.2.2 Expression of surface receptors are increased on B cells by stimulation with poly I:C and Pam3CSK4

We next evaluated the expression of CD40, CD80, CD86, MHC class II, CD25 and CD69 on B cells stimulated for 24 hours with poly I:C, Pam3CSK4 or the combination. These markers are important costimulatory receptors and have been reported to be increased following B cell stimulation with various TLR agonists [227]. Receptor expression was determined by flow cytometry. Stimulated B cells were also stained with respective isotype controls (Appendix Figure B.2). Gates were set around the population of cells that attained highest mean fluorescence intensity (MFI).

The CD40 receptor interacts with CD154 (CD40L) on Th cells to provide critical activation signals to B cells. Expression of CD40 on B cells cultured without adjuvants was 18.52 ± 6.34% (Figure 3.3A). Similar to the preliminary dosing experiment, poly I:C induced expression of CD40 on 51.59 ± 10.74% of the cells and Pam3CSK4 induced expression on 82.12 ± 4.68% of the cells. The combination of poly I:C and Pam3CSK4 resulted in the highest expression of CD40, 88.32 ± 3.16%, although this was not

significantly greater than Pam3CSK4 alone. The MFI results correlated with the percent positive (Figure 3.3B).

CD80 (B7-1) and CD86 (B7-2) are related costimulatory molecules expressed by APCs and interact with CD28 on T cells to provide positive costimulation signals. The percent of B cells cultured without adjuvant expressing these molecules was low, 5.35 ± 0.81% CD80 and 3.95 ± 0.71% CD86 (Figure 3.4A & 3.5A). Poly I:C stimulation alone did not induce a statistically significant increase in CD80 expression, 8.95 ± 1.92%, but Pam3CSK4 increased CD80 on 21.59 ± 5.78% of the B cells (Figure 3.4A). The combination of poly I:C and Pam3CSK4 resulted in a substantial increase in CD80 expression, detected on 45.68 ± 5.37% of the cells, MFI results were consistent with these trends (Figure 3.4B). Expression of CD86 was increased to 53.72 ± 4.31% by poly I:C and to 44.35 ± 7.37% by Pam3CSK4 (Figure 3.5A). Poly I:C also induced a higher CD86 MFI compared to Pam3CSK4 (Figure 3.5B). The combination induced the highest levels of CD86 expression on 85.95 ± 3.23% of the cells.

MHC class II is also upregulated on activated B cells to allow antigen presentation to $CD4^+$ T cells. B cells cultured without adjuvant had low to medium expression of MHC class II (Figure 3.6A), and only 23.28 ± 2.97% of the cells expressed a high level of expression (MFI >500). Poly I:C alone significantly increased the percent of cells expressing high levels of MHC class II, to 77.14 ± 3.25%, as did Pam3CSK4, to 93.97 ± 1.03%. The combination of poly I:C and Pam3CSK4 did not increase the number of cells expressing high levels of MHC class II significantly more than Pam3CSK4 alone, 95.68 ± 0.49%. These results are consistent with the level of expression determined by MFI (Figure 3.6B)

CD25, the high affinity IL-2Rα chain, facilitates proliferation and antibody production by naïve B cells [484, 485]. Expression of CD25 on B cells cultured without adjuvant was low, 1.14 ± 0.19% positive (Figure 3.7A). Alone, poly I:C and Pam3CSK4 each had a modest effect on CD25 expression, increasing it respectively on 19.96 ± 4.54% and 25.50 ± 4.22% of B cells. The combination resulted in a substantial increase in CD25 expression on 77.64 ± 2.28%% of the B cells, which was also reflected in the MFI values (Figure 3.7B).

Expression of CD69 is induced quickly after activation of B cells, as well as T cells and NK cells [486]. CD69 signaling contributes to lymphocyte cytokine expression, migration and proliferation. Expression of CD69 on B cells cultured without adjuvant was low, 5.94 ± 1.10% positive (Figure 3.8A). Poly I:C induced expression of CD69 on 38.37 ± 3.99% of B cells, and Pam3CSK4 induced CD69 expression on 81.51 ± 2.77% of B cells. The combination of poly I:C and Pam3CSK4 resulted in the highest level of expression of CD69 on 94.32 ± 1.32% of the B cells. Similar trends were seen in the expression level as determined by MFI expression (Figure 3.8B).

3.1.2.3 Production of cytokines following B cell stimulation with poly I:C and/ or Pam3CSK4

Stimulation of B cells with TLR agonists has been reported to increase production of several cytokines [227, 250]. We screened for production of cytokines important to B cell survival and proliferation: IL-6, IL-10, IL-21 and TNF-α; Th1 cytokines: IL-12p70 and IFN-γ; and Th2 cytokine: IL-4. Since poly I:C is also known to induce CXCL10 production in other cell types [487] and CpG stimulation of B cells induces CXCL10 [488], we also tested for production of the chemokine CXCL10. We could only detect production of IL-6, TNF-α and CXCL10 (Figure 3.9), the remaining cytokines were below the limits of detection (data not shown). B cells cultured without agonists had undetectable or low levels of IL-6, TNF-α and CXCL10. Neither poly I:C nor Pam3CSK4 induced statistically significant levels of IL-6 production (<30 pg/ mL), but, strikingly, the concentration of IL-6 in the supernatants of combination stimulated B cells was increased to 1078 ± 280 pg/ mL (Figure 3.9A). Production of TNF-α induced by either agonist alone was low, <10 pg/ mL, but the combination induced a statistically significant increase in TNF-α production to 39 ± 6 pg/mL (Figure 3.9B). Poly I:C alone could induce a statistically significant increase in CXCL10 production, 127 ± 16 pg/ mL, however production of CXCL10 to Pam3CSK4 was <10 pg/mL (Figure 3.9C). The combination of poly I:C and Pam3CSK4 resulted in highest levels of CXCL10, at 432 ± 34 pg/ mL.

IL-10, IL-12 and IFN-γ production have been reported to be increased by TLR stimulation of murine B cell by others [227]. To confirm that cytokines were not being

expressed at a later time point 4 day supernatants were also tested; however, these cytokines were still not detected (data not shown).

3.1.2.4 Poly I:C enhances Pam3CSK4 induced B cell proliferation

Pam3CSK4 is known to be a potent inducer of B cell proliferation [299], and poly I:C and Pam3CSK4 induced CD69 expression (Figure 3.8) which is often indicative of proliferation [489]. B cell proliferation was measured by [^{3}H]-TdR uptake after 3 day (72 hour) stimulation with poly I:C at 25 μg/mL and Pam3CSK4 at 1 μg/mL (Figure 3.10). B cells cultured without agonist had low proliferation rate (1,708 ± 200 CPM). The proliferation induced by poly I:C alone was slightly higher (6,200 ± 656 CPM), although was not significant compared to background. Consistent with literature, Pam3CSK4 induced a statistically significant increase in proliferation (85,300 ± 3,628 CPM, $p<0.001$). However, the combination induced the highest level of proliferation (106,500 ± 5,669 CPM), which was statistically significant compared to either adjuvant alone ($p<0.001$).

3.1.3 TLR3 and TLR2 are essential to optimal B cell activation by poly I:C and Pam3CSK4

The major receptor for poly I:C is TLR3 and the major receptor for Pam3CSK4 is TLR1/2; the expression of both of these receptors has been reported on B cells [223, 227]. To determine if poly I:C and Pam3CSK4 were in fact exerting their effects on B cells through these receptors, we stimulated B cells isolated from TLR2 and TLR3 knockout mice. Signaling through TLR1/2 heterodimer is primarily mediated by TLR2, therefore we utilized TLR2$^{-/-}$ mice to confirm stimulation with Pam3CSK4 [298]. B cells were stimulated with poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL), or the combination of poly I:C and Pam3CSK4. Relevant wild-type B cells were stimulated in parallel. To confirm that TLR intracellular signaling pathways remained intact, wild-type and knockout B cells were also stimulated with the TLR4 agonist LPS (10 μg/mL) and TLR9 agonist CpG (25 μg/mL). After 24 hours, B cells were analysed by flow cytometry for expression of CD80, CD86, CD40, MHC class II and CD25. Supernatants were also harvested after 24 hours for detection of cytokines. Proliferation was measured after 3 days by [^{3}H]-TdR uptake.

TLR3$^{-/-}$ B cells exhibited impaired expression of all surface markers in response to poly I:C stimulation, most noticeably CD86 (Figure 3.11C), MHC class II (Figure 3.11D) and CD25 (Figure 3.11E) which were all strongly increased in wild-type B cells. Poly I:C stimulation also failed to induce detectable CXCL10 production by TLR3$^{-/-}$ B cells, although wild-type B cells significantly enhanced production this chemokine in response to poly I:C (Figure 3.12C). Proliferation to poly I:C was low in both TLR3$^{-/-}$ and wild-type B cells (Figure 3.13). In response to the poly I:C and Pam3CSK4 combination, TLR3$^{-/-}$ B cells increased activation markers to a level equivalent to Pam3CSK4 alone (Figure 3.11). Production of IL-6, TNF-α and CXCL10 by TLR3$^{-/-}$ B cells were not enhanced by stimulation with the poly I:C and Pam3CSK4 combination, as observed in wild-type B cells (Figure 3.12). Proliferation of TLR3$^{-/-}$ B cells to poly I:C was low but detectable (3,438 ± 317 CPM), not significantly different from wild-type B cell proliferation to poly I:C (5,864 ± 1,149 CPM). Proliferation to Pam3CSK4 was nearly identical in both B cells (27,490 ± 3,024 CPM in TLR3$^{-/-}$ and 27,180 ± 5,273 CPM in wild type). However, proliferation of TLR3$^{-/-}$ B cells to the poly I:C/ Pam3CSK4 combination was (45,950 ± 2,618 CPM) significantly higher than wild type B cell proliferation to the combination (35,150 ± 2,390 CPM, $p<0.05$). The knockout of TLR3 was confirmed by PCR using spleen samples taken during necropsy (Appendix Figure B.3). Stimulation with LPS and CpG generated comparable receptor expression (Figure 3.17A) and proliferation (Figure 3.17B) in TLR3$^{-/-}$ and wild type B cells, indicating no aberrations in TLR signaling pathways.

TLR2$^{-/-}$ B cells had significantly reduced expression of CD40 (Figure 3.14A), CD80 (Figure 3.14B), MHC class II (Figure 3.14D) and CD25 (Figure 3.14E) activation markers relative to wild-type controls in response to stimulation with Pam3CSK4 alone. CD86 expression in response to Pam3CSK4 expression was reduced in TLR2$^{-/-}$ B cells; however, the result was not statistically significant compared to wild type (Figure 3.14C). This is probably due to variation in response in the wild-type mice. Production of TNF-α (Figure 3.15A) and CXCL10 (Figure 3.15B) by wild-type and TLR2$^{-/-}$ B cells was low in response to Pam3CSK4; IL-6 production was not assessed. While wild type B cells proliferated strongly to Pam3CSK4 (46,890 ± 2,565 CPM), proliferation of TLR2$^{-/-}$ B cells to Pam3CSK4 was not statistically significant compared to the no adjuvant

background (191 ± 11 CPM; Figure 3.16). Likewise, proliferation of TLR2$^{-/-}$ B cells to poly I:C/ Pam3CSK4 combination was not higher than to poly I:C alone (916 ± 281 CPM and 1,113 ± 302 CPM respectively). Stimulation with LPS and CpG generated comparable receptor expression (Figure 3.17C) and proliferation (Figure 3.17D) in TLR2$^{-/-}$ and wild type B cells, indicating no aberrations in TLR signaling pathways

3.1.4 Poly I:C and Pam3CSK4 activate NFκB signaling pathways

NFκB is a major transcription factor activated by TLR signaling that directly and indirectly induces a pro-inflammatory phenotype [490]. Since TLR3 and TLR2 induce NFκB through different cytosolic signaling pathways, namely TRIF and MyD88 respectively, it seemed possible that they were working in concert to increase NFκB activation [307]. A previous study documented that TLR4 and TLR9 signaling of B cells can increase phosphorylation of the inhibitory protein IκBα, leading to phosphorylation of p65 [217]. To determine if this pathway was being activated by poly I:C and Pam3CSK4 combination signaling, we examined the total levels and phosphorylation status of p65 and IκBα using western blot analysis. Cell lysates were prepared at 15, 20, 25 and 30 minutes to detect rapid phosphorylation of each protein which occurs after TLR stimulation. Cell lysates were also prepared at later time points, 1, 2, 3 and 4 hours, in order to monitor degradation and regeneration of total IκBα. Densitometry results were normalized to β-actin, then to levels in unstimulated B cells.

Stimulation with poly I:C, Pam3CSK4 or the combination resulted in rapid phosphorylation of p65 (Figure 3.18A). Level of total p65 remained unchanged during early (not shown) and late time points (Figure 3.18B). Although not statistically significant, Pam3CSK4 tended to induce the quickest and highest levels of phospho-p65, peaking at 25 minutes, although by 30 minutes the level of phospho-p65 was the same for all three treatments.

Pam3CSK4 also induced rapid phosphorylation of IκBα (Figure 3.19A), which peaked at 15 minutes. However, by 30 minutes all three treatments had similar levels of phospho-IκBα. Phosphorylation of IκBα led to subsequent reduction of total IκBα for all three treatment conditions, however the level of total IκBα persisted for the duration of the time points (Figure 3.19B). The data generated from these three experiments did not

reach statistical significance; however, the trends indicate that stimulation with the poly I:C and Pam3CSK4 combination may result in prolonged reduction in the inhibitor IκBα.

3.1.5 Poly I:C and Pam3CSK4 enhance T-dependent B cell activation

TLR stimulation has been reported to synergize with T-dependent B cell activation *in vitro* [217, 258], and is more representative of B cell activation *in vivo*; therefore, we tested the effects of poly I:C and/ or Pam3CSK4 stimulation of B cells activated in a T-dependent manner. T-dependent activation was simulated by adding anti-Ig and anti-CD40 into cultures. After 24 hour incubation, expression of surface receptors was assessed by flow cytometry (Figure 3.20). Poly I:C and Pam3CSK augmented the expression of CD80 (Figure 3.20A), CD86 (Figure 3.20B) and CD25 (Figure 3.20C) and the combination of both agonists induced the highest levels of these receptors. We found that MHC class II (Figure 3.20D) and CD69 (Figure 3.20E) were strongly induced by T-dependent activation, and were not further enhanced by TLR stimulation. The effects on CD40 expression could not be reliably detected due to the blocking effect of the anti-CD40 antibody used for T-dependent activation (data not shown).

Twenty-four hour supernatants were also screened for IL-4, IL-6, IL-10, IL-12p70, IL-21, IFN-γ, TNF-α and CXCL10. As with T-independent B cell stimulation, we could only detect production of IL-6, TNF-α and CXCL10 (Figure 3.21), the remaining cytokines were below the limits of detection (data not shown). Production of IL-6 was not enhanced over background by either agonist alone (<60 pg/mL), but in response to the combination IL-6 levels were increased to 376 ± 125 pg/mL ($p<0.01$; Figure 3.21A). TNF-α was not induced by either agonist alone (<10 pg/mL); however, the combination induced TNF-α production to 53 ± 11 pg/mL ($p<0.001$; Figure 3.21B). Poly I:C induced a statistically significant increase in CXCL10, 101 ± 30 pg/mL, but Pam3CSK4 did not, <10 pg/mL (Figure 3.21C). The combination of poly I:C and Pam3CSK4 induced the highest levels of CXCL10, 164 ± 30 pg/mL, although this was not statistically significant compared to poly I:C alone.

Finally, we measured how the proliferation of B cells with T-dependent activation was affected by the adjuvants (Figure 3.22). After 3 days of culture, the proliferation

induced by T-dependent stimulation in the absence of agonists was higher (19,990 ± 2,195 CPM), although not significant, than proliferation of B cells cultured without stimulation (1,951 ± 222 CPM). Poly I:C did not augment proliferation induced by T-dependet activation (16,580 ± 1,962 CPM). Pam3CSK4 resulted in a statistically significant increase in proliferation, 140,800 ± 8,098 CPM, compared to poly I:C. The proliferation of B cells stimulated with poly I:C and Pam3CSK4 combination (137,300 ± 8,424 CPM) was not statistically higher than Pam3CSK4 alone.

3.1.5.1 Poly I:C and Pam3CSK4 enhance B cell induced activation of $CD4^+$ T cells

Stimulation of B cells with poly I:C and Pam3CSK4 resulted in increased expression of several costimulatory molecules involved in T cell activation, such as CD80 and CD86. We therefore tested whether poly I:C and Pam3CSK4 activated B cells could more efficiently stimulate $CD4^+$ T cell proliferation using an allogeneic model (Figure 3.23). B cells were isolated from a C57BL/6 mouse and cultured overnight with T-dependent activation, provided by anti-Ig and anti-CD40, as well as stimulation with poly I:C and/ or Pam3CSK4, as in preceding experiments. After 24 hours, the B cells were inactivated with mitomycin C treatment, then washed four times with complete RPMI medium. B cells were setup in co-culture with $CD4^+$ T cells isolated from BALB/c mice at ratios of 1:10, 1:25 and 1:50, holding the number of T cells constant. Proliferation of $CD4^+$ T cells was measured after 3 days by [^{3}H]-TdR uptake, and IL-2 detected in supernatant by ELISA. After 24 hours, expression of CD69 and CD25 was measured by flow cytometry.

At the 1:10 ratio, $CD4^+$ T cell proliferation induced by B cells cultured without agonists was 8,578 ± 1,973 CPM (Figure 3.23). B cells stimulated with poly I:C or Pam3CSK4 alone induced T cell proliferation of 29,850 ± 3,611 CPM and 40,730 ± 2,512 CPM, respectively, and B cells stimulated with the combination induced T cell proliferation of 45,300 ± 7,152 CPM. Although not statistically significant, the proliferation induced by B cells activated with the combination of poly I:C and Pam3CSK4 was most efficient at inducing $CD4^+$ T cell proliferation at all three ratios tested. $CD4^+$ T cell activation was confirmed by increased expression of CD69 and

CD25 (Figure 3.24 A&B), as well as levels of IL-2 detected in co-culture supernatants (Figure 3.24C).

3.1.5.2 B cell differentiation into antibody producing plasma cells is enhanced by poly I:C and Pam3CSK4

To determine if poly I:C and Pam3CSK4 could promote B cell differentiation into antibody secreting cells, we looked for markers of plasma cell differentiation on B cells that had been stimulated for 24 hours with poly I:C and/ or Pam3CSK4 with T-dependent activation. The combination treatment resulted in the highest levels of surface receptors associated with plasma cell differentiation, CD138 and TACI (CD267) (Figure 3.25 A&B). Supernatants were harvested after 4 days of proliferation to measure secretion of IgG (Figure 3.25C). B cells activated without TLR agonists or with poly I:C alone induced low levels of IgG, <1 ng/mL. Pam3CSK4 induced a statistically significant increase production of IgG, 37 ± 7 ng/mL ($p<0.05$). The combination of poly I:C and Pam3CSK4 induced the highest production of IgG, 92 ± 13 ng/mL ($p<0.001$). Notably, IgG was not detected in the supernatants of B cells stimulated with poly I:C and/ or Pam3CSK4 without T-dependent activation (data not shown).

3.1.6 Protein vaccines adjuvanted with poly I:C and Pam3CSK4 produce highest levels of antibodies *in vivo*

Having demonstrated that poly I:C and Pam3CSK4 could augment T-dependent B cell activation *in vitro*, resulting in enhanced function and differentiation into antibody secreting cells, we sought to determine if these adjuvants could be used to boost an antibody response to vaccination *in vivo*. We evaluated the poly I:C and Pam3CSK4 adjuvant system by first performing dose-response testing with DepoVax vaccine containing recombinant protective antigen (rPA), the target antigen for anthrax vaccines (DPX-rPA). Poly I:C was tested at 1, 5 and 10 μg doses and Pam3CSK4 at 1, 2.5 and 5 μg doses (Figure 3.26A, B). Serum endpoint titers were determined at 4 and 8 weeks following a single immunization. We found that both adjuvants could enhance antibody production to the vaccine in a dose-dependent manner. We selected a dose of 1 μg for each for a combination study, since this was the lowest dose tested that provided a minimal increase in serum titers after vaccination. We found that vaccines prepared with

the combination of poly I:C and Pam3CSK4 generated significantly enhanced antigen-specific titers at 8 weeks post vaccination, compared to vaccine prepared without adjuvant or with single adjuvants (Figure 3.26C).

We next evaluated poly I:C/ Pam3CSK4 adjuvant system using an influenza recombinant hemagglutinin (rHA) antigen prepared in DepoVax (DPX-rHA) (Figure 3.27). Due to limited supply of antigen, we did not repeat the dosing study but used the 1 μg dose combination that augmented response to the rPA antigen. Again, we found that the poly I:C/ Pam3CSK4 adjuvant system yielded serum titers that were significantly higher than either adjuvant in as little as 4 weeks following a single immunization, and were maintained until 16 weeks when the study was terminated.

3.2 Table & Figures

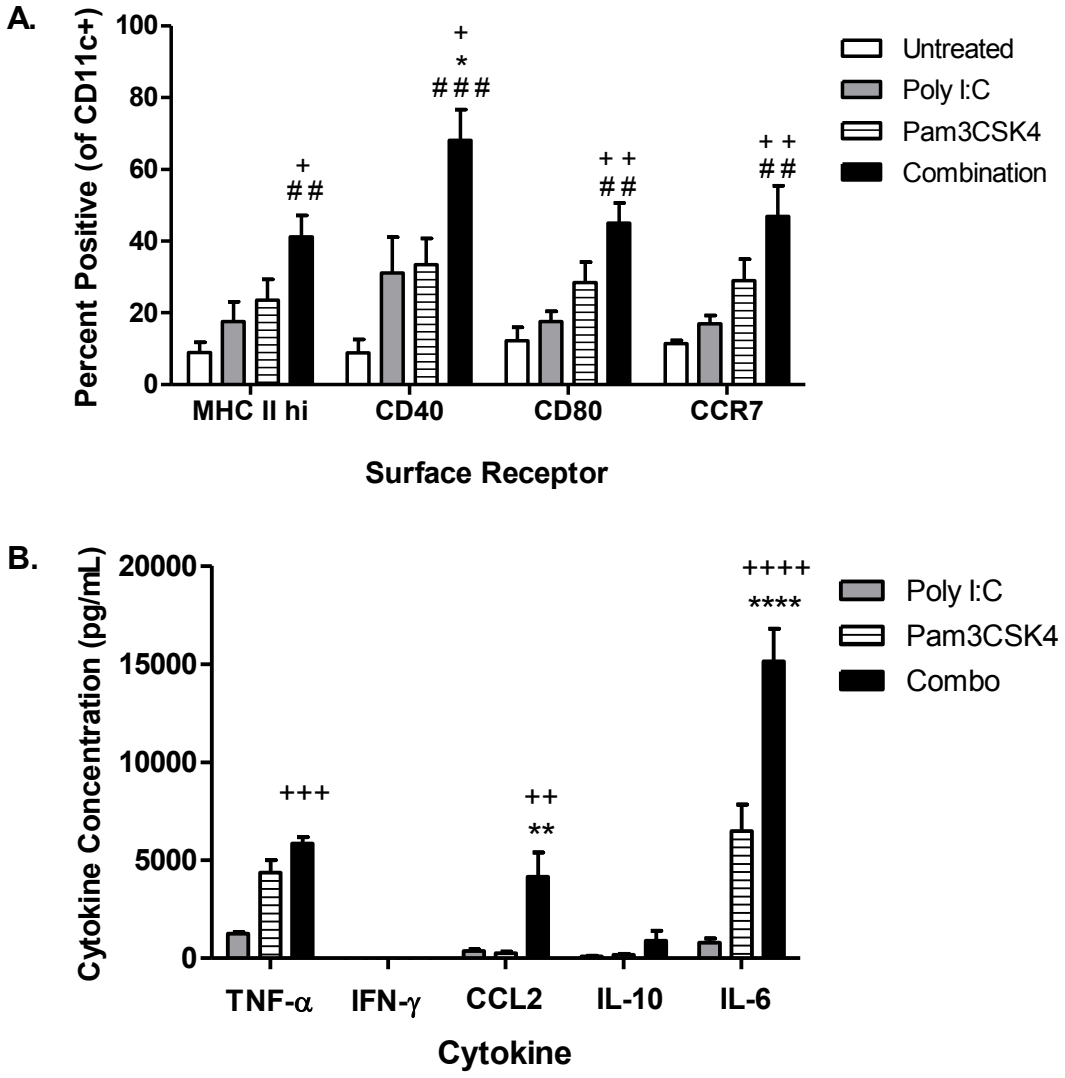


Figure 3.1: Stimulation of dendritic cells with the combination of Poly I:C and Pam3CSK4 increases activation. Dendritic cells (DCs) were cultured from naïve C57BL/6 bone marrow cells and matured for 7 days in the presence of GM-CSF. On day 7, DCs were treated with poly I:C (5 μg/mL), Pam3CSK4 (10 μg/mL) or a combination of both. **(A)** Cells were harvested on day 8 and analysed for surface marker expression by flow cytometry (n=5-7). Cells gated on $CD11c^+$ events. **(B)** Cell supernatants were analysed for cytokine levels using Cytokine Bead Array kit (n=3-4). Data shown as mean ± SEM, statistics by ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C, "*" compared to Pam3CSK4.

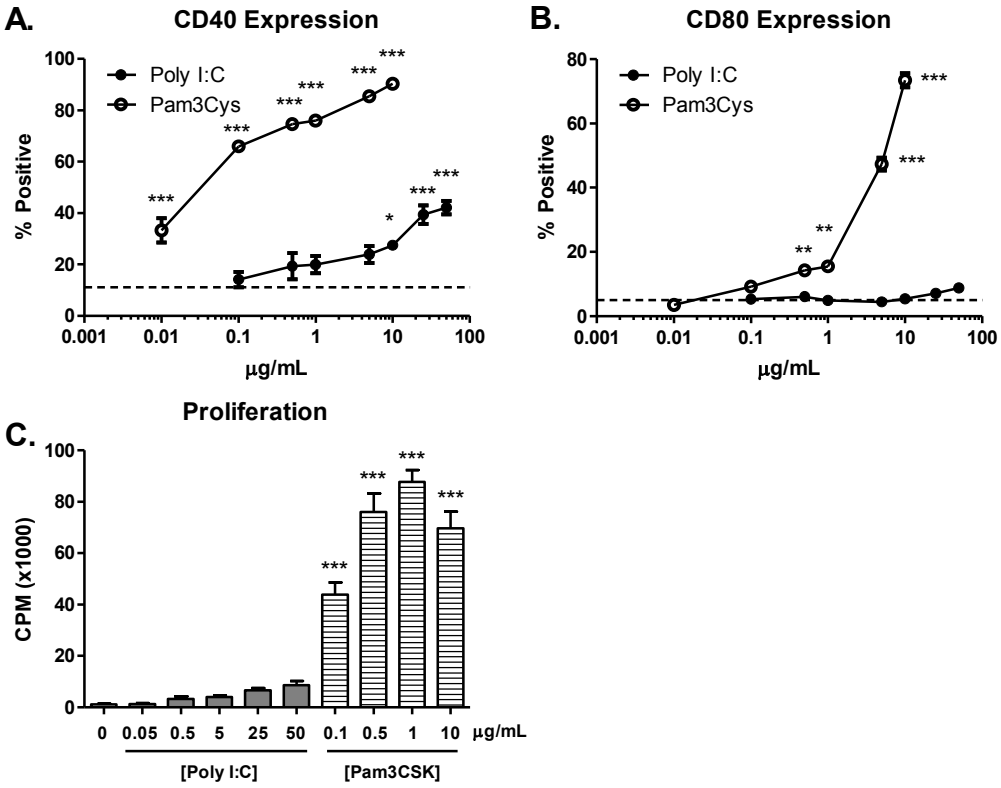


Figure 3.2: Stimulation of B cells with Poly I:C or Pam3CSK4 enhances expression of activation markers and proliferation in a dose-dependent manner. B cells were isolated from the spleens of naïve C57BL/6 mice (n=3) and stimulated with various concentrations of Poly I:C or Pam3CSK4. Expression of CD40 **(A)** and CD80 **(B)** after 24 hours stimulation (n=3), dashed line indicates level of unstimulated B cells. **(C)** Proliferation measured after 3 days by [^{3}H]-TdR uptake (n=2-7). Data shown as mean ± SEM, statistics by one-way ANOVA with Tukey post-test comparing each dose to unstimulated, *p≤0.05, **p<0.01, ***p<0.001.

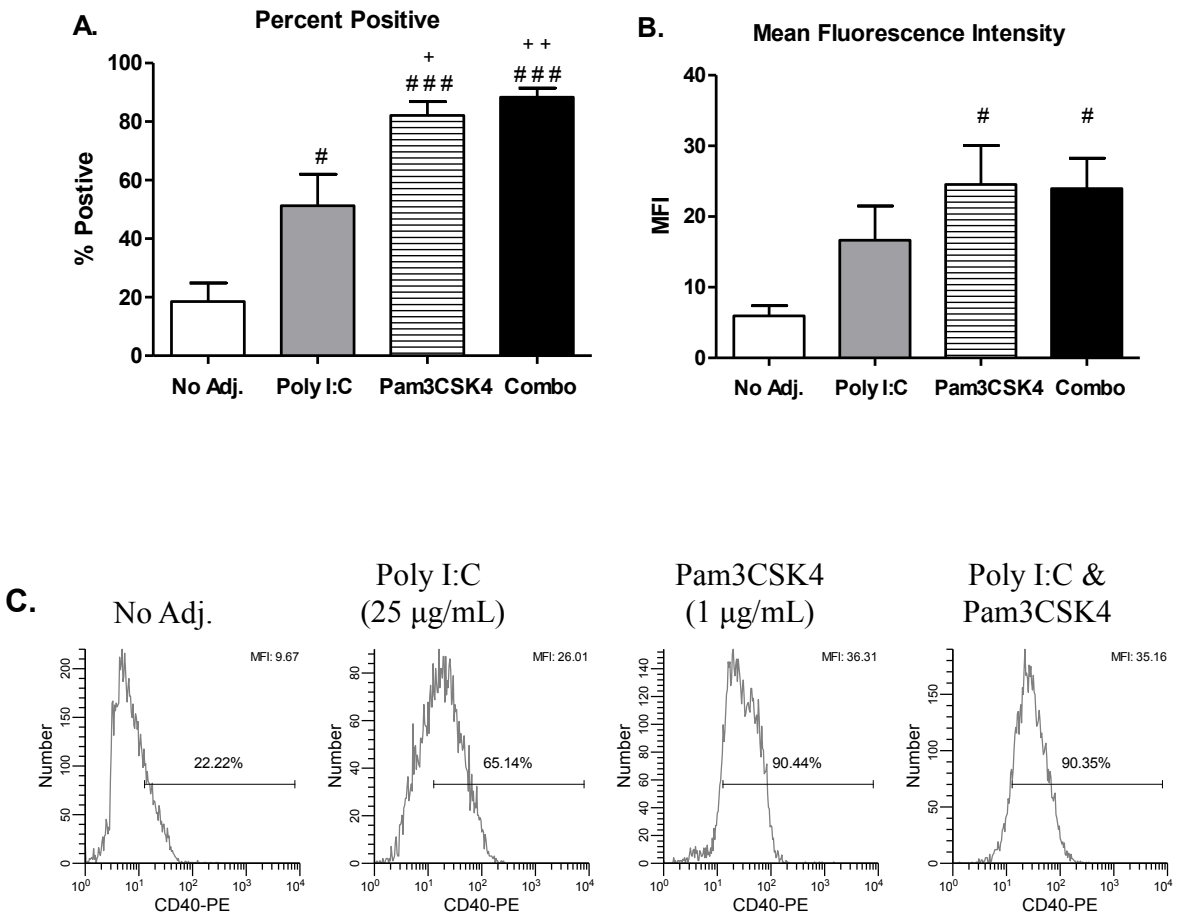


Figure 3.3: B cell expression of CD40 after 24 hour stimulation with poly I:C and/ or Pam3CSK4. B cells were purified from spleen of naïve C57BL/6 mouse and cultured overnight in the presence of adjuvants at indicated concentrations. Surface expression of CD40 was determined by flow cytometry after staining with PE conjugated mAb (clone 1C10). **(A)** Average percent positive and **(B)** average mean fluorescence intensity of 6 samples, data shown as mean ± SEM, statistics by ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant. **(C)** Representative histograms with mean fluorescence intensity of x-axis and percent positive as gated.

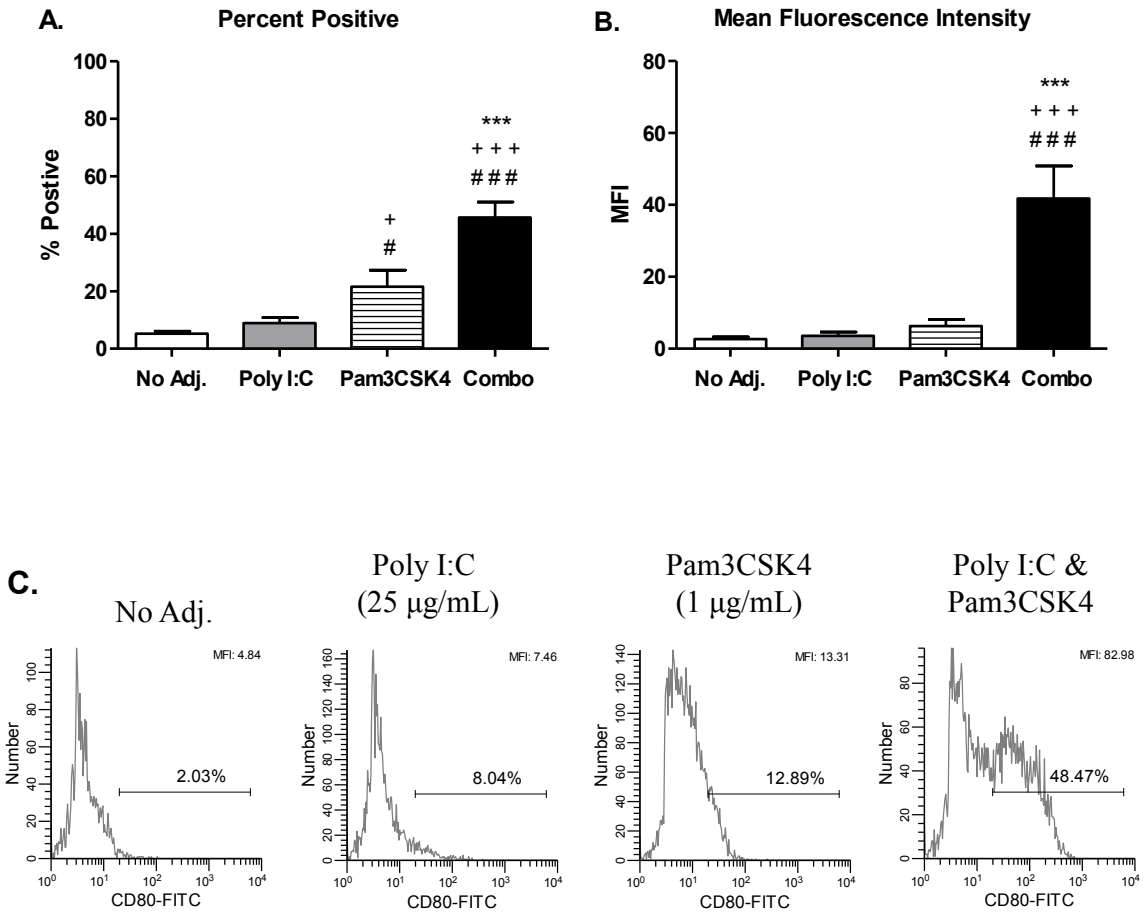


Figure 3.4: B cell expression of CD80 after 24 hour stimulation with poly I:C and/ or Pam3CSK4. B cells were purified from spleen of naïve C57BL/6 mouse and cultured overnight in the presence of adjuvants at indicated concentrations. Surface expression of CD80 was determined by flow cytometry after staining with FITC conjugated mAb (clone 1610A1). **(A)** Average percent positive and **(B)** average mean fluorescence intensity of 7-10 samples, data shown as mean ± SEM, statistics by ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant. **(C)** Representative histograms with mean fluorescence intensity of x-axis and percent positive as gated.

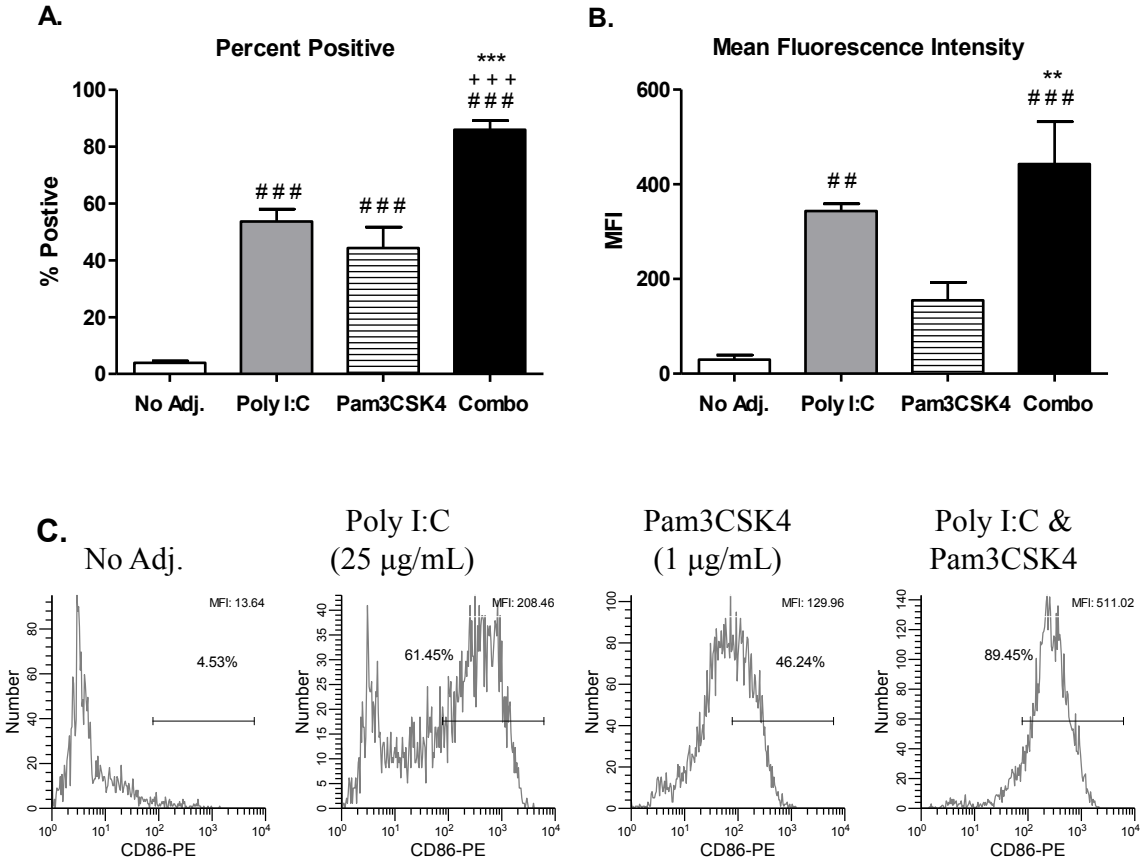


Figure 3.5: B cell expression of CD86 after 24 hour stimulation with poly I:C and/ or Pam3CSK4. B cells were purified from spleen of naïve C57BL/6 mouse and cultured overnight in the presence of adjuvants at indicated concentrations. Surface expression of CD86 was determined by flow cytometry after staining with PE conjugated mAb (clone GL1). **(A)** Average percent positive and **(B)** average mean fluorescence intensity of 5 samples, data shown as mean ± SEM, statistics by ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant. **(C)** Representative histograms with mean fluorescence intensity of x-axis and percent positive as gated.

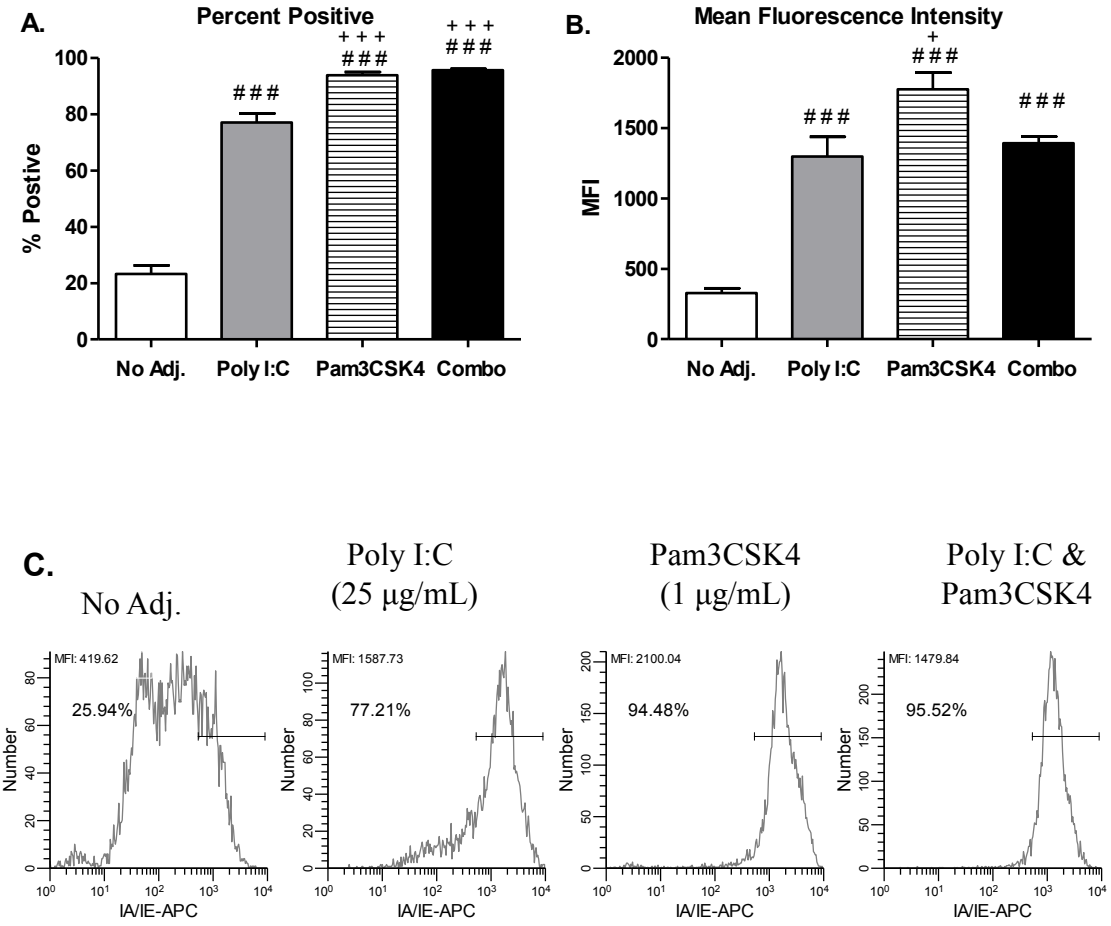


Figure 3.6: B cell expression of MHC class II after 24 hour stimulation with poly I:C and/ or Pam3CSK4. B cells were purified from spleen of naïve C57BL/6 mouse and cultured overnight in the presence of adjuvants at indicated concentrations. Surface expression of MHC II (I-A/I-E) was determined by flow cytometry after staining with APC conjugated mAb (clone M5/114.15.2). **(A)** Average percent positive and **(B)** average mean fluorescence intensity of 5 samples, data shown as mean ± SEM, statistics by ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant. **(C)** Representative histograms with mean fluorescence intensity of x-axis and percent positive as gated.

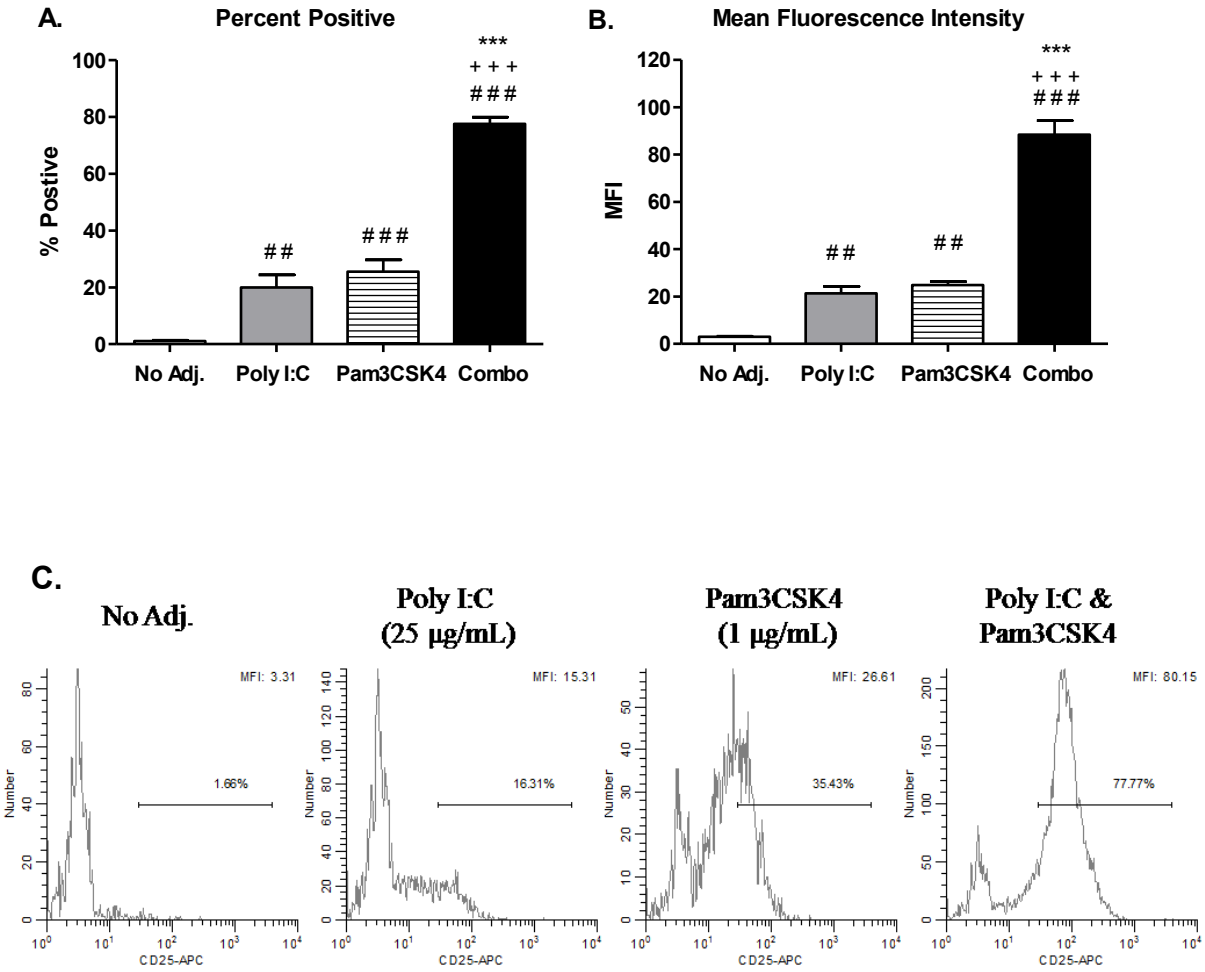


Figure 3.7: B cell expression of CD25 after 24 hour stimulation with poly I:C and/ or Pam3CSK4. B cells were purified from spleen of naïve C57BL/6 mouse and cultured overnight in the presence of adjuvants at indicated concentrations. Surface expression of CD25 was determined by flow cytometry after staining with APC conjugated mAb (clone PC61.5). **(A)** Average percent positive and **(B)** average mean fluorescence intensity of 5 samples, data shown as mean ± SEM, statistics by ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant. **(C)** Representative histograms with mean fluorescence intensity of x-axis and percent positive as gated.

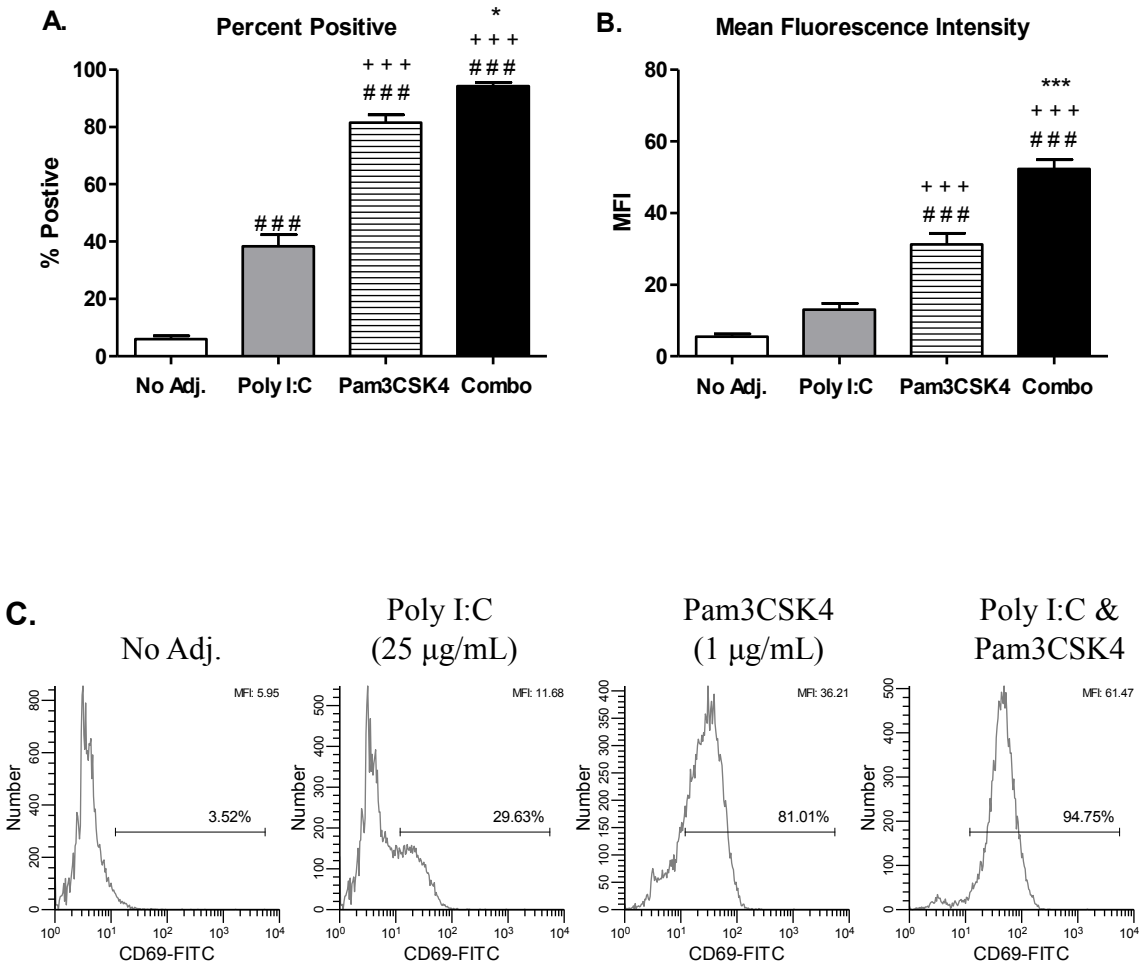


Figure 3.8: B cell expression of CD69 after 24 hour stimulation with poly I:C and/ or Pam3CSK4. B cells were purified from spleen of naïve C57BL/6 mouse and cultured overnight in the presence of adjuvants at indicated concentrations. Surface expression of CD25 was determined by flow cytometry after staining with APC conjugated mAb. **(A)** Average percent positive and **(B)** average mean fluorescence intensity of 6 samples, data shown as mean ± SEM, statistics by ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant. **(C)** Representative histograms with mean fluorescence intensity of x-axis and percent positive as gated.

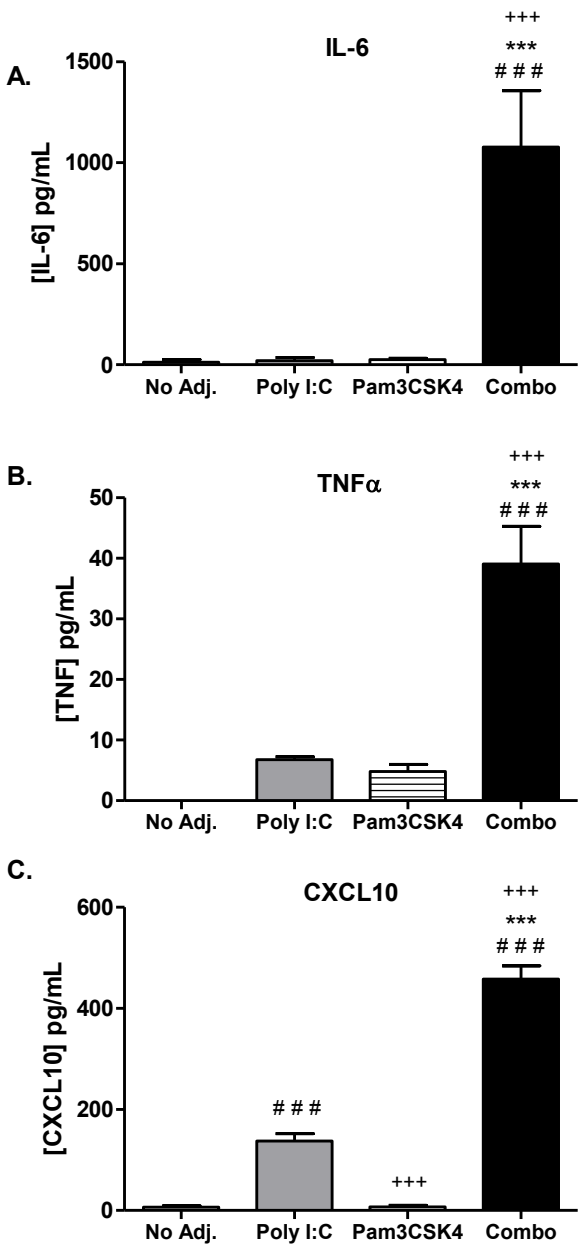


Figure 3.9: Stimulation of B cells with the combination of Poly I:C and Pam3CSK4 increases cytokine and chemokine secretion. B cells were purified from spleen of naïve C57BL/6 mouse and cultured overnight in the presence of poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL) or combination of both. Supernatants harvested after 24 hours and levels of **(A)** IL-6 (n=5), **(B)** TNF-α (n=6), or **(C)** CXCL10 (n=5) were detected by ELISA or CBA. Data shown are mean of separate samples ± SEM, statistics by ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant.

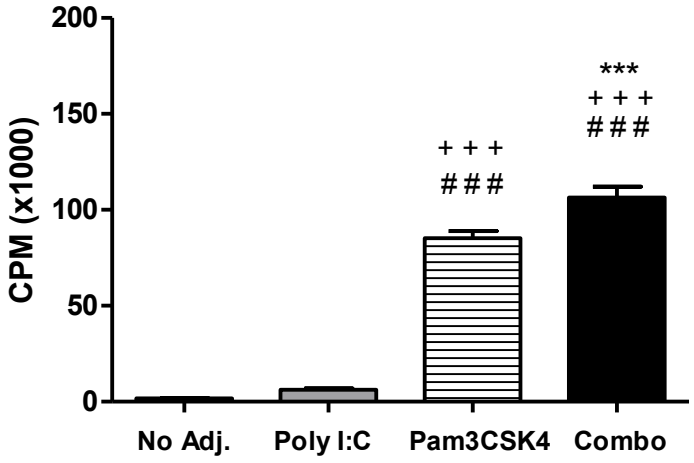


Figure 3.10: Stimulation of B cells with the combination of Poly I:C and Pam3CSK4 increases proliferation. B cells were purified from spleen of naïve C57BL/6 mouse and cultured for 3 days in the presence of poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL) or combination of both. Proliferation measured by [^{3}H]-TdR uptake. Results are shown as mean of 9 separate samples ± SEM, statistics by ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant.

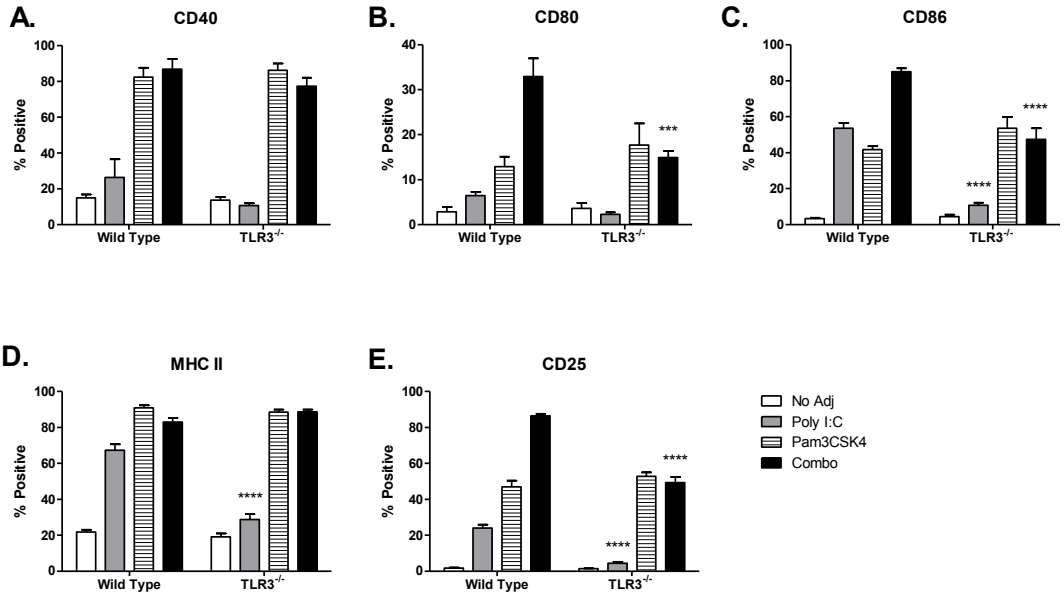


Figure 3.11: Poly I:C induces B cell expression of surface receptors through TLR3. B cells were purified from spleens of wild type (n=4) or TLR3 knockout mice (n=5). B cells were stimulated with poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL), or the combination of poly I:C and Pam3CSK4. After 24 hours, surface receptor expression was detected by flow cytometry. **(A)** CD40 **(B)** CD80 **(C)** CD86 **(D)** MHC class II **(E)** CD25. Data shown are mean ± SEM. Statistics by ANOVA with Bonferroni post-test comparing wild type to TLR3$^{-/-}$ B cell response, ***p<0.001, ****p<0.0001.

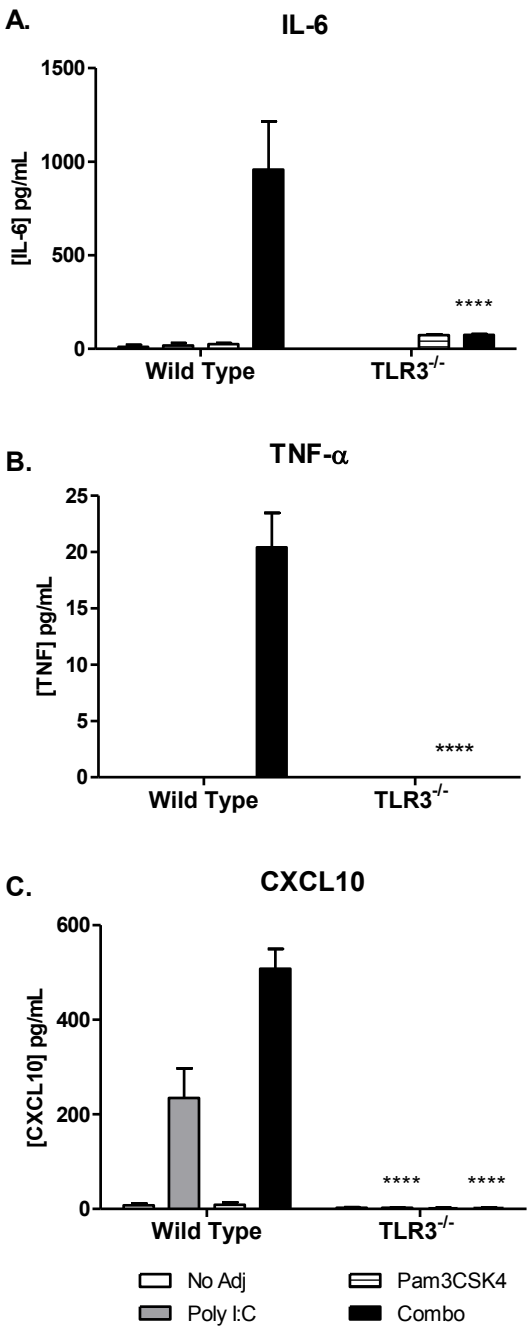


Figure 3.12: Poly I:C induces B cell cytokine production through TLR3. B cells purified from spleens of wild type (n=4-6) or TLR3$^{-/-}$ mice (n=4) were stimulated with poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL) or the combination**.** Supernatants were harvested after 24 hours and levels of **(A)** IL-6, **(B)** TNF-α, and **(C)** CXCL10 measured by ELISA. Data shown are mean ± SEM. Significance by ANOVA comparing wild type to TLR3$^{-/-}$, **** $p<0.0001$.

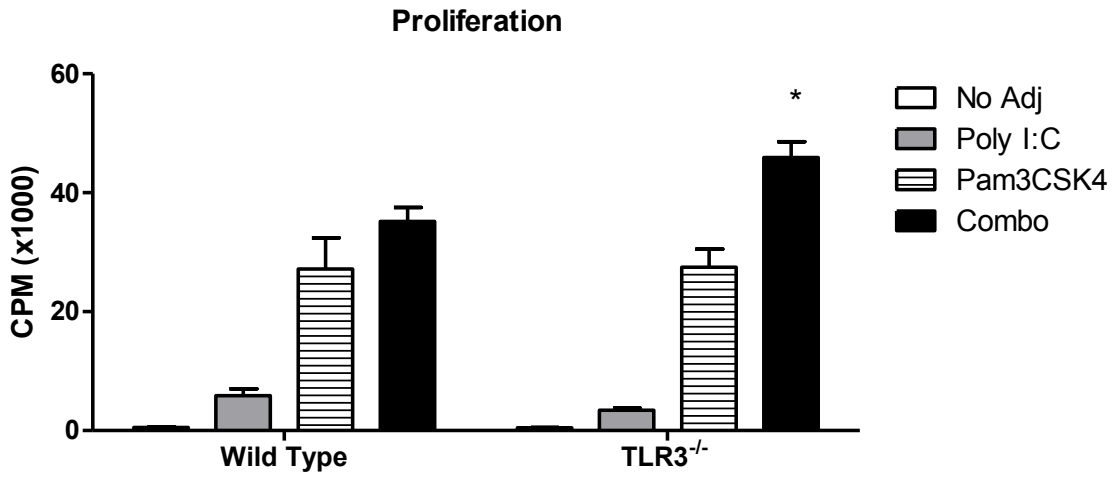


Figure 3.13: Poly I:C Induces B cell proliferation through TLR3. B cells were isolated from spleens of wild type (n=4) or TLR3$^{-/-}$ mice (n=6) and stimulated with poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL), or the combination of poly I:C and Pam3CSK4. Proliferation was assessed by [^{3}H]-TdR incorporation after 3 days. Results pooled from 3 independent experiments, data shown as mean ± SEM. Statistics by ANOVA: *$p<0.05$.

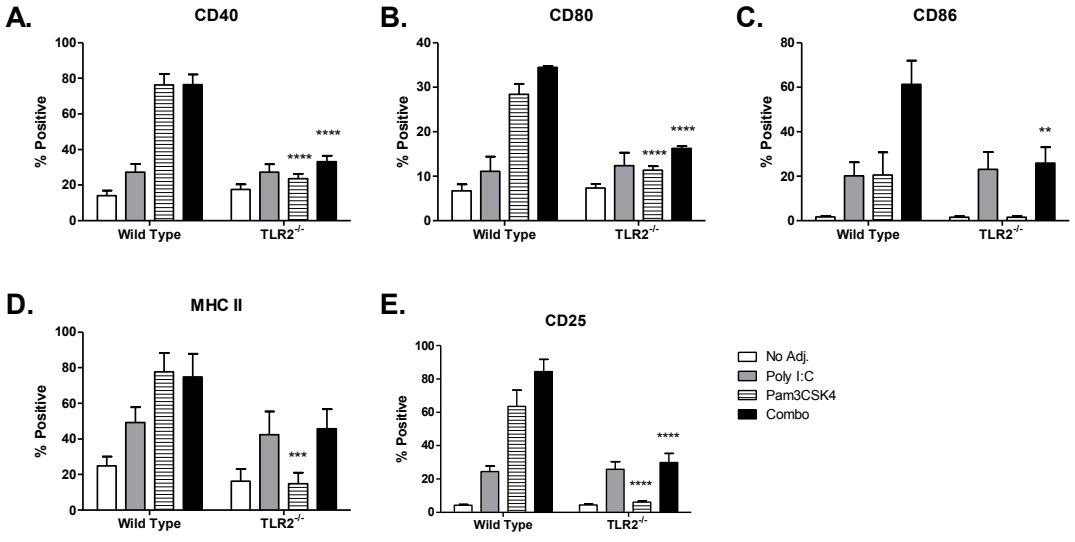


Figure 3.14: Pam3CSK4 induced B cell expression of surface receptors through TLR2. B cells purified from spleens of wild type (n=5) or TLR2$^{-/-}$ mice (n=4) were stimulated with poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL), or the combination of poly I:C and Pam3CSK4. After 24 hours, surface receptor expression was detected by flow cytometry. **(A)** CD40 **(B)** CD80 **(C)** CD86 **(D)** MHC class II **(E)** CD25. Data shown are mean ± SEM. Statistics by two-way ANOVA with Bonferroni post-test comparing wild type to TLR2$^{-/-}$ B cell response, **p<0.01, ***p<0.001, ****p<0.0001.

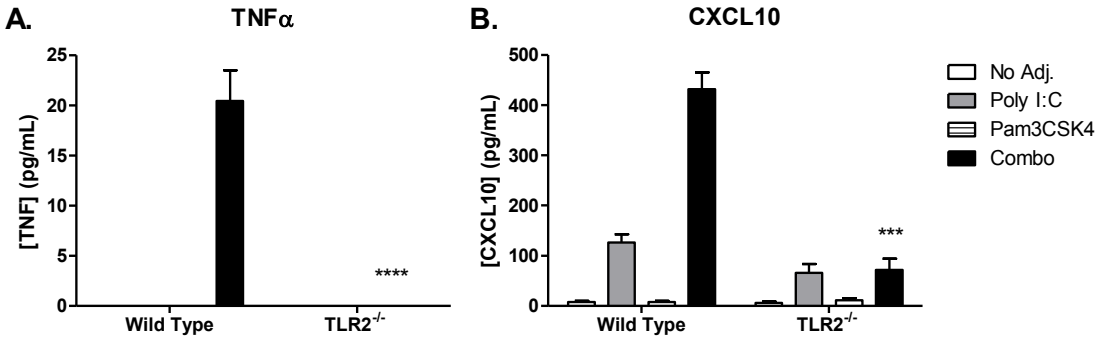


Figure 3.15: Pam3CSK4 induces B cell cytokine production through TLR2. B cells purified from spleens of wild type (n=5) or TLR2$^{-/-}$ mice (n=4) were stimulated with poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL) or the combination**.** Supernatants were harvested after 24 hours and levels of **(A)** TNFα and **(B)** CXCL10 measured by ELISA. Data shown are mean ± SEM. Significance by two-way ANOVA with Bonferroni post-test comparing wild type to TLR2$^{-/-}$, *** $p<0.001$, **** $p<0.0001$.

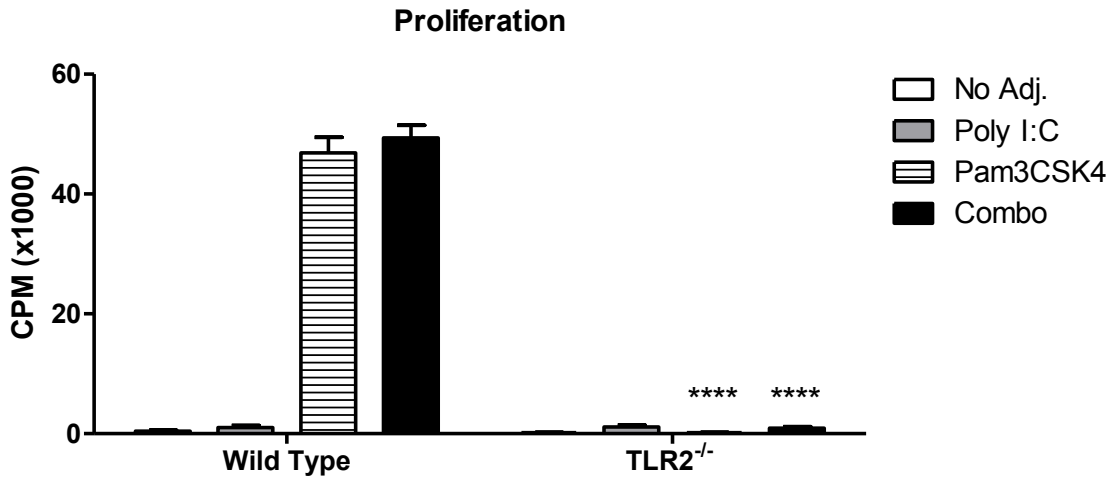


Figure 3.16: Pam3CSK4 induces B cell proliferation through TLR2. B cells purified from spleens of wild type (n=4) or TLR2$^{-/-}$ mice (n=4) were stimulated with poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL), or the combination of poly I:C and Pam3CSK4. Proliferation was assessed by [^{3}H]-TdR incorporation after 3 days. Results pooled from 2 independent experiments, data shown as mean ± SEM. Significance by two-way ANOVA with Bonferroni post-test comparing wild type to TLR2$^{-/-}$, **** $p<0.0001$.

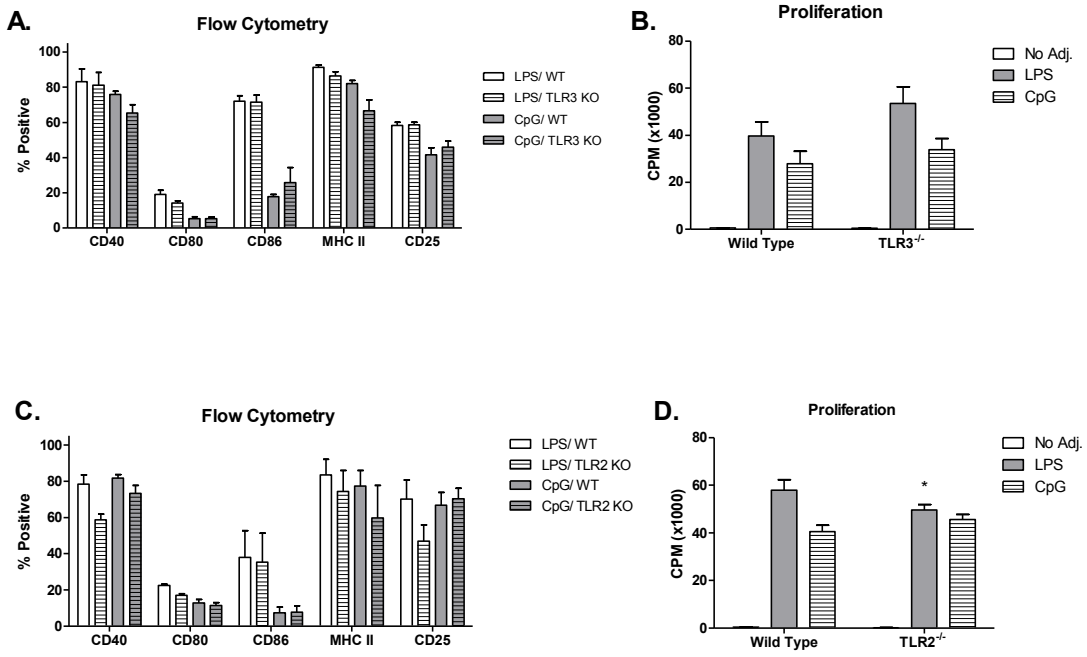


Figure 3.17: TLR3$^{-/-}$, TLR2$^{-/-}$ and wild type B cell response to LPS and CpG stimulation. B cells were isolated from TLR3$^{-/-}$ (n=6), TLR2$^{-/-}$ (n=4) or appropriate wild type control mice (n=4-5). B cells were stimulated with LPS (10 μg/mL) or CpG (25 μg/mL). Surface receptor expression determined by flow cytometry after 24 hour stimulation of TLR3$^{-/-}$ **(A)** and TLR2$^{-/-}$ **(C)** B cells. Proliferation was determined by [^{3}H]-TdR uptake after 3 days in TLR3$^{-/-}$ **(B)** and TLR2$^{-/-}$ **(D)** B cells. Data shown as mean ± SEM. Significance by two-way ANOVA with Bonferroni post-test comparing wild type to knockout, * $p<0.05$.

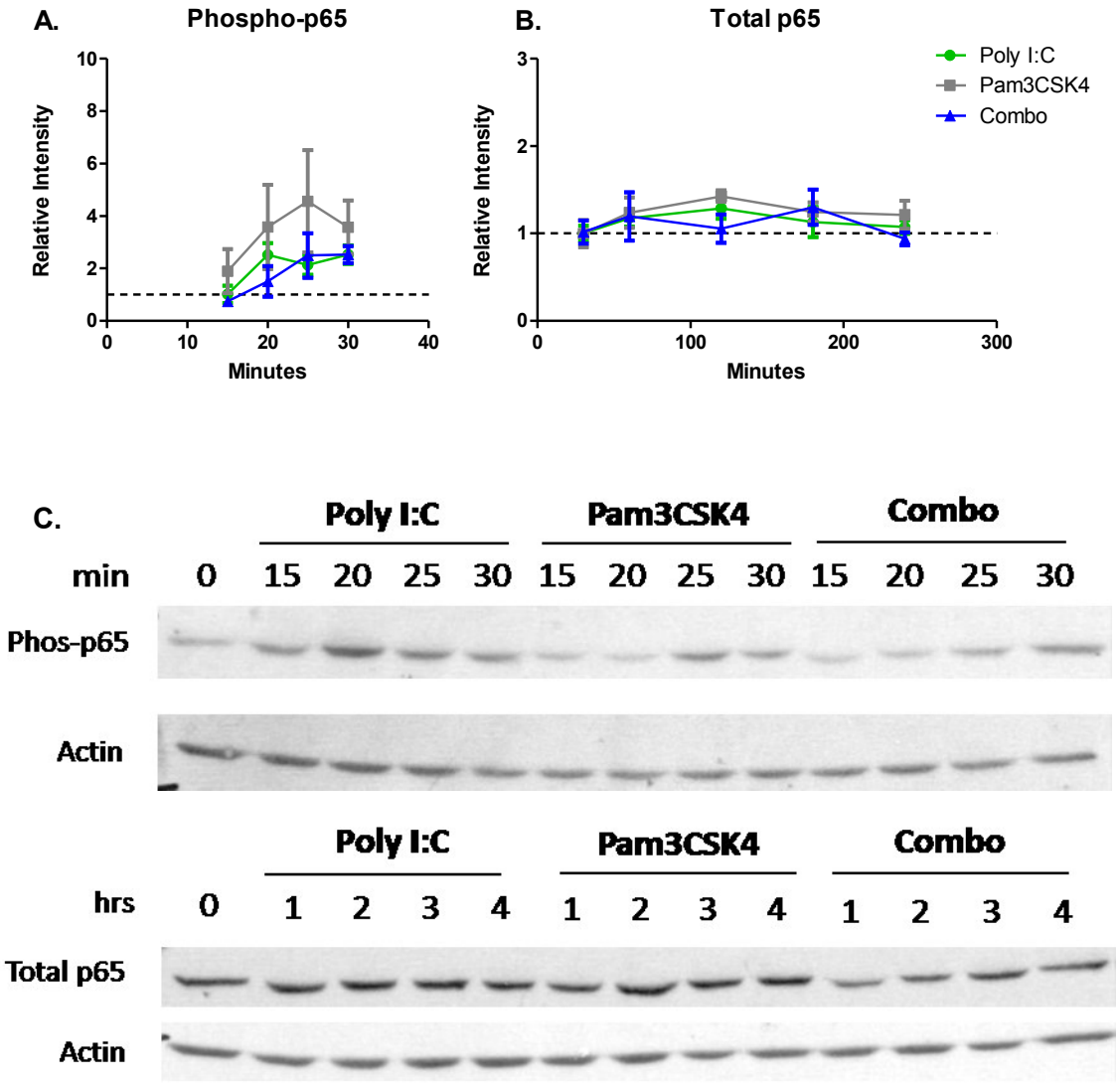


Figure 3.18: Poly I:C and Pam3CSK4 induce phosphorylation of p65. Levels of phosphorylated p65, total p65, and β-actin in B cells stimulated with poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL) or the combination. Levels of phosphorylated p65 **(A)** and total p65 **(B)** were each normalized to the level of total actin then to unstimulated B cells (dashed line). Data shown as average of 3 independent experiments ± SEM. **(C)** Representative blots.

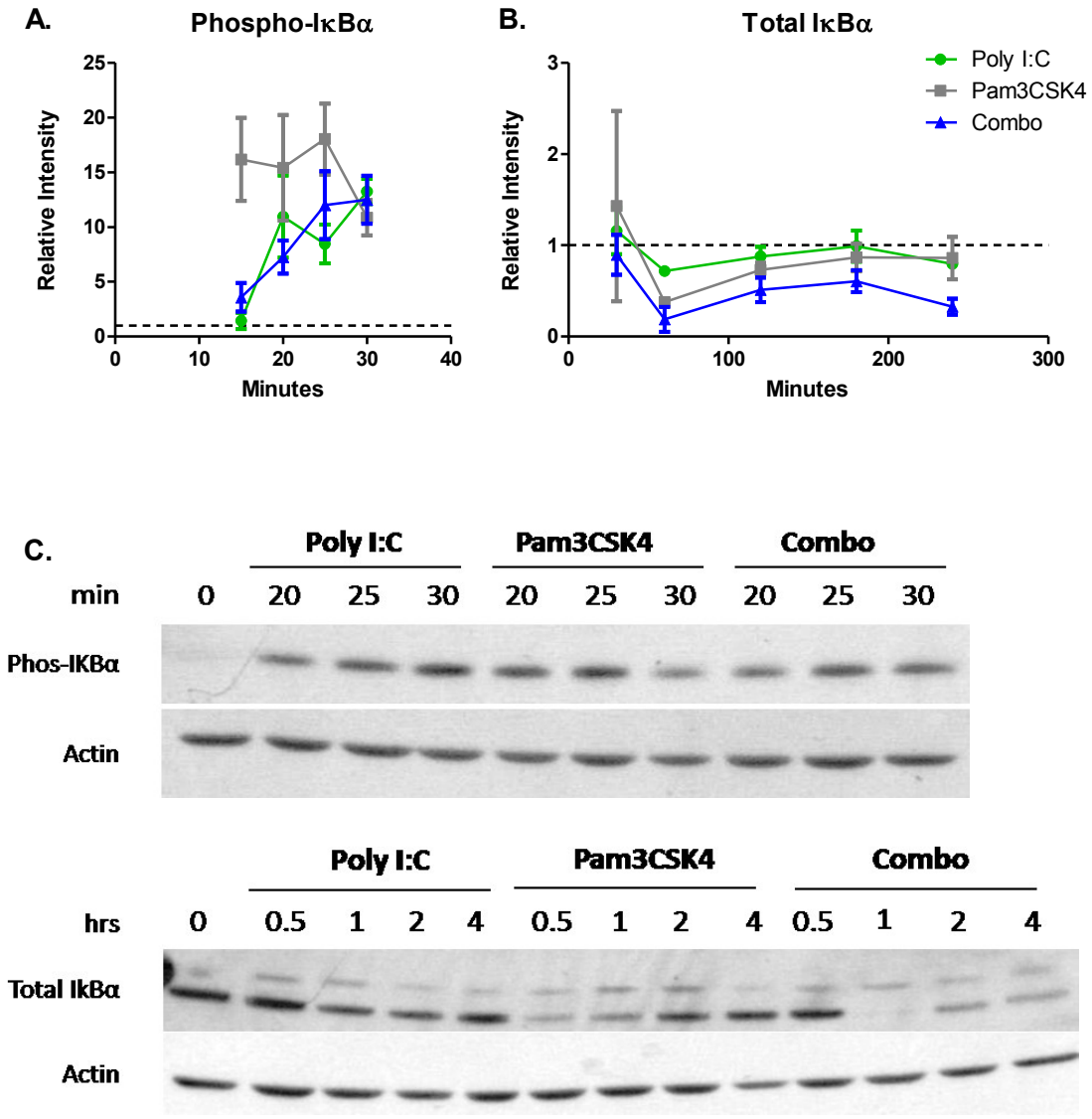


Figure 3.19: Poly I:C and Pam3CSK4 induce phosphorylation and degradation of IKBα. Levels of phosphorylated IκBα, total IκBα, and β-actin in B cells stimulated with poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL) or the combination. Levels of phosphorylated IκBα **(A)** and total IκBα **(B)** were each normalized to the level of total actin then to unstimulated B cells (dashed line). Data shown as average of 3 independent experiments ± SEM. **(C)** Representative blots.

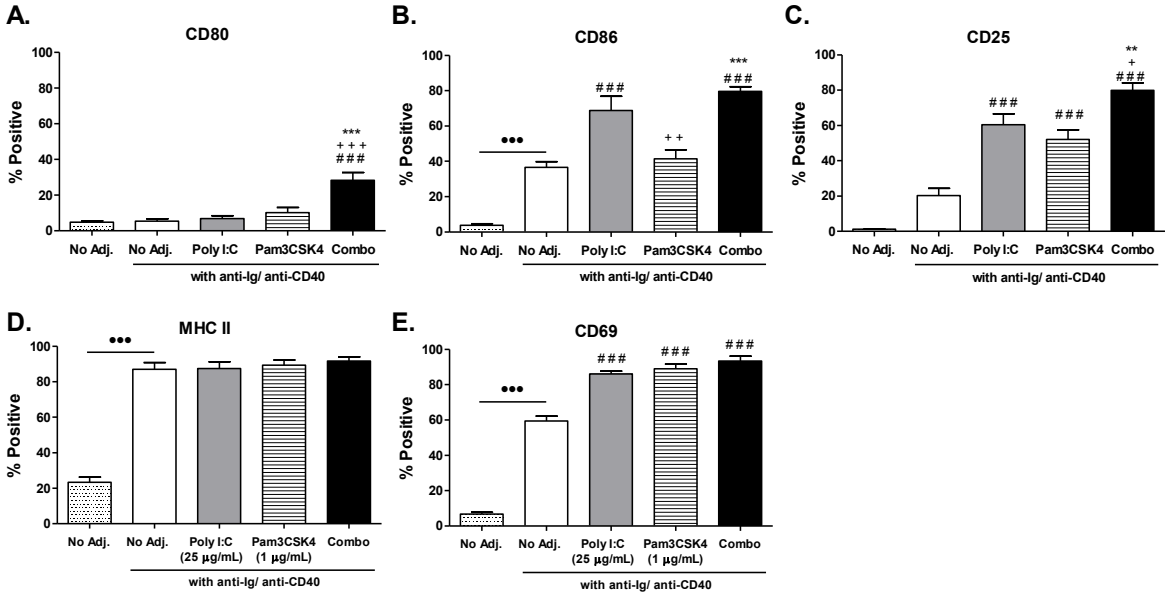


Figure 3.20: Poly I:C and Pam3CSK4 enhance expression of surface receptors following T-dependent B cell activation. Purified B cells were stimulated with anti-Ig (1 µg/mL) and anti-CD40 (2.5 µg/mL) antibody cocktail to simulate T-dependent activation. Poly I:C was added at 25 µg/mL and Pam3CSK4 at 1 µg/mL. After overnight stimulation, cells were stained for **(A)** CD80 (n=7), **(B)** CD86 (n=5), **(C)** CD25 (n=5), **(D)** MHC class II (n=7), **(E)** CD69 (n=3). Data shown as mean ± SEM, statistics by one-way ANOVA with Tukey multiple comparisons post-test: "●" compared to T-independent, no adjuvant background; "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant.

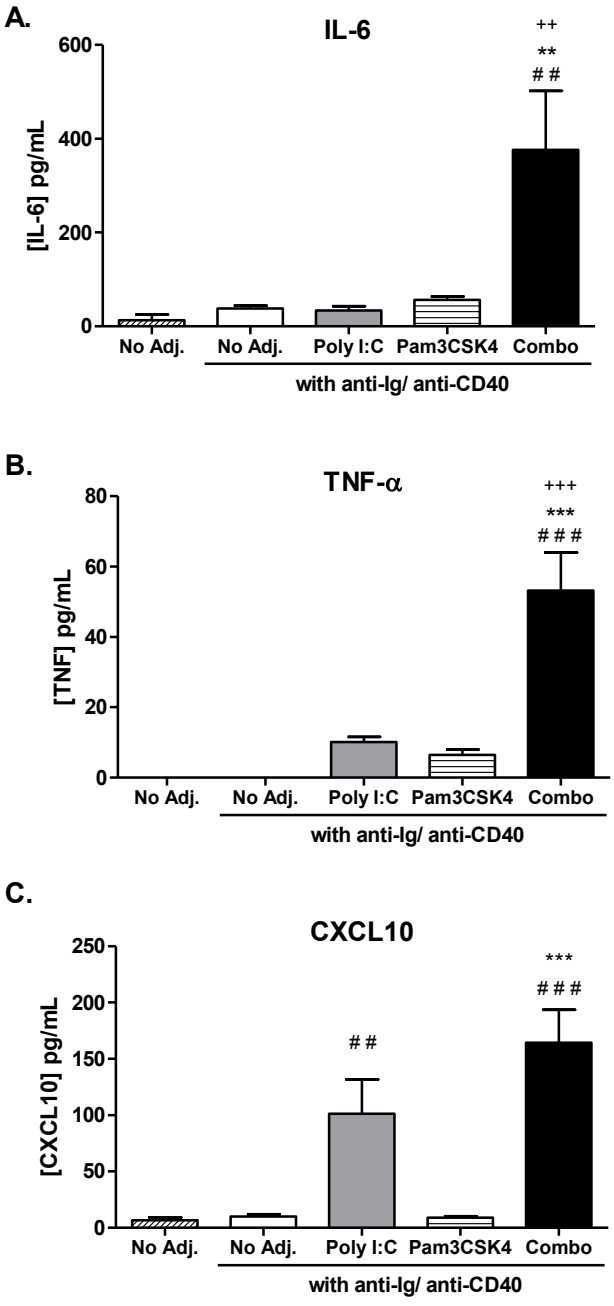


Figure 3.21: Poly I:C and Pam3CSK4 enhance T-dependent B cell cytokine production. Purified B cells were stimulated with anti-Ig (1 μg/mL) and anti-CD40 (2.5 μg/mL) antibody cocktail to simulate T-dependent activation. Poly I:C was added at 25 μg/mL and Pam3CSK4 at 1 μg/mL. After overnight stimulation, supernatants were harvested for cytokine detection by ELISA or CBA. **(A)** IL-6 (n=6), **(B)** TNF-α (n=6), **(C)** CXCL10 (n=8). Data shown as mean ± SEM, statistics by one-way ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant.

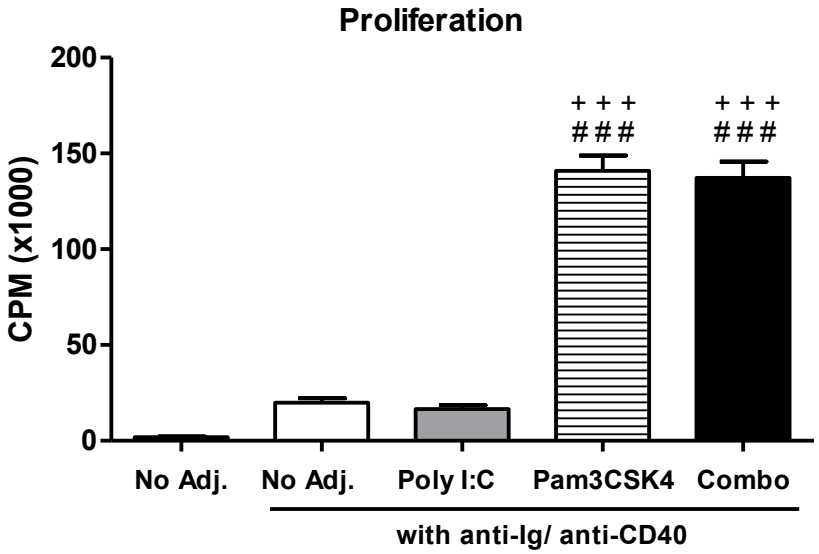


Figure 3.22: Poly I:C and Pam3CSK4 enhance T-dependent B cell proliferation. Purified B cells were stimulated with anti-Ig (1 μg/mL) and anti-CD40 (2.5 μg/mL) antibody cocktail to simulate T-dependent activation. Poly I:C was added at 25 μg/mL and Pam3CSK4 at 1 μg/mL. Proliferation was measured after 3 days by [^{3}H]=TdR uptake. Data shown as mean of 5 samples ± SEM, statistics by one-way ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant.

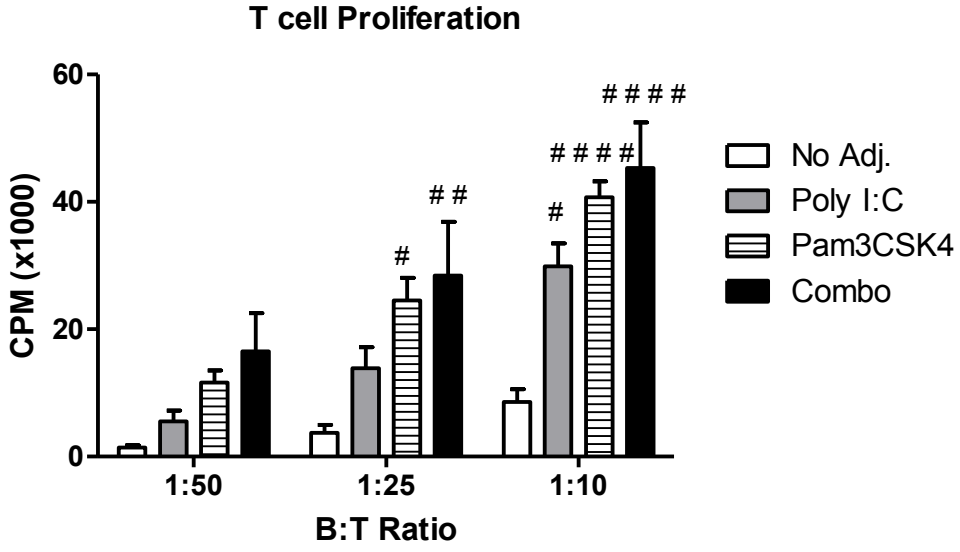


Figure 3.23: B cells activated with poly I:C and Pam3CSK4 induce allogeneic CD4$^+$ T cell proliferation. Purified C57BL/6 B cells were stimulated with anti-Ig (1 μg/mL) and anti-CD40 (2.5 μg/mL) antibody cocktail to simulate T-dependent activation. Poly I:C was added at 25 μg/mL and Pam3CSK4 at 1 μg/mL. After 24 hours, B cells were inactivated by mitomycin c treatment and co-cultured with purified BALBc CD4$^+$ T cells at indicated ratios holding the T cells constant at 10^5 cells/ well. Proliferation was measured after 3 days by [^{3}H]-TdR uptake. Results shown as average of 5 independent experiments ± SEM, statistics by one-way ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant.

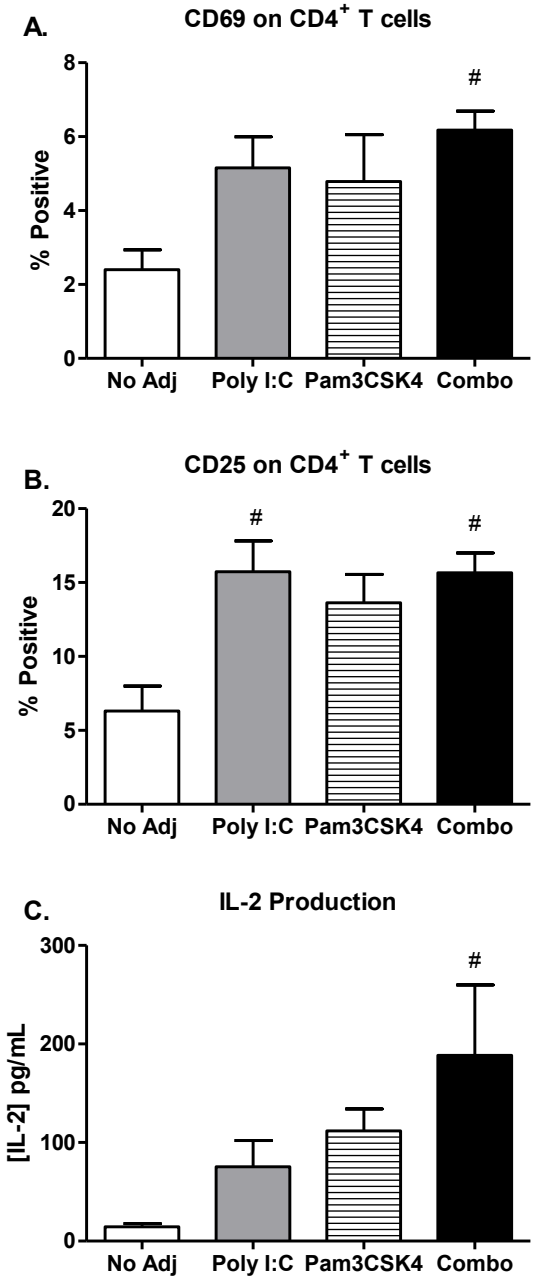


Figure 3.24: Poly I:C and Pam3CSK4 activated T cells induce allogeneic CD4$^+$ T cell activation. Allogeneic B:T co-cultures were setup in 1:10 ratio as in Figure 3.23. After 3 days, cells were harvested and expression of **(A)** CD69 and **(B)** CD25 detected on CD4$^+$ T cells. **(C)** Levels of IL-2 in supernatant harvested at 3 days by ELISA. Results shown as average of 5 independent experiments ± SEM, statistics by one-way ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant.

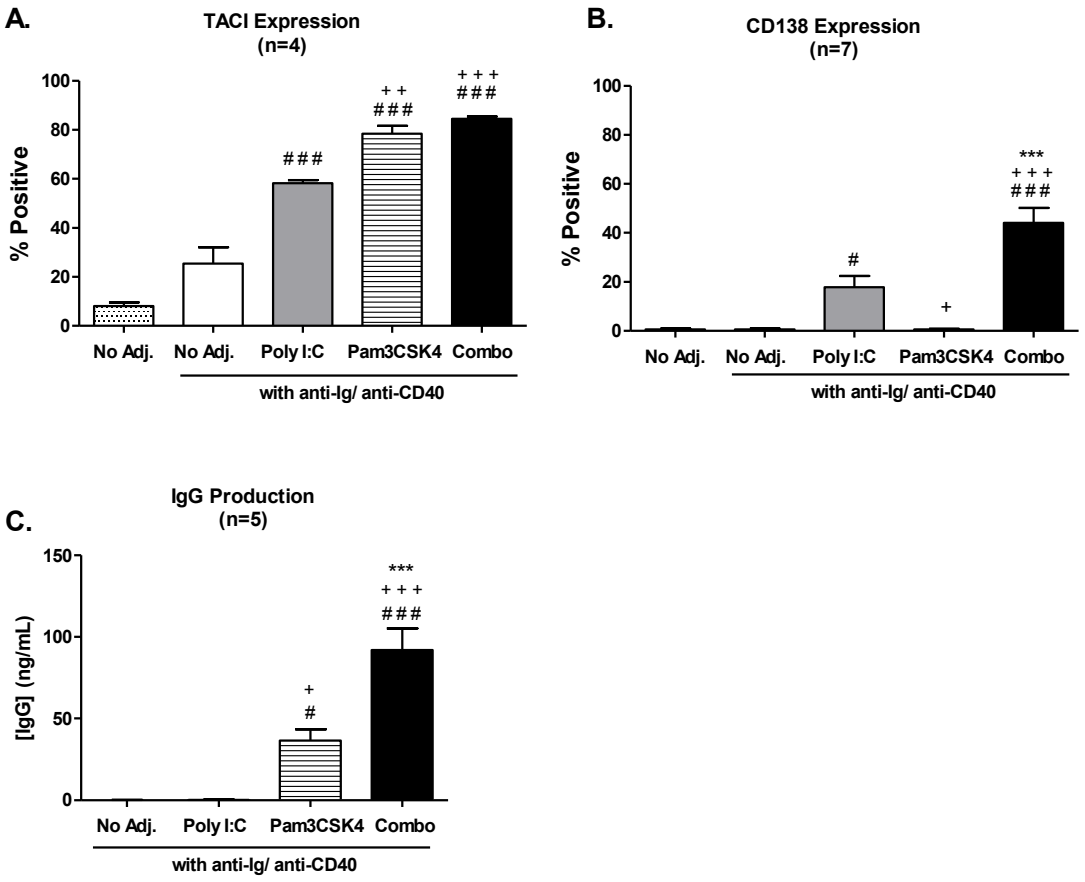


Figure 3.25: Poly I:C and Pam3CSK4 promote B cell differentiation into antibody-secreting plasma cells. Purified B cells were stimulated with anti-Ig (1 μg/mL) and anti-CD40 (2.5 μg/mL) antibody cocktail to simulate T-dependent activation. Poly I:C was added at 25 μg/mL and Pam3CSK4 at 1 μg/mL. After 24 hours, expression of surface **(A)** TACI (n=4) and **(B)** CD138 (n=7) were detected by flow cytometry. **(C)** After 4 days, supernatant harvested and total IgG detected by ELISA (n=5). Data shown as average ± SEM, statistics by one-way ANOVA with Tukey multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant.

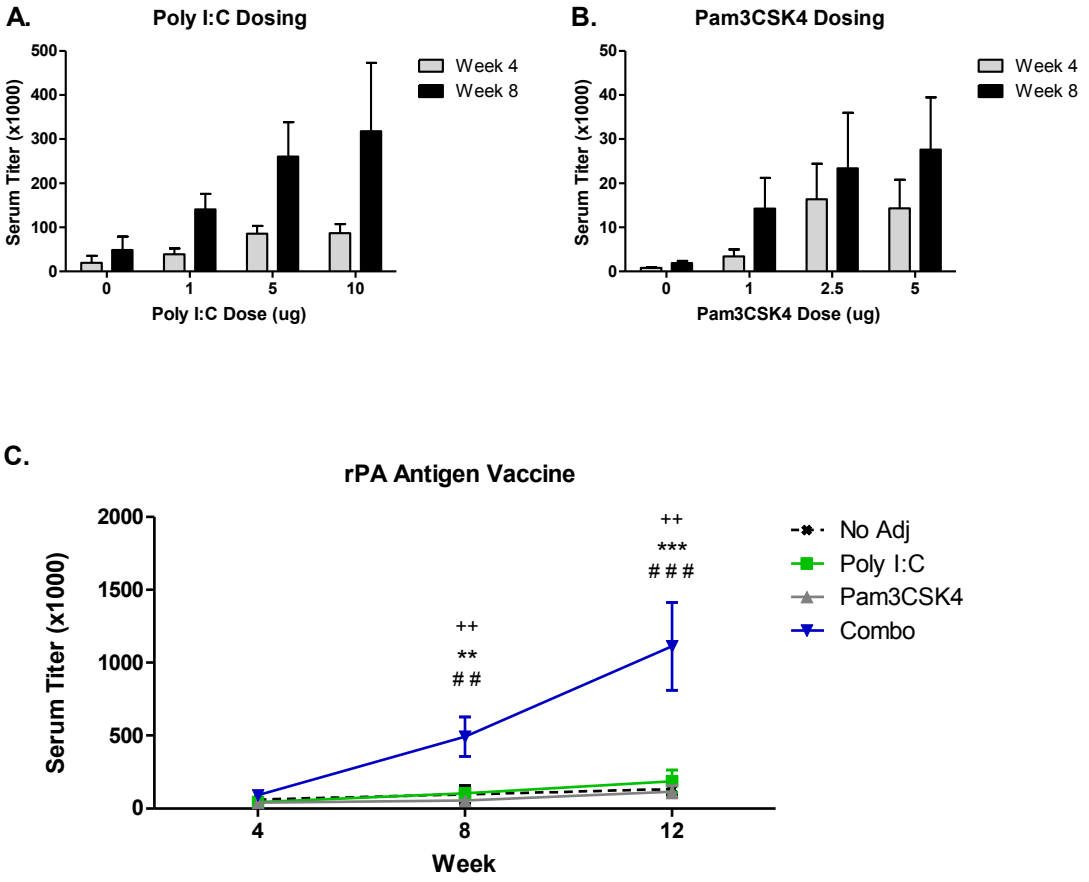


Figure 3.26: Poly I:C and Pam3CSK4 adjuvant combination enhances induction of PA-specific antibodies in mice immunized with anthrax vaccine. (A) CD-1 mice (n=8) were vaccinated with rPA antigen (1 µg/ dose) formulated in DepoVax vaccine containing no adjuvant or poly I:C at 1, 5 or 10 µg/ dose**. (B)** CD-1 mice (n=10) were vaccinated with rPA antigen (1 µg/ dose) formulated in DepoVax vaccine containing no adjuvant or Pam3CSK4at 1, 2.5 or 5 µg/ dose. **(C)** CD-1 mice (n=8) were vaccinated with rPA antigen (1 µg/ dose) formulated in DepoVax vaccine containing no adjuvant, poly I:C (1 µg/ dose), Pam3CSK4 (1 µg/dose) or the combination of both poly I:C and Pam3CSK4. Antigen-specific antibodies were detected in the serum of mice at weeks indicated post immunization and antigen-specific end-point titer determined by ELISA. Statistics by two-way ANOVA with Bonferroni multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant. Results shown as mean endpoint titer ± SEM.

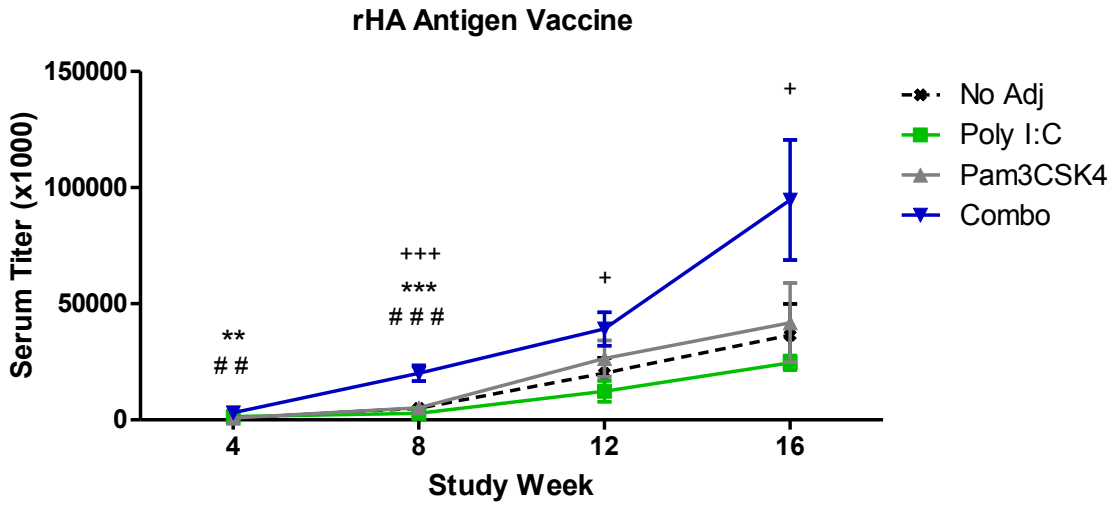


Figure 3.27: Poly I:C and Pam3CSK4 adjuvant combination enhances induction of HA-specific antibodies in mice immunized with influenza vaccine. CD-1 mice (n=8) were vaccinated with rHA antigen (1 μg/ dose) formulated in DepoVax vaccine containing no adjuvant, poly I:C (1 μg/ dose), Pam3CSK4 (1 μg/dose) or the combination of both poly I:C and Pam3CSK4. Antigen-specific antibodies were detected in the serum of mice at weeks 4, 8, 12 and 16 post immunization and end-point titer determined by ELISA. Results shown as mean endpoint titer ± SEM, statistics by two-way ANOVA with Bonferroni multiple comparisons post-test: "+" compared to poly I:C; "*" compared to Pam3CSK4; "#" compared to no adjuvant.

Table 3.1: Summary of Data for T-Independent *in vitro* Stimulation of B cells. Raw data compiled from Figures 3.1-3.10.

Parameter	No Adjuvant	Poly I:C (25 µg/mL)	Pam3CSK4 (1 µg/ mL)	Combo
CD40 (%)	18.52 ± 6.34	51.29 ± 10.74	82.12 ± 4.68	88.32 ± 3.16
CD80 (%)	5.32 ± 0.81	8.95 ± 1.92	21.59 ± 5.78	45.68 ± 5.37
CD86 (%)	3.95 ± 0.71	53.72 ± 4.31	44.35 ± 7.37	85.95 ± 3.23
MHC class II (%)	23.28 ± 2.98	77.14 ± 3.25	93.97 ± 1.03	95.68 ± 0.49
CD25 (%)	1.14 ± 0.19	19.96 ± 4.54	25.50 ± 4.22	77.64 ± 2.28
CD69 (%)	5.94 ± 1.10	38.37 ± 3.99	81.51 ± 2.77	94.32 ± 1.32
IL-6 (pg/ mL)	13.12 ± 11.95	21.19 ± 14.78	25.55 ± 6.67	1078.00 ± 279.8
TNF-α (pg/ mL)	0.00	6.76 ± 0.51	4.80 ± 1.20	39.07 ± 6.18
CXCL10 (pg/ mL)	7.58 ± 2.09	126.70 ± 16.18	7.56 ± 2.30	431.70 ± 33.98
Proliferation (CPM)	1,708 ± 199	6,200 ± 657	85,320 ± 3,628	106,500 ± 5,668

Table 3.2: Summary of Data for T-Dependent *in vitro* Stimulation of B cells. Raw data compiled from Figures 3.20-3.22. ND = Not Detected.

Parameter	No Adjuvant	Poly I:C (25 µg/mL)	Pam3CSK4 (1 µg/ mL)	Combo
CD40 (%)	ND	ND	ND	ND
CD80 (%)	5.41 ± 1.30	6.76 ± 1.73	10.11 ± 2.95	28.32 ± 4.37
CD86 (%)	36.55 ± 3.21	68.76 ± 5.24	41.36 ± 5.24	79.59 ± 2.71
MHC class II (%)	87.08 ± 3.83	87.47 ± 3.81	89.38 ± 2.98	91.71 ± 2.34
CD25 (%)	20.39 ± 4.03	60.41 ± 6.11	52.08 ± 5.42	79.96 ± 4.17
CD69 (%)	59.48 ± 2.79	86.07 ± 1.56	88.92 ± 2.68	93.44 ± 2.66
IL-6 (pg/ mL)	38.38 ± 6.12	33.76 ± 9.09	56.12 ± 7.87	376.40 ± 125..80
TNF-α (pg/ mL)	0.00	10.10 ± 1.56	6.50 ± 1.56	53.26 ± 10.79
CXCL10 (pg/ mL)	10.15 ± 1.93	101.40 ± 30.28	8.78 ± 1.63	164.40 ± 29.26
Proliferation (CPM)	19,990 ± 2,195	16,580 ± 1,962	140,800 ±8,098	137,300 ± 8,424

3.3 Discussion

3.3.1 Poly I:C and Pam3CSK4 enhnace dendritic cell activation

DCs are an important target for vaccination since they can differentiate into highly efficient APCs with the capacity to prime naïve $CD4^+$ T cells. DC activation can be stimulated through sensing of PAMPs, DCs express a variety of PRRs that help them tailor immune responses towards different types of infection through selective induction of cytokines. Previous reports have indicated that DC stimulation can be enhanced by using combinations of two or more TLR agonists [267, 491]. In particular, poly I:C and Pam3CSK4 have been reported to increase activation of human and murine DCs [109, 308, 309] as well as murine macrophages [307].

In this study we confirmed that poly I:C (5 μg/mL) and Pam3CSK4 (10 μg/mL) increased activation of murine DCs after 24 hour stimulation. This was evident by increased expression of MHC class II, CD40, CD80 and CCR7 (Figure 3.1A). MHC class II, CD40 and CD80 are essential receptors involved in priming a naïve $CD4^+$ T cell helper response. CCR7 is a chemokine receptor that allows activated DCs to home to the lymph node in response to CCL19 and CCL21 chemokine gradients. We also detected changes in cytokine secretion patterns, the DCs stimulated with poly I:C and Pam3CSK4 combination had elevated TNF-α, CCL2 and IL-6 levels (Figure 3.1B).

Previous studies on the effects of poly I:C and Pam3CSK4 combination on murine DCs has shown increased secretion of IL-12p40 and TNF-α [308, 309], as well as through increased expression of CD40 and CD80 [309]. Functionally, DCs activated with poly I:C and Pam3CSK4 have demonstrated enhanced capacity to activate $CD4^+$ T cells, with preferential differentiation to Th1 and activation of NK cells [308]. DCs stimulated *in vitro* with poly I:C and Pam3CSK4 and loaded with peptide antigen can also provide effective control of tumour growth *in vivo* [309]. These observations indicate that the poly I:C/ Pam3CSK4 combination may be an effective means to activate APCs.

3.3.2 Poly I:C and Pam3CSK4 cause differential activation of B cells

Poly I:C and Pam3CSK4 have been reported to enhance activation of both DCs and macrophages. Having confirmed this effect in DCs, we sought to determine if B cells could be similarly activated by the agonist combination. We first evaluated each agonist for the capacity to induce B cell activation independent of T cell help. We found that each agonist had the capacity to activate B cells, resulting in unique phenotypic profiles (Table 3.1). A dose-response study of poly I:C and Pam3CSK4 demonstrated that each agonist could induce expression of several receptors important for B:T interactions, namely CD40, CD86, MHC class II and CD25. Generally, Pam3CSK4 had a more potent effect than poly I:C. However, poly I:C has a higher molecular weight (989,486 g/mol) than Pam3CSK4 (1510 g/ mol), meaning that at the doses used the molar concentration of Pam3CSK4 (1 μg/mL = 662 nmol/ L) was 26 times higher than the molarity of poly I:C (25 μg/ mL = 25 nmol/ L). This may explain why such low doses of Pam3CSK4 could induce such strong expression of receptors compared to poly I:C. Another explanation may be that receptor expression of TLR2 and TLR3 was not equivalent. We did not evaluate the expression of the receptors due to lack of specific mAb commercially available for TLR3; however, others have determined quantitative levels of mRNA for each receptor and reported that TLR3 mRNA tends to be low or undetectable in naïve B cells, while expression of TLR2 tends to be higher [227]. If mRNA is indicative of protein expression, then it is feasible that TLR2 is expressed on B cells at a greater level than TLR3.

Individually, neither poly I:C nor Pam3CSK4 could induce expression of CD80, IL-6 or TNF-α. CD80 is related to CD86 as both belong to the CD28 receptor family and interact with CD28 on T cells to provide the second signal of activation [492]. CD86 may be more sensitive to upregulation in the time frame used in these experiments (24 hours) because it is known to be expressed constitutively at low levels on naïve APCs, and is further upregulated after activation; CD80 on the other hand is only expressed after activation [327]. Both IL-6 and TNF-α are important cytokines involved in promoting B cell survival and proliferation [493]. Failure of B cells to produce these cytokines within the time frame tested may be indicative of inefficient long term survival of B cells stimulated with either adjuvant alone.

Two striking differences in poly I:C and Pam3CSK4 induced activation were observed in the production of CXCL10 and proliferation. Despite stimulating lower expression of most surface receptors, poly I:C induced a statistically significant increase in production of CXCL10 while Pam3CSK4 did not induce any detectable production of this chemokine. CpG, an oligonucleotide ligand detected by TLR9, has also been reported to induce CXCL10 production in B cells [488]. CXCL10 is associated with Th1 immune responses and can direct homing of activated $CXCR3^+$ T cells. Hence, the differences between poly I:C and Pam3CSK4 for CXCL10 production is a reflection of differential TLR signaling pathways. To see if B cells were also expressing IFN-γ, thus influencing Th differentiation towards Th1, we also quantified IFN-γ in supernatants but levels were undetectable even after 4 days of culture (data not shown). In contrast, poly I:C was inefficient at inducing substantial B cell proliferation, while Pam3CSK4 induced considerable proliferation in both T-independent and T-dependent culture conditions. These results are consistent with previous reports of mitogenic activity of Pam3CSK4 on B cells [223, 258]. The contrast in poly I:C and Pam3CSK4 effects on CXCL10 production and proliferation demonstrate that each agonist induced a unique response in B cells.

While the activity of Pam3CSK4 on B cells has been previously described, the contribution of poly I:C stimulation to B cell activation remains debatable. Previous reports using murine B cells have reached inconsistent conclusions. Marshall-Clarke *et al* [281] demonstrated that poly I:C at a dose of 50 μg/mL could stimulate murine B cells, resulting in increased CD86, CD40 and MHC class II expression, as well as proliferation. The effect of poly I:C stimulation in that study was comparable to the LPS control. Thus, these authors concluded that poly I:C is an important agonist of B cell activation. In the same year (2007) Barr *et al* published a report in which the expression of all TLRs was quantified in various B cell subsets (B1, B2, FO and MZ) by qPCR. They found TLR3 mRNA, relative to the other TLRs, to be consistently low. They also reported that purified total $CD19^+$ B cells stimulated with 25 μg/mL poly I:C increased expression of CD86 and MHC II, but no increases in CD40 or secretion of IL-6, IL-10 or IL-12p40 could be detected [227]. These authors concluded that relative to the other TLR agonists tested, including Pam3CSK4, poly I:C was a weak stimulator of B cell activation. In

both cases poly I:C weakly increased B cell activation *in vitro*, although the results were interpreted differently based on comparisons to other adjuvants used. Present results are consistent with these studies as they indicate that at a minimum dose of 25 μg/mL poly I:C does activate B cells to increase expression of some surface receptors to a low level, compared to Pam3CSK4.

In summary, these data demonstrates that poly I:C, particularly at doses ≥25 μg/mL, can activate B cells and results in increased expression of CD40, CD86, MHC class II, and CD25 as well as CXCL10 secretion. Pam3CSK4 induced increases in expression of CD40, CD86, MHC class II, and CD25, as well as proliferation, at doses as low as 0.5 μg/ mL. Notably, each adjuvant induced a unique activation profile in B cells, indicating non-redundancy in signaling pathways.

3.3.3 The combination of poly I:C and Pam3CSK4 provides enhanced activation of B cells

Having demonstrated that poly I:C and Pam3CSK4 could each enhance activation of B cells in a non-redundant manner, we examined the effect of the combination on B cell activation. For most of the activation parameters examined, the effect of stimulation with both poly I:C and Pam3CSK4 was greater than either adjuvant alone (Table 3.1). For CD40 (Figure 3.3) and MHC class II (Figure 3.6), Pam3CSK4 alone induced almost maximal expression which was increased slightly, but not significantly, by poly I:C. The expression of CD80 (Figure 3.4) and CD25 (Figure 3.7), as well as secretion of IL-6 (Figure 3.8A) and CXCL10 (Figure 3.8B), was increased by the combination treatment to a level that exceeded the level predicted by adding the level induced by each adjuvant separately, indicating a synergistic effect. The expression of CD86 (Figure 3.5) and proliferation (Figure 3.9) were increased additively by stimulation with the adjuvant combination.

Vanhoutte *et al* documented a synergistic effect of poly I:C (at 1 μg/mL) and Pam3CSK4 (at 0.5 μg/mL) on DCs based on the increased production of cytokines induced by the combination of these agonists *in vitro* [308]. However, they also reported that, at the doses tested, poly I:C and Pam3CSK4 had a cross-inhibitory effect on the expression of CXCL9, CXCL10 and IFN-β. This was measured by decreased mRNA in

DCs stimulated with the two adjuvants. No such effect was observed in our study of B cells at the doses used, however it is conceivable that these two agonists could have cross-regulatory effects that include both increased and decreased sensitivity depending on dose and cell type used.

The surface receptors that were increased on the B cells stimulated with the poly I:C/ Pam3CSK4 combination (CD40, CD80, CD86, MHC class II, CD25) are important in T cell interactions and expressed by efficient APCs. Most notably, CD80 expression was increased by the adjuvant combination after 24 hours, but not increased significantly by either adjuvant alone (Figure 3.4). CD80 and CD86 are members of the B7 family of co-signaling receptors and are primarily expressed on APCs. Both receptors interact with CD28, a costimulatory receptor on T cells, and CTLA-4, a co-inhibitory receptor on T cells, to help regulate T cell expansion and contraction phases during active immune responses [327]. CD86 is constitutively expressed on B cells at low levels and increased after activation, whereas CD80 is only expressed upon activation [494]. Although they bind to the same ligands, intracellular signaling of CD80 and CD86 is non-redundant and they each contribute to effective B cell differentiation into antibody-producing plasma cells [495, 496]. Furthermore, the rapid increase in both CD80 and CD86 induced by the poly I:C and Pam3CSK4 combination implies that these B cells would be more effective APCs as they will likely interact with $CD28^+$ T cells during early immune response, before CTLA-4 is unregulated [497].

CD25 expression on B cells was also substantially augmented in response to poly I:C and Pam3CSK4 stimulation. CD25, the high affinity α chain of the IL-2 receptor, is a survival and proliferation factor for T cells and B cells [485, 498]. T cells are the most significant producers of IL-2 which acts in an autocrine fashion to support their expansion [498]. B cells are not known to be a significant producer of IL-2, but CD25 expression is detected on activated B cells and IL-2 signaling promotes their proliferation and antibody production [485]. This could be an important positive feedback loop created during B cell and $CD4^+$ T cell interaction. Amu *et al* has recently suggested that $CD25^+$ B cells are a phenotypically and functionally distinct B cell subset with inherent APC capacity [499, 500]. $CD19^+CD25^+$ B cells, comprising about 2% of the spleen, were isolated from mice and cultured *in vitro* with a mixture of TLR agonists

including CpG, LPS and Pam3CSK4. These $CD25^+$ B cells were found to secrete higher levels of cytokines (IL-6, IL-10 and IFN-γ) as well as higher expression of costimulatory receptors, CD80 and CD86. After BCR crosslinking induced with anti-Ig, $CD25^+$ B cells produced a higher level of spontaneous production of IgM, IgG and IgA antibody than $CD25^-$ B cells. The $CD25^+$ B cells also performed better as APCs in a mixed lymphocyte reaction. In our study, over 75% of the B cells were induced to express CD25 with poly I:C/ Pam3CSK4 stimulation. Poly I:C/ Pam3CSK4 stimulated B cells also shared many of the same features as the $CD25^+$ B cells freshly isolated by Amu *et al*, such as increased CD80/ CD86 expression, IL-6 production, secretion of IgG and $CD4^+$ T cell costimulatory activity. This may indicate that stimulation of B cells with poly I:C and Pam3CSK4 promotes expansion of B cells with enhanced APC function.

Another possibility that was considered is that the $CD25^+$ B cells have regulatory activity, similar to CD25-expressing Tregs. Tregs express high levels of CD25 in order to remove excess IL-2 and prevent it from acting on effector T cells [501]. However, while the majority of the B cells expressed CD25, expression was low on a per cell basis compared to what we normally detect on Tregs (personal experience). Furthermore, we could not detect any IL-10 in culture supernatants, which is a key cytokine defining the regulatory B cell population [229].

Poly I:C and Pam3CSK4 induced a significant increase in IL-6, TNF-α and CXCL10 secretion by B cells. IL-6 and TNF-α are important cytokines involved in promoting B cell survival and proliferation [214, 493]. CXCL10 is a chemokine recognized by CXCR3, an important chemokine receptor involved in migration of activated T cells during Th1-type immune responses [502]. Poly I:C is known to induce expression of CXCL10 in other cell types [487]. Expression of CXCL10 by activated B cells could therefore promote B and T cell interactions by recruiting activated T cells expressing CXCR3. Others have reported production of IL-10, IFN-γ and IL-12p40 by B cells in response to TLR stimulation, however these were not detected in our system even after 4 days of culture (data not shown) [227, 250].

Lastly, we evaluated the effect of poly I:C and Pam3CSK4 stimulation on B cell proliferation. Expression of CD69, an early activation marker, correlated with enhanced proliferation of lymphocytes [489], which indicated that poly I:C and Pam3CSK4

combination could increase B cell proliferation. Proliferation was assessed after 3 day culture by [^{3}H]-TdR uptake and was indeed enhanced by the agonist combination. B cell proliferation is an important feature necessary for the formation of germinal centres *in vivo* and contributes to development of affinity maturation in B cells [195].

3.3.4 TLR2 and TLR3 are required for B cell activation enhancement by poly I:C and Pam3CSK4

Results using TLR2$^{-/-}$ B cells clearly demonstrated that Pam3CSK4 was signaling through this receptor as surface receptor expression (Figure 3.13), cytokine/ chemokine production (Figure 3.14) and proliferation (Figure 3.15) were all ablated in these cells in response to Pam3CSK4 stimulation. Likewise, stimulation of TLR2$^{-/-}$ B cells with poly I:C and Pam3CSK4 combination produced responses comparable to poly I:C alone with no enhancement of any parameter studied.

TLR3$^{-/-}$ B cells also had a significant reduction in surface receptor expression (Figure 3.10) and cytokine/ chemokine production (Figure 3.11) in response to poly I:C or poly I:C/ Pam3CSK4 combination. However, proliferation of TLR3$^{-/-}$ B cells to poly I:C and Pam3CSK4 combination was actually enhanced, although proliferation to poly I:C alone remained negligible (Figure 3.12). As proliferation was measured after 3 days of incubation and the other parameters after 24 hour stimulation, it is possible that the B cells upregulated an alternate receptor for poly I:C that could mediate proliferation in combination with Pam3CSK4.

Two alternate receptors for poly I:C have been identified that can stimulate activation signals similar to TLR3: RIG-I and MDA-5 [273, 274]. MDA5 and RIG-I mRNA expression is upregulated in macrophages in response to viral infection, reaching maximal levels 24 hours after exposure to modified vaccinia virus Ankara (MVA) [503]. Response to MVA is also dependent upon TLR2 expression, which suggests there could be some cooperation between these two receptors [503]. Therefore, it is possible that Pam3CSK4 is inducing expression of MDA5 and/ or RIG-I in B cells between 24 hours and 3 days of stimulation to enable response to poly I:C, which may be one mechanism through which Pam3CSK4 enhances responses to poly I:C.

3.3.5 Changes to the kinetics of NFκB activation in B cells following stimulation with poly I:C and/ or Pam3CSK4

The analysis of total and phosphorylated p65 and IκBα indicated that while all three treatments could effectively induce phosphorylation of p65 and IκBα, the combination treatment maintained prolonged degradation of IκBα. The results did not reach significance; however, the trends were consistent across three experiments indicating that statistical significance may have been attained with additional experimentation.

Protein levels of IκBα are closely related to activity of NFκB; IκBα knockout mice have increased NFκB activity [504] and overexpression of IκBα inhibits NFκB activation [505]. One of the target genes for activated p65 is IκBα, which replenishes the cytosolic levels of this inhibitor protein and provides feedback regulation to control NFκB activity [506]. However, the translational activity of p65 can be influenced by its acetylation and phosphorylation [120]. In this study, the phosphorylation of p65 was assessed at serine 536 only, but there are three other serine sites known to undergo modification in response to different stimuli [507]. It is possible that the activation of p65 in response to poly I:C and Pam3CSK4 combined stimulation resulted in differences in post-translational modifications that influenced its target gene specificity.

Other intracellular signaling events may also be influenced by combined poly I:C and Pam3CSK4 stimulation that contribute to the enhanced activation of B cells observed in this study. TLR3, as well as other dsRNA sensing receptors, RIG-I and MDA-5, activate IRF transcription factors that control expression of type 1 IFN genes independent of NFκB [508]. Certainly, the proliferation induced by the poly I:C and Pam3CSK4 combination in $TLR3^{-/-}$ B cells suggest that Pam3CSK4 could stimulate increased expression of other poly I:C receptors, which could enhance B cell activation independent of NFκB activation.

3.3.6 Poly I:C and Pam3CSK4 enhance T-dependent B cell activation

Co-stimulation of B cells through CD40 is a necessary signal controlling T-dependent B cell activation, leading to class-switch recombination and plasma cell differentiation [208, 209]. Multimerization of CD40 through interactions with CD154

ligand on the surface of T cells leads to activation of p52-RelB subunits of NFκB through intracellular signaling events known as the non-canonical pathway [210]. This is distinct from TLR-induced NFκB activation, which primarily results in activation of the canonical pathway mediated by the p65 subunit [509, 510]. Stimulation of TLR4 and TLR9 has been previously shown to synergize with CD40 signaling in B cells, resulting in class switch recombination and antibody production [217, 258].

In our system, stimulation of B cells with poly I:C and/ or Pam3CSK4 during T-dependent activation resulted in similar expression of surface receptors (Figure 3.20), cytokine/ chemokine production (Figure 3.21) and proliferation (Figure 3.22) as was observed after B cell stimulation independent of T cell activation, with some exceptions (see summary in Table 3.2). T-dependent activation of B cells without TLR stimulation increased the expression of CD86, CD69 and MHC class II; only CD86 and CD69 could be further enhanced with TLR stimulation in these conditions. T-dependent B cell proliferation was enhanced by Pam3CSK4 alone and not further enhanced with the poly I:C and Pam3CSK4 combination. These observations support that co-stimulation of B cells provided by CD40 ligation strongly promotes B cell survival and activation. However, CD40 engagement alone may not be sufficient for full B cell activation, since stimulation provided by poly I:C, Pam3CSK4 or the combination resulted in upregulation of more surface receptors, cytokine production and enhanced B cell proliferation.

We noted that in some instances the level of activation induced in T-dependent B cell cultures in response to poly I:C and Pam3CSK4 combination was lower than that induced in T-independent cultures. For example, mean expression of CD80 induced by poly I:C/ Pam3CSK4 combination was 45% in T-independent cultures (Figure 3.4) and only 28% in T-dependent cultures (Figure 3.20A), a statistically significant difference ($p<0.05$ by Student's t-test). Likewise, expression of IL-6 and CXCL10 induced by the combination treatment were also lower in T-dependent cultures (Figure 3.12) compared to T-independent (Figure 3.20), $p<0.05$ and $p<0.001$ by Student's t-test, respectively. CD40 can interact with TRAF1, 2, 3, 5 and 6, and by doing so instructs differential down stream signaling leading to activation [210]. Variations in the recruitment of these TRAFs to CD40 can lead to changes in signaling pathways and ultimately activation of NFκB [511]. TLR2 and TLR3 interact with TRAF6 and TRAF3 [27], and therefore the

differences in B cell phenotype observed after poly I:C and Pam3CSK4 stimulation in T-independent or T-dependent culture conditions may be a reflection of the availability and activation status of different TRAF signaling proteins.

The enhanced T-dependent activation of B cells stimulated with poly I:C and Pam3CSK4 ultimately manifested as increased capacity to elicit T cell activation and proliferation in an allogeneic system (Figures 3.23 & 3.24). This could be due to the differences in co-receptor expression, such as CD80 and CD86, since MHC class II expression was maximally increased by anti-CD40/ anti-Ig T-dependent culture conditions (Figure 3.20D). Notably, poly I:C and Pam3CSK4 stimulation of B cells enhanced the expression of CD25, the high affinity IL-2 receptor (Figure 3.20C). Likewise, T cells stimulated with poly I:C/ Pam3CSK4 activated B cells also induced expression of CD25 and production of IL-2. This may indicate a mechanism through which B cell activation is amplified during a T-dependent antigen response. As discussed above, $CD25^+$ B cells isolated from murine splenocytes have been demonstrated by others to have enhanced ability to stimulate T cells *in vitro* [499].

Class switch recombination and antibody production are hallmarks of T-dependent B cell activation [203]. In this study, we confirmed these events were enhanced by poly I:C and Pam3CSK4 stimulation by looking at the expression of TACI and CD138 as well as polyclonal IgG production (Figure 3.25). TACI and CD138 are surface receptors commonly expressed by antibody producing plasma cells. TACI is a TNF superfamily receptor member expressed only by B lymphocytes. TACI can respond to B cell survival factors BAFF and APRIL, which are primarily produced by non-B cells in order to regulate B cell survival and differentiation during B cell maturation. Previously, TACI has been shown to be increased on murine B cells in response to TLR stimulation by LPS and CpG [512, 513]. TACI itself interacts with intracellular MyD88 and converges with signaling induced by TLR and CD40 to promote class switch recombination in B cells [514]. CD138, also known as syndecan-1, is a heparin sulfate-bearing proteoglycan that acts as a co-receptor to facilitate binding of cytokines, including the TACI ligand APRIL [515].

In our studies, antibody production could only be induced during T-dependent activation of B cells, and was augmented by poly I:C and Pam3CSK4 combination

treatment. In this respect, the agonist combination is unlike LPS which can induce antibody production by murine B cells in the absence of T help [203]. Although it is unlikely that TACI signaling participated in this event *in vitro* since BAFF and APRIL are not produced by B cells, it is interesting to speculate that increased TACI expression promoted by poly I:C and Pam3CSK4 might contribute to more efficient plasma cell differentiation *in vivo*.

3.3.7 Summary of *in vitro* studies

The *in vitro* studies described herein demonstrate for the first time that the combination of poly I:C and Pam3CSK4 can efficiently enhance stimulation of B cells, characterized by increased expression of surface receptors associated with activation, cytokine/ chemokine production and proliferation. Poly I:C and Pam3CSK4 further enhance T-dependent B cell activation, promoting differentiation into antibody-producing plasma cells with increased capacity to stimulate $CD4^+$ T cells. These data suggest that the combination of poly I:C and Pam3CSK4 could serve as a potent adjuvant system to boost antibody responses to protein vaccines. We therefore tested the adjuvant system using two model antigens, rPA (anthrax) and rHA (influenza). Protective responses to these indications are associated with antibody responses; however, current vaccine formulations for each are inadequate.

3.3.8 Use of poly I:C and Pam3CSK4 as an adjuvant system *in vivo*

3.3.8.1 Anthrax (rPA) Vaccine

When used as an adjuvant system *in vivo*, poly I:C and Pam3CSK4 boosted antigen-specific antibody titers to rPA protein antigen formulated in the DepoVax vaccine (Figure 3.26). Antibodies towards PA are correlated with protection from anthrax [516], which is caused by the bacterium *Bacillus anthracis*. *B. anthracis* produces a lethal tripartite toxin comprised of lethal factor (LF), edema factor (EF) and protective antigen (PA) [517]. PA is the cell-binding component that facilitates entry of the LF and EF into the cell. *B. anthracis* forms highly resilient spores that are conducive to their use as a bioterrorism agent. A bioterrorism anthrax attack could potentially expose a large number of people to the pathogen within a short amount of time.

Prophylaxis of those potentially exposed involves intensive 60-day antibiotic regimen [518]; therefore, development of a prophylactic vaccine could greatly reduce the threat of this disease.

There are two vaccines currently licensed: AVA (used in the United States) and AVP (used in the United Kingdom), which are prepared from the supernatants of *B. anthracis* culture that are formalin-inactivated and admixed with alum adjuvant [518]. The current AVA vaccination schedule consists of five 0.5 mL intramuscular injections at 0 and 4 weeks and 6, 12, and 18 months, with annual boosters [517]. This regimen induces neutralizing and long-lasting serum antibody titers, but is hardly sufficient to meet the need of rapid immunity in the event of sudden exposure [517]. Additionally, the repeated vaccinations required with these vaccines are associated with significant reactogenicity, which precludes wide-spread immunization of the general public [519, 520]. In this respect, the DPX-rPA vaccine prepared with the poly I:C and Pam3CSK4 merits further exploration into its potential as an anthrax vaccine candidate. The vaccine tested herein generated strong antibody titers within 8 weeks of a single vaccination. The low dose of adjuvants coupled with strong antigenicity could mean less immunizations required, which could reduce side effects and enable wide-spread application. Further work would need to be performed to validate this vaccine for consideration in humans, including testing against current anthrax vaccines and determination of minimal immunizations required. Potency of anthrax vaccines can be assessed in pre-clinical mouse models using a toxin-neutralization assay. Challenge models for anthrax vaccine testing are performed in monkeys [521].

3.3.8.2 Influenza (rHA) Vaccine

The poly I:C/ Pam3CSK4 adjuvant system also boosted responses to influenza rHA protein antigen, yielding significantly higher titers by four weeks post immunization (Figure 3.27). HA-specific antibody titers are one parameter that is correlated with protection from seasonal as well as pandemic influenza [522]. Seasonal influenza is caused annually by recurring strains that are known to transit between humans, and therefore seasonal influenza vaccines are prepared from antigens of currently circulating virus strains [523]. Pandemic influenza arises from new strains of influenza virus that

have adapted from another animal species for human transmission. Since pandemic strains can be quite different from seasonal influenza strains, they carry the capacity to cause large scale human infection and potentially devastating pathology. Predicting which strains possess the capacity to inflict such damage is impossible [524]; therefore, vaccines that can be quickly formulated and provide rapid antibody-mediated immunity are critical to mitigating this threat [525]. Seasonal influenza vaccines are prepared from inactivated influenza virus mass produced in cultures [523]. Subunit vaccines such as DepoVax containing recombinant proteins are promising for pandemic influenza since they can be rapidly manufactured once appropriate antigen target is identified. The DepoVax vaccine prepared with poly I:C/ Pam3CSK4 is a promising candidate for development of pandemic influenza vaccines, but requires additional testing to demonstrate vaccine efficacy. Additional testing that can be performed in mice include: hemagglutination-inhibition assay, virus neutralization, and class-specific ELISA testing [522]. In addition, induction of cytotoxic $CD8^+$ T cell immunity can be assessed by multimer flow cytometry to detect antigen-specific $CD8^+$ T cells, ELISPOT assay, cellular proliferation and cytotoxicity.

3.3.8.3 Summary of in vivo studies

The *in vivo* studies described herein demonstrate for the first time that a low dose combination of poly I:C and Pam3CSK4 enhanced antibody production to an extent that was equal to or greater than that induced by either adjuvant alone at higher doses, using two different model systems. Anthrax and influenza vaccines each have their own unique challenges in vaccine development, and the results of these studies provide justification for further testing. There are two significant aspects of this adjuvant system that could have applicability to many indications besides anthrax and influenza. First, the amplified serum antibody titers obtained by using two adjuvants at low doses could represent a significant adjuvant sparing effect and reduce the cost of these vaccines. Second, by using low doses of immune-stimulatory adjuvants, we mitigate the risk of side effects that may be associated with using higher doses of single adjuvants. Another consideration that was not fully explored in this study is the potential for reducing boosting requirements by using a potent adjuvant system. In this study we did not compare the

DepoVax formulated vaccines to the currently used vaccines for anthrax or influenza. However, it is notable that the enhanced antibody titers were obtained in this study after only a single immunization.

It remains unclear if the *in vivo* antibody boosting effects of the poly I:C/ Pam3CSK4 adjuvant system were due to direct B cell stimulation or indirect B cell activation through other APCs, such as DCs and macrophages. The role of intrinsic TLR stimulation on B cell responses to vaccination *in vivo* has been controversial [259, 280]. We and others have demonstrated that poly I:C and Pam3CSK4 can increase activation of both DCs and macrophages *in vitro* [307-309]. These cells have been shown to be involved in generating antibody responses to vaccines [23, 196, 238]. Therefore, it is plausible that in our immune competent animal model, stimulation of DCs and macrophages by the poly I:C and Pam3CSK4 adjuvant system contributed to the enhanced antibody responses detected. Previous studies have indicated that the prominence of B cells to vaccine-induced responses may be contingent on the type of antigen [240, 264]. The general consensus is that B cells are integral to priming responses towards particulate antigens, such as viruses and aggregate proteins [526]. B cell interaction with antigen in the lymph node is facilitated by phagocytes that can transport intact antigens from the periphery using non-degradative intracellular compartments [22, 526]. However, in the context of vaccines containing separate TLR agonist adjuvants and antigens, little is known about how TLR agonists may arrive in the lymph node to interact with B cells at the same time as antigen.

In these studies we utilized a novel vaccine platform system, DepoVax, to formulate the antigens and adjuvants. The DepoVax vaccine platform facilitates active phagocytosis of the vaccine components, which we have demonstrated in a separate study by monitoring the *in vivo* migration of iron-labeled antigens over time by magnetic resonance imaging [173]. The results indicated that, unlike emulsion vaccine formulations, the antigens in DepoVax do not freely diffuse from the site of immunization, and are carried to the lymph node by active transport. Although we did not monitor the movement of adjuvants, it stands to reason that the same phagocytic process could also carry adjuvants incorporated into DepoVax to the lymph node where

they may interact with B cells. Certainly, this warrants further investigation into the distribution of adjuvants *in vivo* after vaccination.

3.3.9 Experiment limitations

3.3.9.1 In vitro B cell cultures

The *in vitro* experiments performed on B cells in this study demonstrate a previously undescribed sensitivity to combined stimulation of TLR3 and TLR2. These results may contribute to better understanding of the role that intrinsic TLR stimulation of B cells has to their activation. However, *in vitro* experiments do not always translate equivalently to *in vivo* systems. In these experiments, the *in vitro* culture system of purified B cells does not recapitulate the complex cellular environment of the lymph node where B cell activation normally occurs. In the lymph node, B cells interact with DCs, macrophages, neutrophils and different types of $CD4^+$ and $CD8^+$ T cells, all of which contribute to B cell activation and will influence the course of the immune response [23].

The B cells used in these studies were of the B-2 subset, since they were isolated from the spleens and were $CD19^+$/ $B220^+$ (Appendix Figure B.1B). This B cell population was primarily FO phenotype, as evidenced by CD23 expression on 75% of the cells and high IgD expression (Appendix Figure B.1C). We did not evaluate FO and MZ subsets individually, as others have done, because both contribute to antibody-mediated immunity *in vivo* [52]. Furthermore, the MZ subset in humans has phenotypic and functional differences from the mouse MZ subset, questioning the translational relevance of the study [527].

Comparing T-dependent B cell activation with other studies is difficult due to the diversity in strategies used to initiate CD40 signaling. In our studies we used anti-mouse CD40 IgM, a pentameric Ig complex that facilitated multimerization of CD40 receptor. CD40 multimerization and higher order clustering affect the signal strength imparted by CD40 signaling [528, 529]. Other options, such as the CD40 ligand fraction used by Boeglin *et al.* [258] or mCD154 expression on insect cells by Pone *et al.* [217], may not induce the same degree of CD40 multimerzation as the polyvalent IgM mAb used in our study.

Phosphorylated p65 is often taken as an indication of NFκB activation, but activity should be confirmed by demonstrating nuclear translocation. This may be confirmed by separating cytoplasmic and nuclear protein fractions then probing for phospho-p65. The electrophoretic mobility shift assay can be used to confirm binding of p65 to promoter regions on DNA.

3.3.9.2 In vivo vaccine studies

Our *in vivo* studies enabled us to determine that poly I:C and Pam3CSK4 effectively boosted an antibody response towards protein vaccines. The doses of adjuvant selected were based on *in vivo* dosing of each poly I:C and Pam3CSK4 in the rPA system and the ratio of poly I:C:Pam3CSK4 selected for *in vivo* studies (1:1) did not reflect the ratio used in the *in vitro* studies (25:1). This may be a reflection of the fact that in *in vivo* other cell types may be stimulated by this adjuvant combination, contributing to the overall enhancement of antibody production. Because we used fully immunocompetent mice, we could not rule out the contribution of DCs and macrophages to the ensuing antibody response.

Antibody titers were determined using a Protein G detection system in the *in vivo* experiments. Protein G is an immunoglobulin binding protein expressed by *Streptococcal* bacteria. It binds with varying affinity to mouse antibody isotypes, binding preferentially to IgG1 and IgG2a, and to a lesser extent IgG2b and IgG3. Protein G does not bind IgM, IgA or IgE. The use of Protein G allowed us to quickly assess relative levels of antigen-specific, class switched antibodies, but does not provide an understanding of the type of immune response induced, nor could it detect increases in IgA or IgE responses which may have important implications for application of this adjuvant system.

Although serum antibody titers are an important screen to identify promising vaccine candidates, it does not necessary provide an indication of the effectivenss of the antibodies induced. For both anthrax and influenza there are established neutralization-based assays that are commonly used to provide a measurement of functional activity of vaccine induced antibodies.

3.3.10 Future directions

3.3.10.1 Continuation of in vitro studies

Effect on different B cell subsets, differentiation states

Three distinct B cell subsets have been identified in the mouse: B-1, FO and MZ. Each subset has differential expression of TLRs and response to TLR agonists [223, 227]. Due to the specific functions each of these subsets is reported to contribute to immune responses, it would be of interest to investigate their sensitivity to poly I:C/ Pam3CSK4 stimulation. In particular, MZ cells have an important role in mediating antibody responses to carbohydrate antigens; therefore, understanding how they become more efficiently activated may be useful in developing more effective carbohydrate-conjugate vaccines [226].

There is also variation in TLR expression and responsiveness between naïve and memory B cell phenotypes. Naïve B cells from humans and mice express low levels of TLRs [203, 223, 251, 252]. Naïve murine B cells are more responsive to TLR stimulation than human naïve B cells, but both are enhanced by costimulation provided through BCR and CD40 ligation. Memory human and murine B cells have higher and more diverse expression of TLRs and are more responsive, even without costimulation. Notably, we confirmed that poly I:C and Pam3CSK4 could enhance human B cell proliferation, but not CD40 expression (Appendix Figure B.4). In these studies, the majority of murine B cells used may be considered naïve since they were isolated from 6 week old mice that had not been vaccinated and had minimum exposure to environmental microbial factors [530]. Study of memory B cells in mice is challenging because the memory markers do not correlate with humans and, since they are kept in a pathogen-free environment, they do not naturally generate a diverse memory population as humans do [530, 531]. A naïve versus memory response to poly I:C/ Pam3CSK4 stimulation may be more easily evaluated *in vitro* using human B cells, which can be differentiated in the blood based on the memory surface marker CD27 [232].

Intracellular signaling pathways triggered by poly I:C and Pam3CSK4

This study indicated that the enhanced effects of poly I:C and Pam3CSK4 could be partially attributed to changes in intracellular signaling leading to enhanced activation

of NFκB. Understanding how these signals integrate may provide insight into optimal activation of B cells, and perhaps may be useful in designing other agonist combinations. There are several intracellular signaling proteins that may be of interest for further investigation, including activation of IRF3 and IRF7, phosphorylation of other sites on p65 besides Ser539 and activation of MAPK pathway (ERK, JNK, p38). It was also postulated that the prolonged degradation of IκBα may allow prolonged activity of p65. This may be confirmed by conducting a time course experiment to measure interaction of p65 with DNA by electrophoretic mobility shift assay. This assay may also be used to investigate the binding of p65 to specific promoter regions on the DNA, and determine if there were changes as a result of poly I:C and Pam3CSK4 stimulation.

Poly I:C and Pam3CSK4 stimulation of B cells had slightly different effects in the context of T-dependent activation. CD40 signaling also results in activation of NFκB through the non-canonical pathway. We speculated that stimulation through CD40 as well as TLRs altered the availability of TRAF proteins which are shared between these pathways. It would be of interest to investigate how the recruitment of TRAF proteins differs under these two activation conditions, using immunoprecipitation methods. This could provide insight into how B cell activation is regulated through TLR signaling during T-dependent activation.

Indirect B cell activation

DCs and macrophages both contribute to B cell activation [526] and have been shown by us and others to respond to poly I:C/ Pam3CSK4 combined stimulation *in vitro* [307-309]. To evaluate if the ability of DCs and macrophages to activate B cells is enhanced by the agonist combination, an antigen-specific system such as hen egg lysosome (HEL) could be used. DCs or macrophages from wild type mice could be exposed to HEL antigen in the presence of poly I:C and/ or Pam3CSK4. After thorough washing, the DCs or macrophages would then be co-cultured with B cells isolated from transgenic mice bearing BCR specific to HEL. B cell activation could be measured by proliferation and receptor expression. Addition of anti-CD40 to the culture may be necessary to promote B cell antibody production, but class switch recombination would be influenced by the cytokine secretions of the DCs or macrophages; it would also be of interest to determine the isotype promoted by the cytokine milieu [532]. Additionally,

DCs and macrophages can produce the TACI ligands APRIL and BAFF [278, 533], which may also impact the activation and differentiation of B cells.

3.3.10.2 Continuation of in vivo studies

Role of B cells in priming response to DepoVax protein vaccines

One question we did not address is the role of B cells in the priming of the immune response *in vivo*. Our *in vitro* studies certainly demonstrate that poly I:C and Pam3CSK4 signaling promoted enhanced activation of B cells, and the *in vivo* vaccination studies demonstrate that poly I:C and Pam3CSK4 can boost antibody production. However, the contribution of direct B cell TLR stimulation to the *in vivo* response is unknown. We postulated that the unique features of the DepoVax vaccine platform may enable active transport of antigens and adjuvants to the lymph node for exposure to naïve B cells, and that this stimulation of B cells leads to their more efficient activation and differentiation into antibody-producing plasma cells. This theory may be tested simply by vaccinating mice with two separate vaccines, one containing the antigen and the other containing the adjuvants, at two separate sites. This theory could be further explored by generating conditional knockout mice in which TLR signaling in B cells is ablated by flanking both TLR3 and TLR2 with Lox sequences and placing Cre under control of CD19. Alternatively, chimeric mice could be generated using a mixture of μMT and $TLR2^{-/-}$ or $TLR3^{-/-}$ leukocytes for reconstitution. The impact of limiting or removing TLR signaling in B cells could be assessed by measuring antibody responses in serum by endpoint ELISA as described.

Development of poly I:C/ Pam3CSK4 adjuvanted anthrax and influenza vaccines

The *in vivo* studies indicated that poly I:C and Pam3CSK4 enhanced antibody production towards anthrax and influenza protein antigens. Although only used as a model system, novel vaccines for these indications are being actively studied by Immunovaccine and others. In order to justify translational research in humans, more characterization must be performed using animal models. A number of studies can be done in preparation for this. Antibody function can be assessed by the toxin-neutralization assay for anthrax. Briefly, sera obtained from vaccinated mice are pre-incubated with PA toxin, which is then added to a murine cell line susceptible to killing

by anthrax toxin [534]. After an incubation period, cell death is detected using a standard MTT colourmetric assay. The toxin neutralization assay has been correlated with protection in monkeys [521]. Further optimization of this vaccine may include testing against current standard vaccines, as well as varying dose volume and testing the number of booster shots needed. One key aspect of developing new anthrax vaccines is demonstrating safety, therefore reactogenicity of the vaccine would be closely monitored in mice by performing detailed clinical evolutions of vaccine immunization sites during long-term studies. Ultimately, vaccine efficacy would be tested in challenge studies performed in monkeys before being considered for use in humans [521].

Development of an influenza vaccine candidate would proceed upon similar lines. Functional evaluation of serum antibodies could be assessed using a hemagglutination inhibition assay. This assay measures the capacity of antisera to inhibit the hemagglutination reaction, which is a lattice structure that forms between red blood cells and surface HA proteins of influenza virus. In the inhibition assay, dilutions of serum are added to wells containing red blood cells and virus and the titer is defined as the lowest dilution capable of inhibiting lattice formation. Generation of cytotoxic T lymphocytes (CTLs) is another aspect considered in developing influenza vaccines, as CTLs can recognize and kill viral infected cells. Induction of CTLs can be measured using IFN-γ ELISPOT assay. Briefly, splenocytes from vaccinated mice are stimulated with HA protein or peptide on ELISPOT plates pre-coated with anti-IFN-γ. After set incubation time, usually overnight, the plates are washed and bound IFN-γ is detected using a secondary HRP-coupled antibody. Efficacy of influenza vaccine may be measured using the ferret model [181].

CHAPTER 4:
IN VIVO DEVELOPMENT OF A COMBINATORIAL IMMUNOTHERAPY FOR CANCER

Portions of this chapter have been accepted for publication:

Weir GM, Hrytsenko O, Stanford MM, Berinstein NL, Karkada M, Liwski RS, Mansour M. Metronomic cyclophosphamide enhances HPV16E7 peptide vaccine induced antigen-specific and cytotoxic T-cell mediated antitumour immune response. (2014) *Oncoimmunology*, 3:8. **Open Access**.

Contributions:

GW participated in project conception, designed and executed experiments, analysed and interpreted data, and prepared manuscript. OH optimized and performed qPCR analysis. MS, NB, MK, RL and MM all contributed to project conception, experiment design, data interpretation and manuscript revision. Technical assistance to perform experiments was provided by V. Kaliaperumal, R. Rajagopalan, A. MacKay, T. Quinton, and B. Ray (acknowledged).

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4.1 Results

4.1.1 mCPA and sbCPA in combination with a peptide-based therapeutic vaccine provide enhanced tumour control

Immune modulation with low dose cyclophosphamide (CPA) administered as single bolus (100 mg/kg IV; sbCPA) was compared to metronomic administration (20 mg/kg/day PO; mCPA) in combination with a DepoVax based peptide vaccine containing R9F peptide antigen (DPX-R9F). Mice were implanted subcutaneously with C3 tumours and vaccinated every three weeks starting 12 days after implantation. sbCPA was given every three weeks, one day before each vaccination and mCPA treatment was given one week on/ one week off starting on day 5. Starting vaccination on day 12 enabled us to test the combination of low dose CPA with vaccine with established tumours which are difficult to control with vaccine or low dose CPA monotherapy.

Tumor growth of mice treated with mCPA or sbCPA combinations are shown in Figures 4.1. All groups were part of the same experiment, and the vaccine alone and PBS control groups are the same in each panel. Repeated DPX-R9F vaccine monotherapy delayed tumour growth, as expected, but was ultimately unable to control tumour growth. Both mCPA and sbCPA also delayed tumour growth when given as a monotherapy, but sbCPA provided a significantly longer delay in tumour growth than mCPA ($p<0.0001$). Both mCPA and sbCPA in combination with DPX-R9F vaccine provided durable tumour growth control with no significant differences between the two combinations in the long term.

The presence of an active immune response was confirmed in each group eight days after the final vaccination (Day 62 of the study) by terminating the mice and performing IFN-γ ELISPOT using lymph node cells (LNC) isolated from the vaccine draining inguinal lymph node. The ELISPOT results shown in Figure 4.2A indicate that the mice implanted with C3 tumours and treated with either mCPA or sbCPA in combination with DPX-R9F vaccination generated a strong antigen-specific immune response. There is non-specific IFN-γ background in these groups as well which has been reported by others after CPA treatment which has been associated with increased proliferation of T cells [535]. Dextramer staining for R9F-specific $CD8^+$ T cells also

confirmed the presence of antigen-specific $CD8^+$ T cells (Figure 4.2B). Similar effects were seen in the spleen (% R9F-specific CD8/ Total spleen CD8: DPX-R9F only = 5.05%, DPX-R9F & mCPA = 6.30%, n=3).

4.1.2 Timing of mCPA relative to vaccination does not significantly affect efficacy or immunogenicity

A disadvantage to sbCPA treatment is that the timing of vaccination relative to CPA administration is very sensitive [464, 466]. To test whether the timing of mCPA relative to vaccination affected the efficacy of treatment, we performed a tumour challenge experiment in which mice were consistently vaccinated at the end or beginning of one week mCPA administration. To determine how late treatment could be initiated after tumour challenge and still provide protection, treatment was begun either one week (day 7) or two weeks (day 14) after tumour implantation. All groups were part of one study and were treated in parallel, allowing comparison between treatment groups (Figure 4.3).

In this study, combination therapy provided significantly better protection from tumour growth compared to monotherapy when started early on day 7 (Figure 4.3, A&B). When treatment was started later, on day 14, combination therapy provided no additional therapeutic benefit than either treatment alone (Figure 4.3, C&D). The effectiveness of combination therapy when vaccination was provided both at the start (Figure 4.3A) and at the end (Figure 4.3B) of each mCPA cycle indicates that providing mCPA before or after vaccination does not reduce immunogenicity of vaccine. We also confirmed that mCPA given for one week before or after vaccination did not impact vaccine immunogenicity by performing IFN-γ ELISPOT with lymph nodes of treated mice (Figure 4.4).

4.1.3 Immune responses in the lymph node

4.1.3.1 mCPA enhances immune responses in lymph node detected by IFN-γ ELISPOT after a single round of treatment

To investigate the effects on the immune system induced by the combination of mCPA with DPX-R9F in treatment of C3 tumour bearing mice, and to ensure all groups were alive at the time of analysis, we tested mice implanted with tumours and treated

with a single week-long cycle of mCPA starting on day 14 followed by a single vaccination with DPX-R9F on day 21 (Figure 4.5A). This treatment schedule was used for all the following studies. On day 29, eight days after vaccination, mice were terminated and vaccine-draining inguinal lymph nodes and spleens were collected and counted (Figure 4.5B). At this time point, total lymph node counts were significantly reduced in most groups treated with mCPA relative to the non-mCPA control groups ($p<0.05$). The group treated with mCPA alone did not significantly reduce lymph node size compared to untreated tumour bearing mice ($p=0.683$). The lymph nodes in the non-tumour bearing mice treated with mCPA alone were too small for analysis. We determined the relative antigen-specific immune responses in the lymph nodes by performing IFN-γ ELISPOT (Figure 4.6). Despite having fewer lymph node cells, the antigen-specific immune response in tumour bearing mice was enhanced in the group treated with mCPA and DPX-R9F vaccine. In non-tumour bearing mice, IFN-γ ELISPOT responses were the same in the groups vaccinated with or without mCPA treatment.

4.1.3.2 Antigen-specific $CD8^+$ T cells are enriched in lymph nodes of mice treated with vaccination and mCPA

To determine if the increased IFN-γ ELISPOT responses were due to increased generation of antigen-specific $CD8^+$ T cells, we performed flow cytometry on the lymph node cells using a dextramer reagent that can detect R9F-specific TCR. We found that in both tumour bearing and non-tumour bearing mice, mCPA treatment caused a significant reduction in number of $CD8^+$ T cells (Figure 4.7). However, the number of antigen-specific $CD8^+$ T cells induced by the vaccine remained constant, with and without mCPA treatment

4.1.4 Immune Responses in the Spleen

4.1.4.1 mCPA enhances immune responses in the spleen after a single round of treatment

The spleen is more representative of a systemic immune response than the vaccine-draining lymph node. Immune responses in spleens of mice (treated as shown in

Figure 4.5A) were tested by IFN-γ ELISPOT (Figure 4.8A). Similar to the vaccine draining lymph node, the response was increased in the spleens of mice treated with combination therapy compared to vaccine alone, in response to R9F peptide stimulation, however it was not statistically different ($p=0.097$). Non-tumour bearing mice treated with the vaccine and mCPA generated a significantly higher IFN-γ ELISPOT response than non-tumour bearing mice treated with vaccine only.

To evaluate the possibility of epitope spreading, we stimulated splenocytes from treated tumour bearing mice with C3 cells and detected response by IFN-γ ELISPOT (Figure 4.8B). We found that untreated mice and mice treated with mCPA had low responses to C3 cell stimulation (<10 SFU), but mice vaccinated without (SFU 24 ± 6) and with mCPA treatment (SFU 48 ± 13) generated significantly higher responses than untreated mice ($p<0.05$).

4.1.4.2 mCPA enhances antigen-specific CTL activity induced by DPX-R9F

An *in vivo* cytotoxic T lymphocyte (CTL) assay was performed to measure the functional activity of the cytotoxic T cells induced by the vaccine. In this experiment, mice were vaccinated with DPX containing R9F and the T-helper F21E antigens instead of the conjugated R9F-PADRE antigen used for other experiments. This was to allow proper comparison to groups vaccinated with a control vaccine containing an irrelevant antigen, S9L ($TRP2_{180\text{-}188}$), and F21E. Previous experiments have demonstrated that DPX formulated with R9F-PADRE or R9F + F21E generate comparable IFN-γ ELISPOT responses (data not shown).

Correlating with the increase in IFN-γ ELISPOT responses, antigen-specific killing of R9F-loaded target cells was significantly increased by DPX-R9F vaccination in tumour bearing and non-tumour bearing mice (Figure 4.9A). Antigen-specific killing induced by mCPA in combination with vaccine tended to be higher, but was not statistically higher than vaccine alone (tumour bearing: $p=0.801$, non-tumour bearing: $p=0.401$). Mice vaccinated with the irrelevant control vaccine did not display antigen-specific killing of the R9F-loaded target cells, even with mCPA combination treatment (Figure 4.9B).

4.1.4.3 Adoptive transfer of splenic T cells imparts passive immunity

We noted that even transient and delayed treatment resulted in significantly smaller tumours in the mCPA only or combination treated groups by the end of the study period on Day 29 (Figure 4.10A). To determine if immunity could be transferred using T cells derived from treated animals, we isolated spleens from tumour-bearing mice on day 29 after treatment with mCPA, DPX-R9F or the combination. Purified total T cells ($CD3^+$) were then transferred into recipient mice bearing 3-day old tumours. The antigen-specific immune reactivity of the donor splenocytes was confirmed by IFN-γ ELISPOT (Figure 4.10B) before isolating total T cells. T cell purity was confirmed by immunofluorescence to be >85%, with <5% NK T cells (Appendix Figure B.5). As a positive control, one group of recipient mice did not receive any T cells but was immunized with DPX-R9F at the same time, and a negative control group was treated with PBS.

As shown in Figure 4.10C, only the T cells isolated from mCPA and DPX-R9F treated donors significantly reduced tumour growth in recipient mice ($p<0.001$). We repeated this experiment but transferred purified $CD8^+$T cells (Figure 4.10D) from mCPA treated or mCPA and DPX-R9F treated donors. In this study, only the $CD8^+$ T cells isolated from the combination treated donors significantly reduced tumour volume ($p<0.05$).

4.1.5 Combination therapy increases cytotoxic T cells in the tumour microenvironment

To determine if the antigen-specific $CD8^+$ T cells generated by the treatment could be detected in the tumour, we isolated tumours on day 29 for flow cytometry analysis. We found that mice treated with DPX-R9F vaccine and mCPA had significantly higher percentage of total $CD8^+$ T cells within the tumour ($p<0.01$; Figure 4.11A). Of the $CD8^+$ T cells detected within the $CD45^+$ population of cells in the tumour, mice vaccinated with or without mCPA treatment had approximately the same level of antigen-specific $CD8^+$ T cells as detected using the R9F-dextramer reagent (~1.5% of $CD8^+$/ $CD45^+$; Figure 4.11B).

To measure several markers at once within the tumour microenvironment, we extracted total RNA from tumours of mice on day 29 and performed RT-qPCR (Figure 4.12). We assessed markers associated with cytotoxic T cell activity, *CD8α* (CD8α), *Gznb* (Granzyme B), and *Ifng* (IFN-γ) as well as the NK gene *Klrc1* (killer cell lectin-like receptor subfamily C). We also looked for *Cd4* (CD4) expression as well as the Treg associated genes *FoxP3* (FoxP3) and *Il10* (IL-10). Levels of *Il4* (IL-4) and *Cd19* (CD19) were measured to determine if there was any indication of Th2 or B cell responses in the tumour microenvironment. We assessed mRNA expression of the co-inhibitory proteins PD-1 (*Pdcd1*) and CTLA-4 (*Ctla4*), which are known to be increased on tumour infiltrating lymphocytes which could dampen their activity [536]. Low dose CPA treatment has been associated with an anti-angiogenic effect, therefore we measured *Vegf* mRNA [456].

Corresponding with the increased detection of $CD8^+$/ $CD45^+$ T cells by flow cytometry (Figure 4.11A), the level of *Cd8* mRNA was significantly increased in tumour of mice treated with mCPA/ DPX combination (Figure 4.12A). Coinciding with increased CD8 T cell gene signatures were increased expression of the CTL cytokine markers IFN-γ (Figure 4.12B) and granzyme B (Figure 4.12C), while levels of IL-4 (Figure 4.12I) were low across all groups, indicating a skew towards Th1 phenotype within the tumour microenvironment. Expression of Treg markers FoxP3 (Figure 4.12E) and IL-10 (Figure 4.12F) were both increased in the combination treated group. B cell levels, as measured by CD19 mRNA (Figure 4.12H) were low in all groups. The expression of co-inhibitory markers, PD-1 (Figure 4.12J) and CTLA-4 (Figure 4.12K), were significantly increased in tumour of mice treated with the combination of mCPA and vaccine. Levels of VEGF (Figure 4.12L) were low in all groups.

4.1.6 Effects of mCPA treatment on splenocyte immune phenotypes

The adoptive transfer of T cells or $CD8^+$ T cells was only able to confer partial immunity to recipient mice, indicating that the mCPA may have other effects on the immune system besides enhancing CTL activity. The spleen has been reported to be an important reservoir for tumour-induced immune suppressive cells [537], therefore we conducted an extensive survey on spleen immune populations by flow cytometry. In

order to assess how the tumour was affecting immune responses, we treated both tumour bearing and non-tumour bearing mice. Single bolus CPA has been reported to have a transient effect on immune cells, which reach nadir 4 days after treatment before rebounding [434, 460], but immune response to vaccination peak at day 8 following vaccination (personal observations). Therefore, we looked at the levels of several cell types 4 days and 8 days after treatment by flow cytometry (days 25 and 29 of study, respectively).

On day 25, four days after vaccination or mCPA treatment ended, we found that while the total number of splenocytes was not different between groups (Figure 4.13A), $CD8^+$ T cells (Figure 4.13B), $CD4^+$ T cells (Figure 4.13C) and B cells (Figure 4.13D) were significantly reduced in groups treated with mCPA compared to non-mCPA treated groups. No significant differences were detected in total NK cells (data not shown). There was a statistically significant reduction in total Tregs in the combination treated group compared to the vaccine only treated group, but the decline in Tregs of the mCPA only treated group was not significant compared to untreated tumour bearing mice (Figure 4.13E). We also did not detect any differences in number of myeloid derived suppressor cells (MDSCs) at this time (Figure 4.13F).

On day 29, eight days after vaccination or mCPA treatment ended, we evaluated the spleen populations of tumour bearing and non-tumour bearing mice (Figure 4.14). By this time, the total numbers of $CD8^+$ (Figure 4.14B) and $CD4^+$ T cells (Figure 4.14C) were similar in all groups. Total B cells remained significantly low in the combination treated mice, compared to mice treated with mCPA or vaccine alone (Figure 4.14D), but total Tregs in the combination group were not significantly lower (Figure 4.14E). Notably, total MDSCs in tumour bearing mice treated with mCPA alone were selectively increased, but not in non-tumour bearing mice treated with mCPA (Figure 4.14F). Tumor bearing mice treated with mCPA in combination with vaccination also did not have elevated MDSCs levels.

To confirm that these MDSCs had functional activity, they were isolated from the spleens of each tumour bearing group on day 29 and used in a suppression assay by co-culturing them 2:1 with purified T cells stimulated with anti-CD3/ CD28 beads. The MDSCs from all groups equally suppressed proliferation of T cells (Figure 4.15).

4.2 Figures

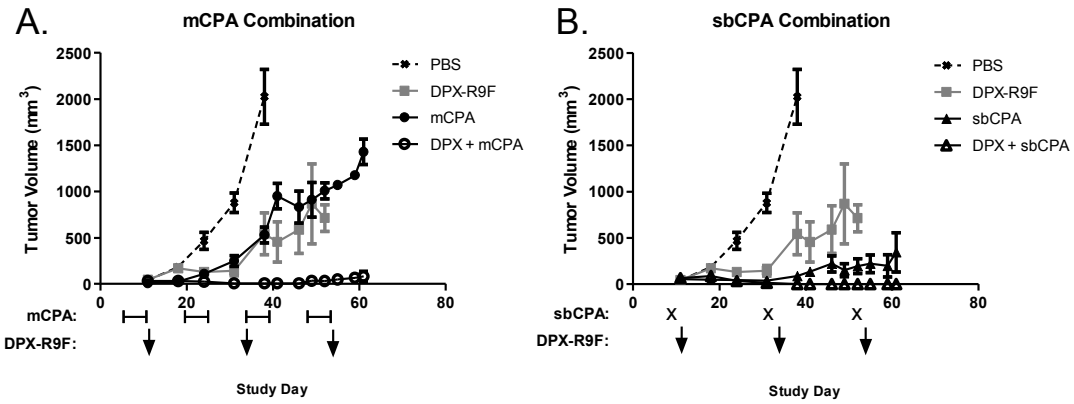

Figure 4.1: Low dose cyclophosphamide provided as mCPA or sbCPA enhance tumour control in combination with DPX-R9F vaccine. Mice (n=8) were implanted with tumours on day 0 and treated with mCPA (20 mg/kg/day PO) starting on day 5 in a one week on/ one week off schedule **(A)** or received a sbCPA (100mg/kg IV) one day preceding each vaccination **(B).** Vaccinations with DepoVax containing 10 μg R9F-PADRE antigen (DPX-R9F) commenced on day 12 and repeated every three weeks (days 33 and 54). Results are representative of two separate experiments.

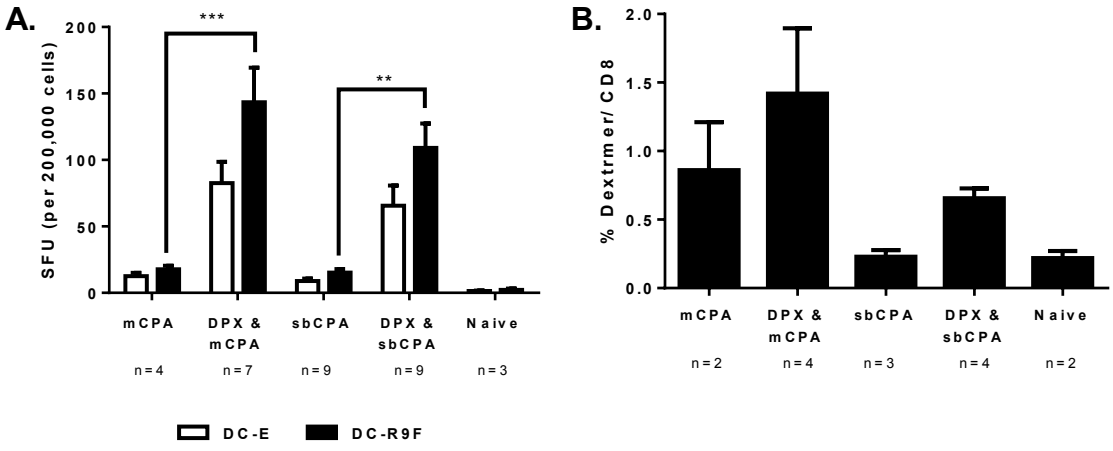

Figure 4.2: Mice treated with vaccine and low dose cyclophosphamide develop antigen-specific immune responses. Mice were treated as described in Figure 4.1. Eight days after the last vaccination (day 62), mice in remaining groups were terminated and vaccine-draining lymph nodes collected. **(A)** Immune response measured by IFN-γ ELISPOT. Lymph node cells were stimulated with syngeneic DCs unloaded (DC-E) or loaded with R9F peptide (DC-R9F), data are pooled from two separate experiments. **(B)** LNC were also stained for CD8 T cells and R9F-specific CD8 T cells using a dextramer, data are from one experiment only. Statistics by one-way ANOVA with Tukey post test, **$p<0.01$, ***$p<0.001$

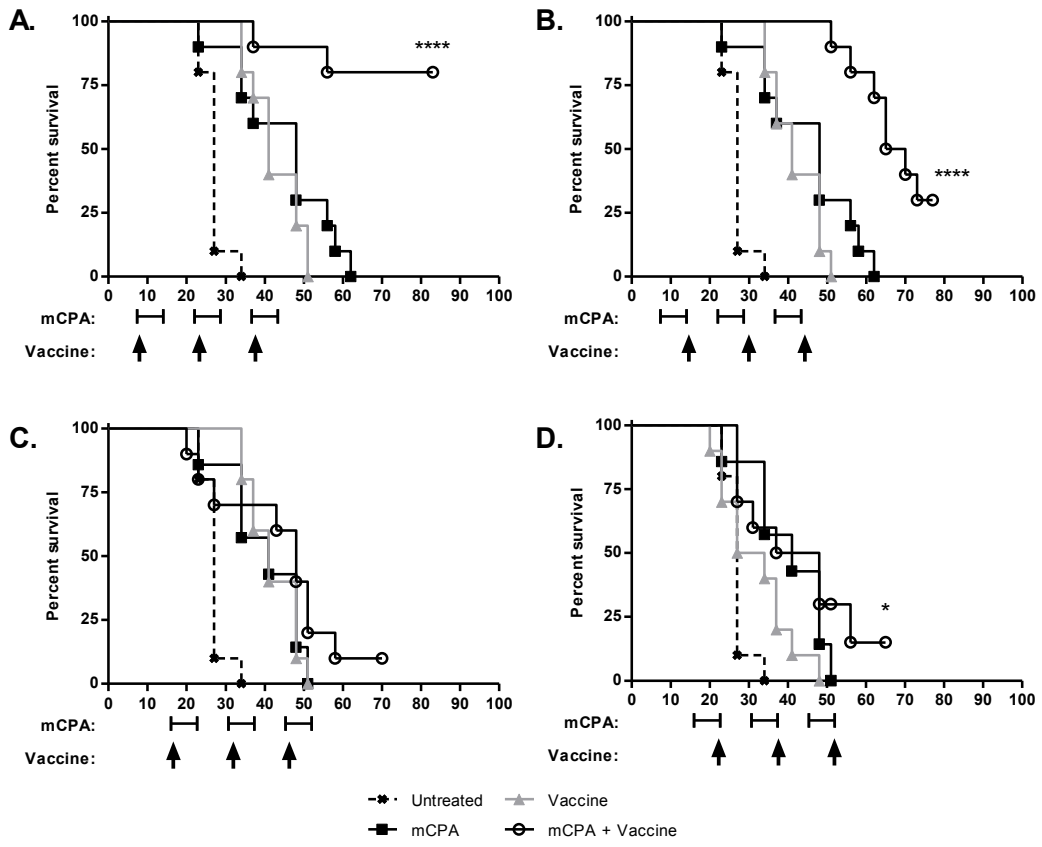

Figure 4.3: Best tumour control is provided with early vaccination and mCPA treatment. Mice (n=10) were implanted with C3 tumours on study day 0. Groups were treated with mCPA only, vaccine (DPX-R9F) only or a combination of mCPA & vaccine, one group of mice was vaccinated with PBS as a negative control. All groups were part of the same experiment and were treated in parallel. Treatment commenced one week (Day 7; **A, B**) or two weeks (Day 15; **C, D**) after implantation. mCPA was administered every other week for 7 consecutive days and vaccine was given corresponding to the first day of each mCPA treatment **(A, C)** or the last day **(B, D)**. Mice were terminated humanely when tumour size reached 2000 mm^3. Statistics by log-rank (Mantel-Cox) test, comparing vaccine only with combination therapy: *p<0.05, ****p<0.0001.

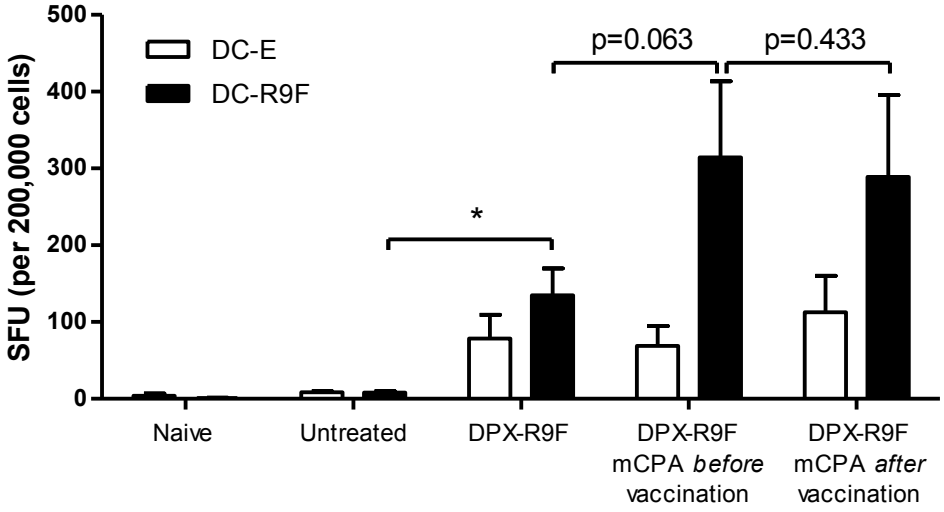

Figure 4.4: Metronomic cyclophosphamide provided before or after vaccination enhances antigen-specific immune responses. Mice (n=5) were implanted with C3 tumours on day 0 and then vaccinated with DPX-R9F on day 21. mCPA was administered for one week *before* vaccination (days 14-21) or one week *after* vaccination (days 21-28). Mice were terminated on day 29 and immune responses in the vaccine draining lymph nodes to DCs loaded with R9F peptide or unloaded were assessed by IFN-γ ELISPOT. Statistics by students t-test, *$p<0.05$.

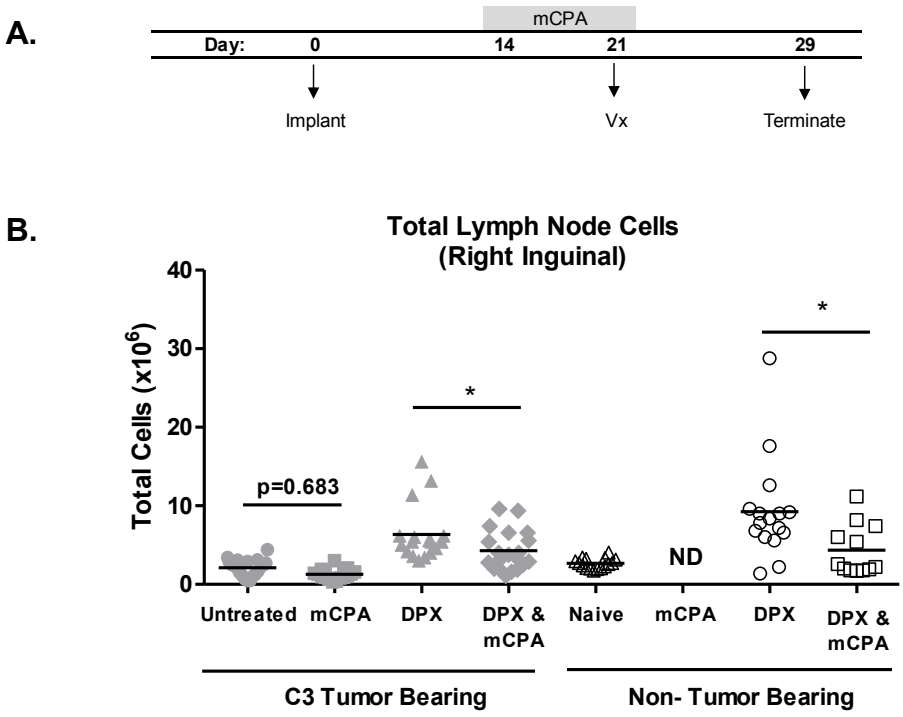

Figure 4.5: Treatment with mCPA reduces lymph node cells. **(A)** Depiction of treatment schedule. Briefly, mice were implanted with C3 tumours on day 0 and treated for 1 week with mCPA between days 14-21. Mice were vaccinated on day 21 with DPX-R9F and terminated on day 29 for immunological analysis. Non-tumour bearing mice did not receive tumour implantation, but were treated with mCPA and/ or DPX-R9F in parallel. **(B)** Total lymph nodes collected from right inguinal vaccine-draining lymph node. Data are shown as mean of 5-25 individual mice ± SEM. Statistics between indicated groups by one-way ANOVA with Tukey post-test, *$p<0.05$, **$p<0.01$. ND: not detected. Results are pooled from 5 separate experiments.

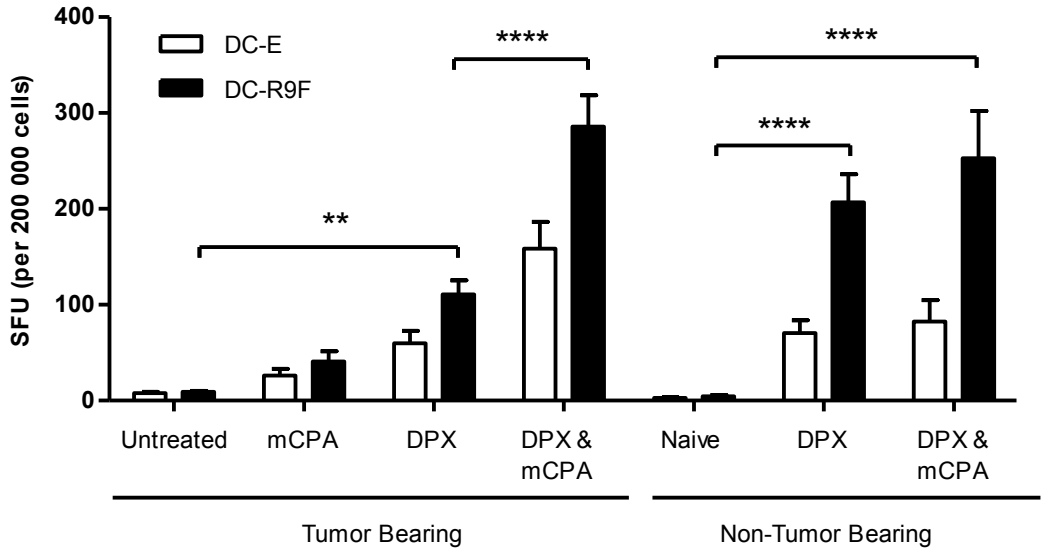

Figure 4.6: Metronomic cyclophosphamide does not reduce vaccine-induced immune response in lymph node. Mice (n=12-30) were treated as shown in Figure 4.5A. On day 29, mice were terminated and IFN-γ ELISPOT performed with lymph node cells (200,000 cells per well) stimulated with syngeneic dendritic cells unloaded (DC-E) or loaded with R9F peptide (DC-R9F). Data shown as mean of individual mice ± SEM. Statistics by one-way ANOVA with Tukey multiple comparisons post-test. *$p<0.05$, ***$p<0.001$. Results are pooled from 5 separate experiments.

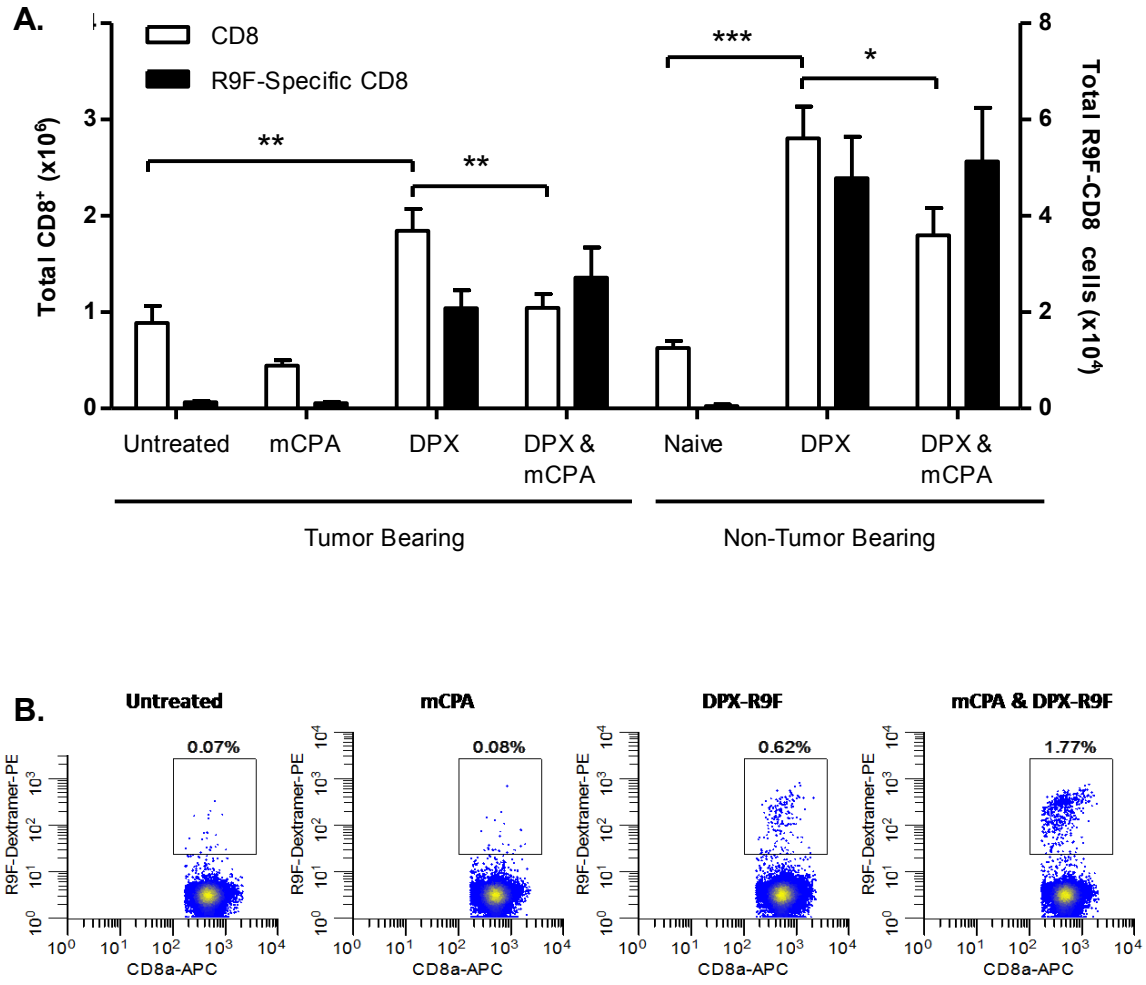

Figure 4.7: mCPA enriches antigen-specific CD8 T cells in the lymph node. **(A)** Mice were treated as in Figure 4.5A. Antigen-specific $CD8^+$ T cells were detected by flow cytometry after staining with R9F-dextramer and anti-CD8α. **(B)** Representative dot plots. Data shown as mean of individual mice ± SEM. Statistics between indicated groups by one-way ANOVA with Tukey post-test, *p<0.05, **p<0.01. Results are pooled from 2 separate experiments.

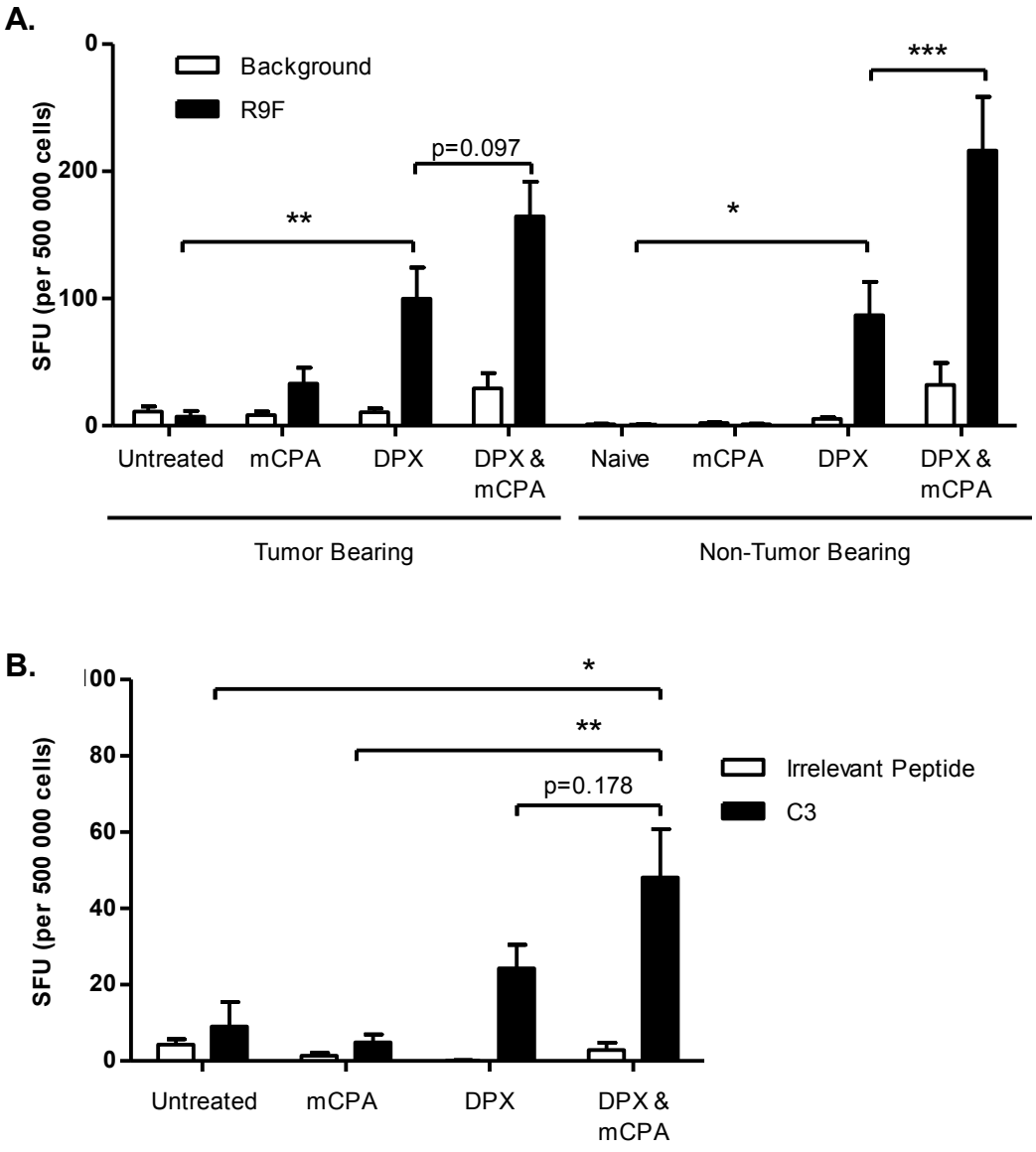

Figure 4.8: mCPA combination with DPX-R9F vaccination results in systemic increase in immune response. Spleens were removed from mice treated as in Figure 4.5A. **(A)** IFN-γ ELISPOT using splenocytes stimulated with R9F peptide or unstimulated (background), n=23-25, mice pooled from 6 separate experiments; **(B)** IFN-γ ELISPOT using splenocytes stimulated with an irrelevant control peptide or C3 tumour cells, n=5 from one experiment. Statistics by one-way ANOVA with Tukey post-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

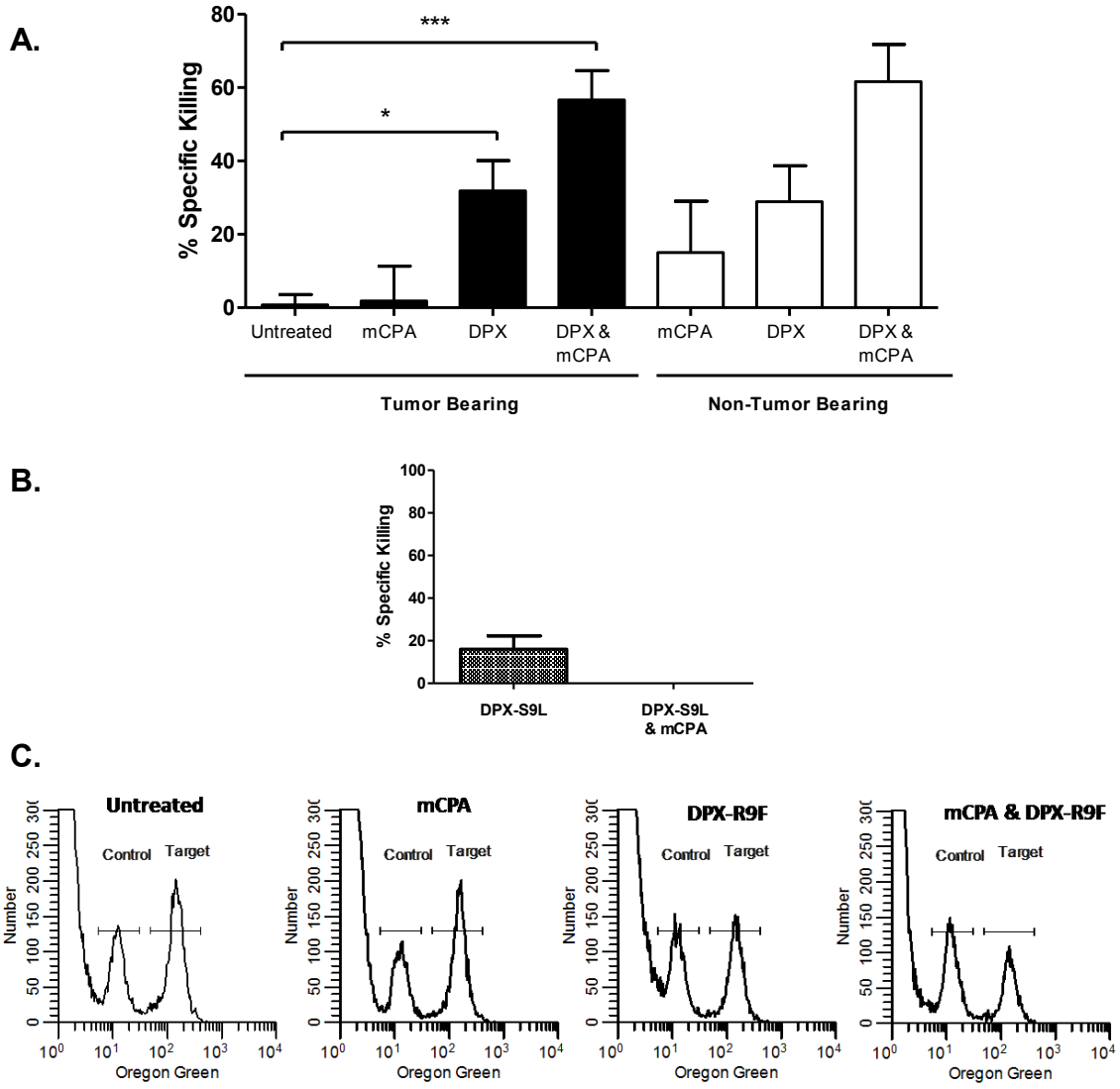

Figure 4.9: Treatment with mCPA enhances cytotoxic T cell activity induced by vaccination. (A) Mice were treated as in Figure 4.5A. In this study, DPX-R9F vaccine was prepared with R9F + F21E instead of R9F-PADRE. On day 28, mice were injected with a 1:1 mixture of Oregon Green 488 labeled unloaded target cells or R9F loaded target cells. Next day, percent specific killing was detected in the spleen by flow cytometry. Results pooled from 3 separate experiments, n as indicated. **(B)** Control mice bearing C3 tumours were vaccinated with an irrelevant peptide S9L + F21E in DPX (DPX-S9L) and injected with the R9F-loaded target mixture (n=3). **(C)** Representative histograms showing the control (unloaded) and target (R9F loaded) populations. Data shown as mean of individual mice ± SEM. Statistics by one-way ANOVA with Tukey post-test, *$p<0.05$, **$p<0.01$.

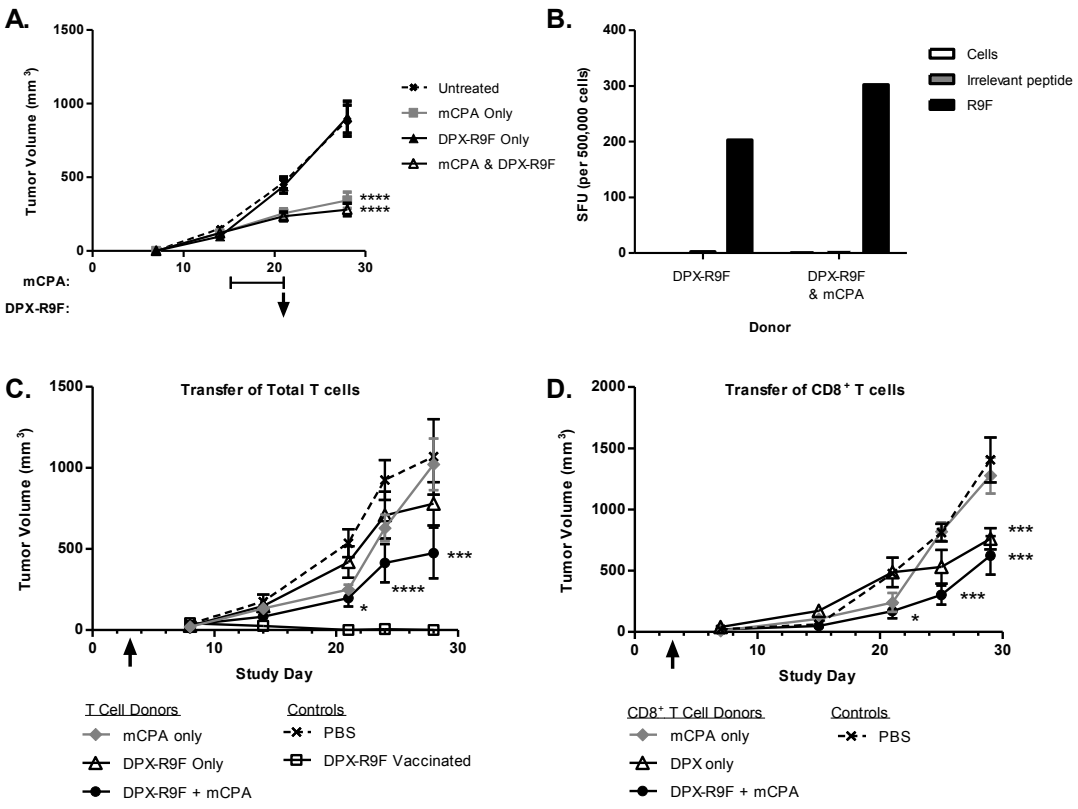

Figure 4.10: Protective immunity is partially transferred through T cells from tumour bearing, mCPA & DPX-R9F treated donor mice. Donor mice were treated as in Figure 4.5A. **(A)** Tumor growth of donor mice until termination. On day 29, donor mice were terminated and spleens and LN collected and pooled from each group. Total T cells were purified by magnetic separation and injected IV into recipient mice which had been implanted with tumours three days before adoptive transfer. **(B)** Antigen-specific immune response of donor cells was confirmed by IFN-γ ELISPOT before transfer to recipient mice, representative data from a single experiment. **(C)** Tumor growth of mice that were transferred (on day 3, indicated with arrow) with total T cells (n=5-15). **(D)** Tumor growth of mice that were transferred (on day 3, indicated with arrow) with $CD8^+$ T cells (n=5-10). Results pooled from 2-3 separate experiments. Statistics by two-way ANOVA with Bonferroni post-test vs. PBS, ***p<0.001, ****p<0.0001.

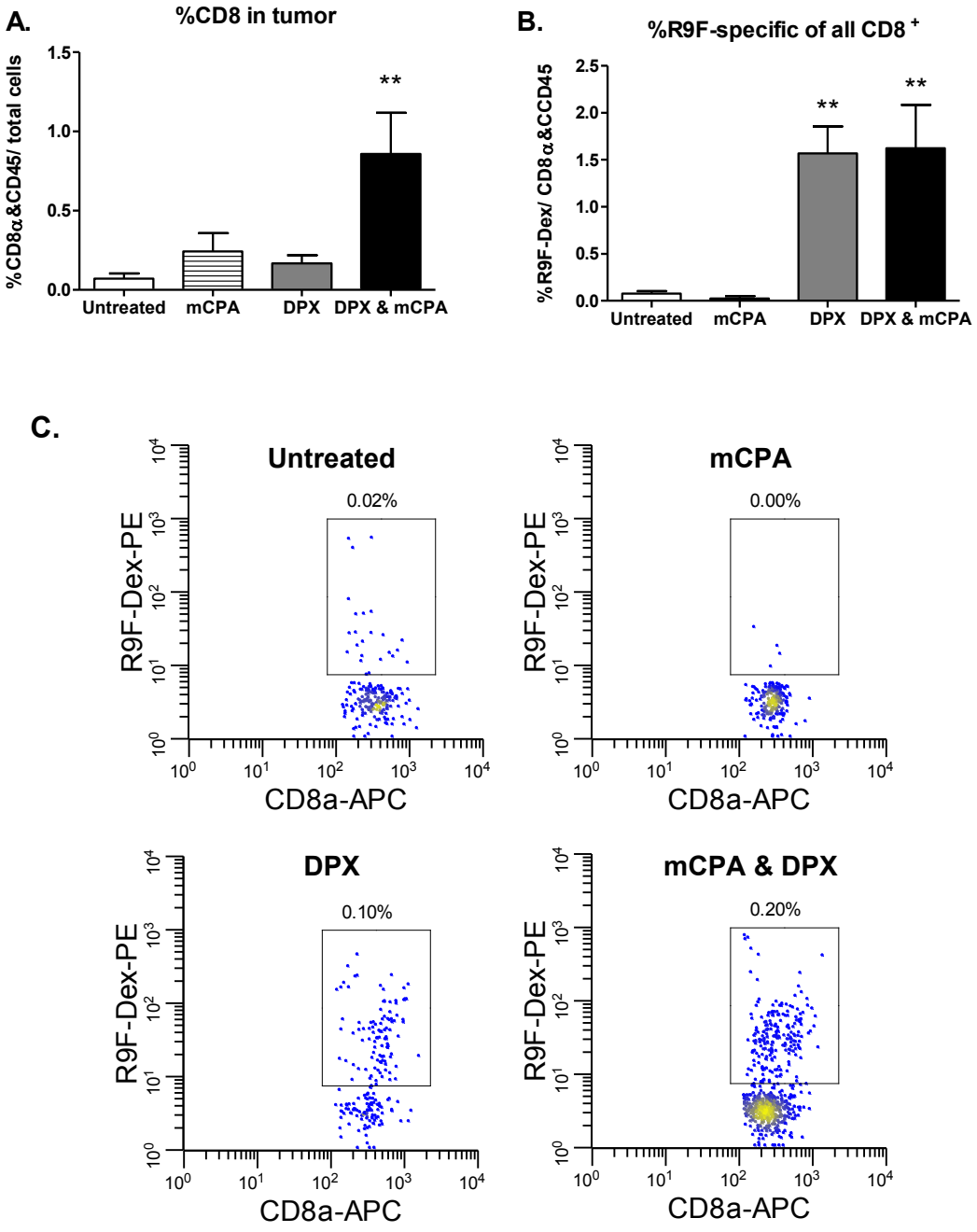

Figure 4.11: Detection of CD8$^+$ and antigen-specific CD8$^+$ T cells in tumour. Mice were treated as in Figure 4.5A. On day 29, tumours were removed and dissociated using enzymatic digestion. Tumor cells were stained with CD45, CD8α and R9F-dextramer reagent to detect positive cells by flow cytometry. **(A)** Percent of CD8α$^+$ & CD45$^+$ T cells of all tumour cells. **(B)** Percent R9F-specific out of CD8α$^+$ & CD45$^+$ T cells. N=4-9 pooled from 2 separate experiments. Statistics by one-way ANOVA with Tukey post-test comparing each bar to untreated, *p<0.05, **p<0.01. **(C)** Representative dot plots showing CD8α and R9F-dextramer double staining of CD45$^+$ events, percentage indicates percent of total cells.

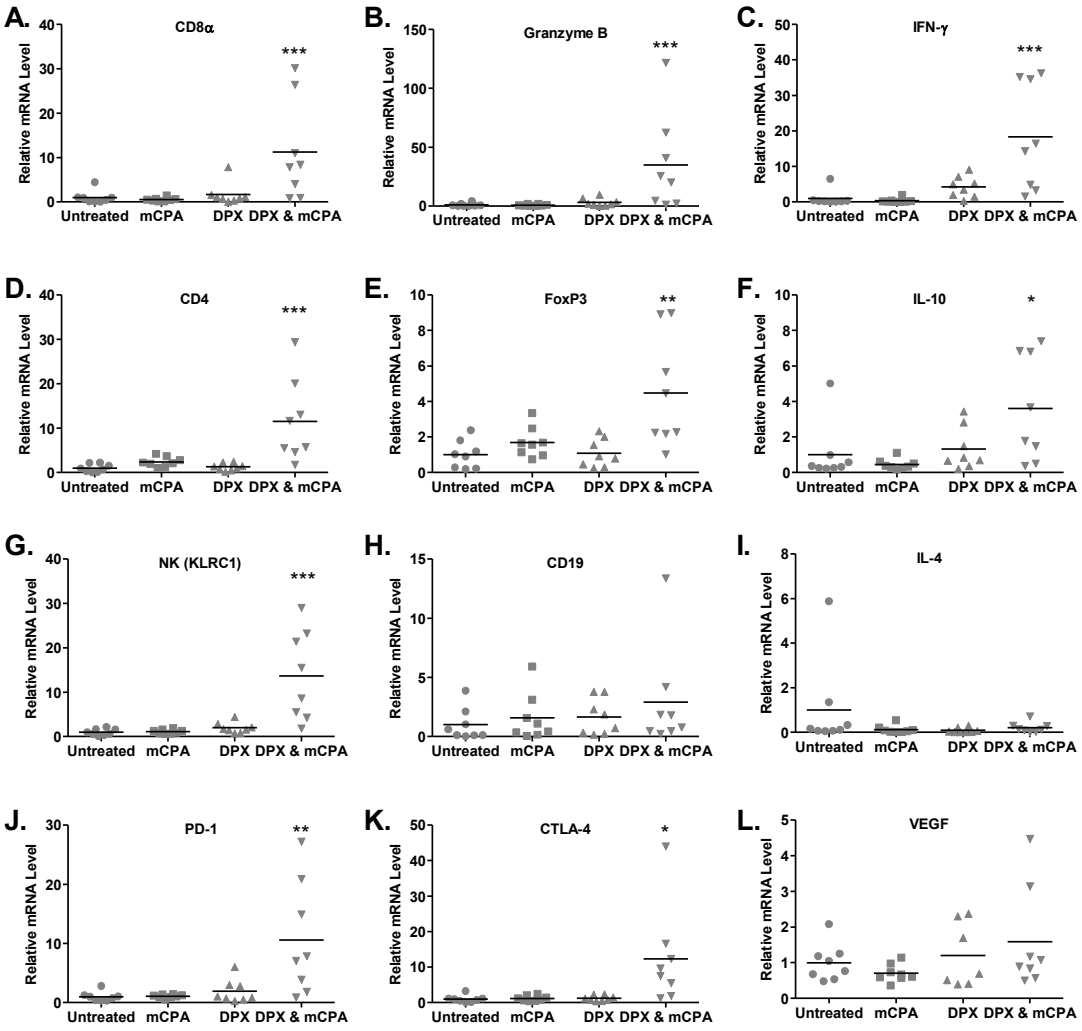

Figure 4.12: Treatment with mCPA & vaccination increases the intratumoural expression of several genes associated with activated cytotoxic T cells. Mice were treated as in Figure 4.5A. On day 29, tumours were removed for RT-qPCR analysis and mRNA expression levels determined relative to *Tbp* (TATA-binding protein) control. Relative levels of transcript are shown for: **(A)** CD8α (*Cd8a*), **(B)** Granzyme B (*Grnb*), **(C)** IFN-γ (*Ifng*), **(D)** CD4 (*Cd4*), **(E)** FoxP3 (*Foxp3*), **(F)** IL-10 (*Il10*), **(G)** NKG2A (*Klrc1*), **(H)** CD19 (*Cd19*), **(I)** IL-4 (*Il4*), **(J)** PD-1 (*Pdcd1*), **(K)** CTLA-4 (*Ctla4*), **(L)** VEGF (*Vegf*). Data shown as individual mice (n=8) with mean indicated by bar, results pooled from two separate experiments. Statistics by one-way ANOVA with Tukey post-test compared to untreated, *p<0.05, **p<0.01, ***p<0.001. RT-qPCR data was obtained and analysed by O. Hrytsenko.

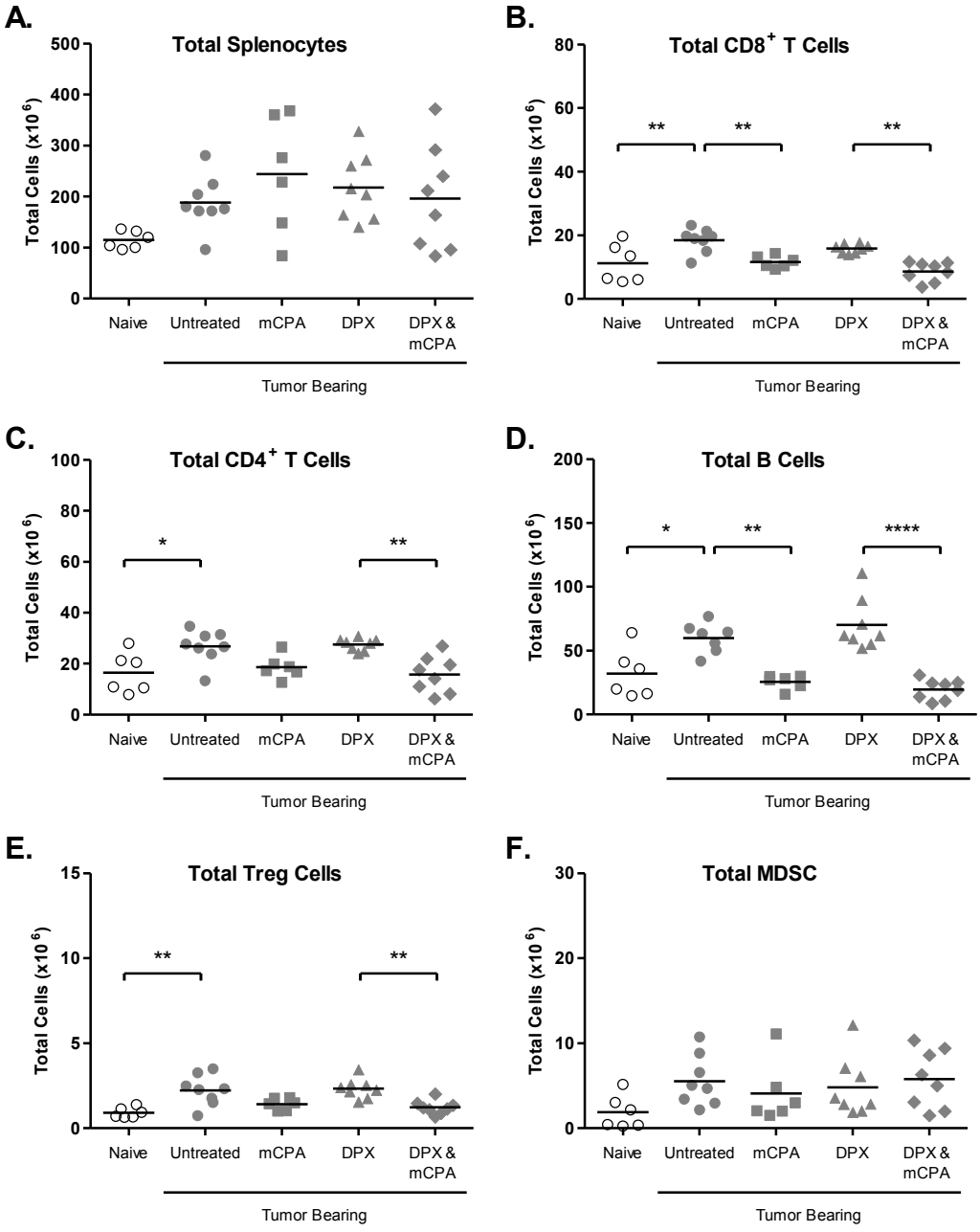

Figure 4.13: Treatment with mCPA alters the immune cell profile in the spleen. Mice were treated as in Figure 4.5A. On day 25, mice were terminated and spleens collected. Total spleen counts were determined and spleen populations were detected by flow cytometry. **(A)** total splenocytes; **(B)** total $CD8^+$ T cells ($CD8^+$); **(C)** total $CD4^+$ T cells ($CD4^+$); **(D)** total B cells ($CD19^+$); **(E)** total Treg cells ($CD4^+$/ $FoxP3^+$/ $CD25^{hi}$); **(F)** total MDSC ($CD11b^+$/ $GR\text{-}1^{hi}$). N=6-8 mice pooled from two separate experiments. Statistics by one-way ANOVA with Tukey post-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

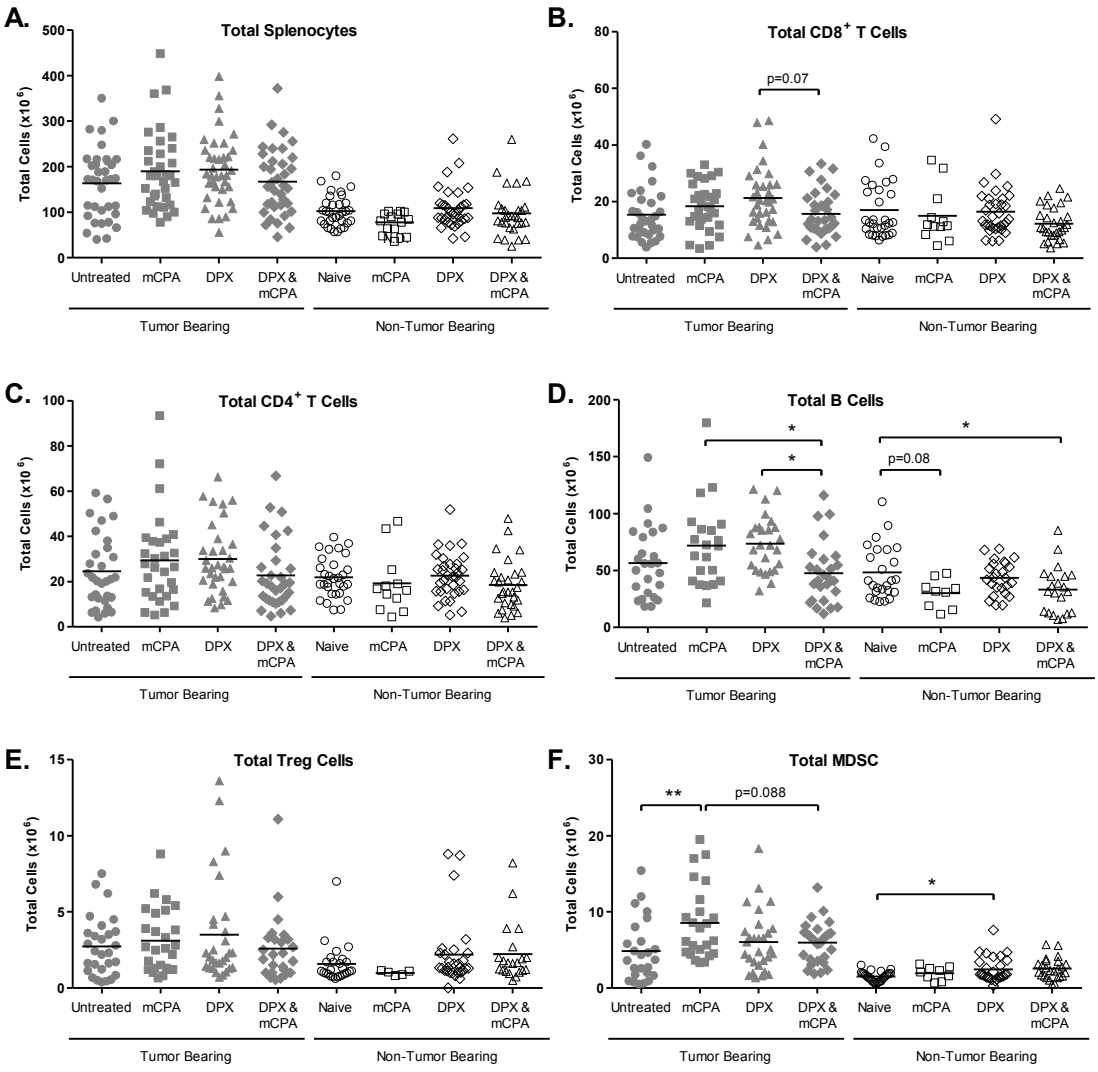

Figure 4.14: DPX-R9F vaccination attenuates mCPA-induced enrichment of immunosuppressive cells in the spleen. Mice were treated as in Figure 4.5A. Naïve mice without tumour challenge were treated in parallel. On day 29, mice were terminated and spleens collected. Total spleen counts were determined and spleen populations were detected by flow cytometry. (A) total splenocytes; (B) total $CD8^+$ T cells ($CD8^+$); (C) total $CD4^+$ T cells ($CD4^+$); (D) total B cells ($CD19^+$); (E) total Treg cells ($CD4^+$/ $FoxP3^+$/ $CD25^{hi}$); (F) total MDSC ($CD11b^+$/ $GR\text{-}1^{hi}$). Tumor bearing mice: N=34-38 mice pooled from 9 separate experiments; non-tumour bearing mice: N=14-33 pooled from 8 separate experiments. Statistics by one-way ANOVA with Tukey post-test. *p<0.05, **p<0.01.

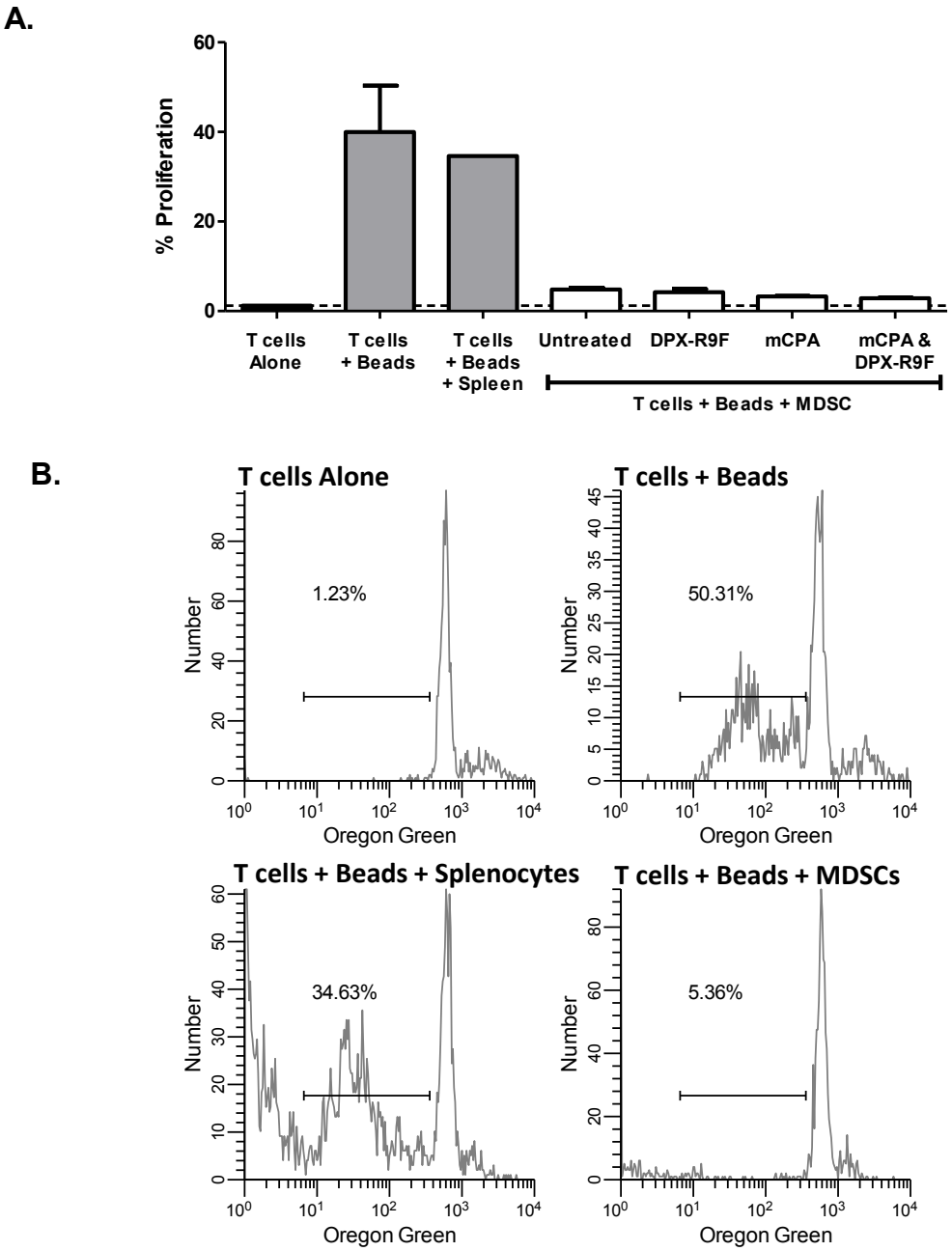

Figure 4.15: MDSC from treated tumour bearing mice have equivalent suppressor activity. (A) Mice were treated as in Figure 4.5A. On day 29, mice were terminated and spleens removed. MDSCs were purified from each mouse (n=3) by MACS immuno-magnetic separation using anti-Gr-1. MDSCs were incubated with Oregon green 488 labeled T cells and anti-CD28/CD3 beads for 3 days in 2:1 ratio. Proliferation was measured by Oregon green dilution detected by flow cytometry. Data not statistically different by one-way ANOVA. **(B)** Representative histograms showing T cell proliferation with and without MDSCs. Results representative of 2 separate experiments.

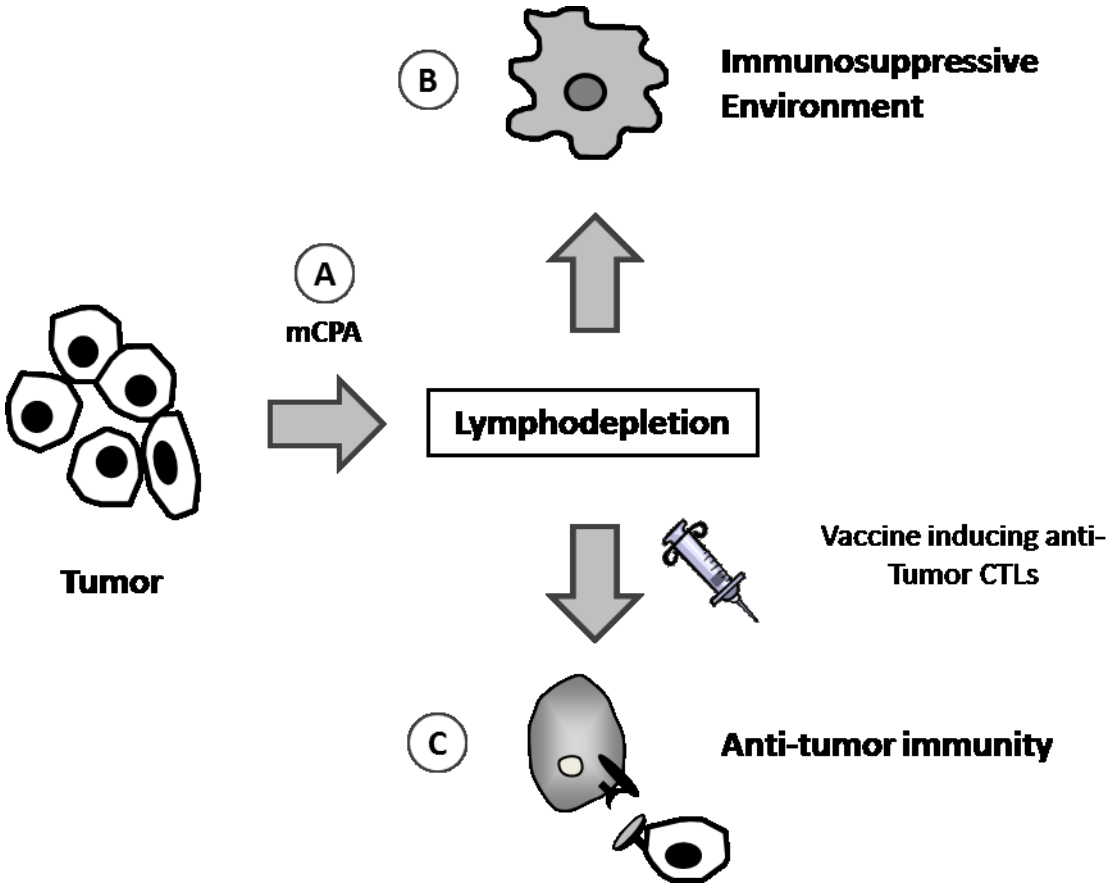

Figure 4.16: Proposed mechanism of anti-tumor immune responses induced by vaccine and enhanced by mCPA. **(A)** Metronomic CPA treatment induces transient, general lymphodepletion. **(B)** In the presence of a tumor, the subsequent re-population of the immune response is skewed towards immunosuppression marked by incrased MDSCs. **(C)** However, when an active anti-tumor immune response is induced by vaccination during the lymphodepleted state then re-population of the immune system is skewed towards anti-tumor immunity.

4.3 Discussion

4.3.1 Metronomic cyclophosphamide enhances efficacy of cancer vaccine

The majority of reported pre-clinical and clinical studies to date that have evaluated low dose CPA as an immune modulator for vaccination have used single bolus administration of CPA (50-100 mg/kg, intravenous, in mice). This form of administration has had poor translational efficacy, which may be due in part to its sensitivity to timing of CPA administration and vaccination [464, 466, 471]. In contrast, daily low dose metronomic CPA administration (20 mg/kg/day, oral, in mice) may allow more flexibility in timing of CPA and vaccination, as well as the added convenience of oral administration. In this study, we have evaluated metronomic low dose CPA in combination with a peptide vaccine for cancer therapy, focusing specifically on immune functionality as well as relevant cell populations.

In our model, both forms of low dose CPA administration enhanced the efficacy of a peptide based vaccine targeting HPV16-expressing tumours (Figure 4.1). Monotherapy with sbCPA provided better protection than mCPA monotherapy, reflecting inherent differences in these two forms of low dose CPA treatment. In both combination groups, an antigen-specific immune response to the vaccine peptide could be detected (Figure 4.2), indicating that the increased efficacy may be due in part to enhancement of vaccine immunogenicity. These results are consistent with the findings of Peng *et al.* who found that both forms of low dose CPA can enhance the efficacy of a DNA HPV16 vaccine, based on tumour growth data and flow cytometric detection of IFN-γ producing $CD8^+$ T cells following treatment [538]. Having confirmed that mCPA could enhance immune responses and result in better control of tumours, we sought to understand how the scheduling of mCPA and vaccine impacted efficacy of treatment and identify the mechanisms through which mCPA enhance vaccine induced immunity.

4.3.2 Metronomic cyclophosphamide delivered before or after vaccination does not reduce vaccine efficacy or immunogenicity

One of the disadvantages with sbCPA/ vaccine combination is that it is very sensitive to timing of CPA administration relative to vaccination. In mouse models, if the vaccine is administered too early after sbCPA (i.e. within 6 hours) it could result in

increased systemic toxicity [464], yet delivered too late (i.e. sbCPA 7 days after vaccination) abrogates any synergistic effects [466]. The therapeutic window appears to be vaccination 1 to 3 days after sbCPA administration. Since mCPA therapy is administered over a longer period of time, it may prolong the therapeutic window and allow more flexibility in timing of vaccination relative to immune modulation. To test this, we investigated whether vaccine efficacy was compromised if given at the beginning or end of a weekly mCPA treatment (Figure 4.3). It was difficult to compare directly because the rapid growth of tumours and sensitivity to both forms of treatment meant that tumour burden was inherently different when mice were treated at different time points. However, significant protection was provided to the combination group which received vaccination and mCPA starting 7 days after tumour implantation (mCPA *after* vaccination; Figure 4.3A) as well as the combination group which received vaccination on day 14 and mCPA starting on day 7 (mCPA *before* vaccination; Figure 4.3B). While early vaccination is a significant contributor to the anti-tumour effect seen in Figure 4.3A, it is important to note that this effect was enhanced rather than reduced whether mCPA was provided before or after vaccination. Supporting this, the immunogenicity of the vaccine tested by IFN-γ ELISPOT was the same in either group (Figure 4.4). These results indicate that vaccination may benefit from concurrent mCPA treatment with less timing constraints which is a considerable improvement on scheduling flexibility compared to sbCPA.

4.3.3 mCPA enhances the antigen-specific immune response induced by vaccination

To understand the mechanism of how mCPA was enhancing vaccine efficacy, the immune response was evaluated in the vaccine-draining lymph nodes and spleens of tumour bearing mice after a single round of treatment with mCPA and/ or vaccine. IFN-γ ELISPOT responses in the lymph node and spleen were highest in mice treated with mCPA and vaccine (Figure 4.6 and 4.8). In the lymph node, we observed a relative increase in antigen-specific $CD8^+$ T cells to total $CD8^+$ T cells compared to mice that were vaccinated without mCPA treatment (Figure 4.7). In the spleen, we detected highest CTL activity in mice treated with mCPA and vaccine (Figure 4.9). These

observations indicate that mCPA-induced enrichment of antigen-specific $CD8^+$ T cells in the lymph node allows more efficient proliferation and migration of vaccine-induced immune response to the spleen, ultimately resulting in better systemic CTL activity. Notably, mCPA did not accelerate the induction of immune responses, as we tested LNC and spleen responses on day 25 (Appendix Figure B.6) and found no significant antigen-specific responses at this early time point. The fact that mCPA does not reduce vaccine induced antigen-specific $CD8^+$ T cells supports that this form of immune modulation is particularly suitable for combination with vaccination. Notably, we also detected increased infiltration of $CD8^+$ T cells in the tumour in response to mCPA and DPX-R9F treatment (Figure 4.11A), indicating that the analysis of lymph node and spleen may be extended to the tumour microenvironment.

Immune responses induced by mCPA monotherapy were low, even though mCPA could induce tumour growth delay (Figure 4.1A, 4.10A). To determine if mCPA was enhancing natural immune recognition of the tumour, splenocytes were stimulated in an IFN-γ ELISPOT assay with C3 tumour cells (Figure 4.8B), responses were not detected above untreated control. These observations suggest that that protection imparted by mCPA is either due to direct cytotoxicity on tumours or through modulation of other types of immune cells.

Others have also reported that sbCPA or mCPA can enhance antigen specific immune responses elicited by vaccination. Taieb *et al* used a DC-derived exosome (DEX) vaccine where exosomes were loaded with TAAs and mixed with CpG adjuvant [465]. A single bolus injection of CPA delivered 6 days before vaccination in tumour bearing mice could enhance the number of antigen-specific $CD8^+$ T cells detected by dextramer staining. This treatment also provided significantly better therapeutic protection from tumour growth. However, when mice were vaccinated with DEX without adjuvant and treated with sbCPA, not only was it ineffective at tumour control, but no increase in antigen-specific $CD8^+$ T cells could be detected. In our model, DPX-R9F alone can generate significant antigen-specific immune responses that are further enhanced by mCPA combination. However, the timing of treatment relative to tumour implantation is a key variable that affects the efficacy of treatment.

4.3.4 mCPA and vaccination modulates immune cell populations in the presence of a tumour

The adoptive transfer experiment demonstrated that tumour control can be partially transferred to recipient mice by total T cells or $CD8^+$ T cells from combination treated donor mice (Figure 4.10C,D). These observations support the concept that mCPA is increasing the functionality of antigen-specific CTLs induced by vaccination. However, lack of complete efficacy could be attributed to mCPA having a direct effect on the tumour or it could be providing immune modulation in some other form. The significant reduction in lymph node sizes in response to mCPA therapy detected eight days after treatment indicated that mCPA was capable of inducing prolonged lymphodepletion (Figure 4.5B). Therefore we investigated immune cell populations in the tumour by RT-qPCR and in the spleen by flow cytometry.

4.3.4.1 Immune modulation of tumour microenvironment

We detected a significant increase in $CD8^+$ T cells in the tumour and mice treated with DPX and mCPA combination, of which a significant proportion were R9F-specific (Figure 4.11A). We analysed the mRNA levels of several genes associated with cytotoxic T cells responses by RT-qPCR. This approach was most feasible since the tumours of combination treated mice were small ($<500mm^3$, Figure 4.10A). Although a limitation of these data is the inability to correlate mRNA expression with cell type in this mixed cell population, the increase in expression of several genes associated with CTL activity is highly suggestive that such expression is due to increased leukocyte populations rather than tumour expression.

In response to treatment with mCPA and vaccine, several genes associated with cytotoxic activity were significantly increased: *CD8a*, *Grnb* and *Ifng*. Additionally, levels of the NK cell associated gene, *Klrc1*, were also enhanced in the combination treated mice. Increased levels of tumour infiltrating $CD8^+$ T cells is a positive prognostic factor in several human cancers, such as triple negative breast cancer, ovarian cancer and colorectal cancer [539-541]. However, genes associated with Treg function, *Cd4*, *FoxP3* and *Il10*, were also increased in the combo treated group and are negative prognostic factors [542, 543]. The genes for the co-inhibitory receptors PD-1 and CTLA-4 were also

elevated in response to combination treatment and are indicative of exhausted T cells [327]. Altogether, these data indicate that while mCPA and DPX-R9F combination treatment enhances recruitment of cytotoxic, antigen-specific $CD8^+$ T cells to the tumour, the immunosuppressive microenvironment may promote their suppression and counteract efficacy. This effect may be the contributing factor to the reduced efficacy observed in treating later stage C3 tumours (Figure 4.3C, D).

PD-1 expression also contributes to T cell anergy and is induced by the tumour microenvironment [544, 545]. Tumor expression of PDL-1, the ligand for PD-1, is increased by IFN-γ, thereby creating a feedback loop to inhibit T cells that recognize tumour cells (Appendix Figure B.7 and [546]). CTLA-4 is primarily expressed by activated CD4 and CD8 T cells and is an inhibitory counterpart to CD28 [327]. Both CTLA-4 and CD28 bind to the costimulatory receptors B7-1 and B7-2 on APCs, but ligation of CTLA-4 promotes T cell anergy and apoptosis, while CD28 reinforces activation [547]. The significance of these molecules in tumour immune evasion has been well established and monoclonal antibodies blocking PD-1 and CTLA-4 are promising therapies in clinical development [348, 349]. Due to the increase in mRNA expression of both these molecules within the tumour in response to treatment with mCPA and DPX vaccine, it seems likely that therapy with anti-PD1 and/ or anti- CTLA-4 may be complimentary.

Of note, while the tumour sizes of groups treated with mCPA only and mCPA & DPX-R9F were comparable at this time point, we detected no evidence of enhanced cytotoxic T cell gene signatures in the tumours of mice treated with mCPA only. Metronomic CPA is generally regarded to have anti-angiogenic activity, a mechanistic feature that may explain the anti-tumour effect of mCPA monotherapy [463]. VEGF mRNA expression has been correlated with level of angiogenesis in some cancers [548]. However, no changes were detected in VEGF mRNA expression in response to mCPA treatment, or in any other group.

4.3.4.1.1 Immune modulation in the spleen microenvironment

The abundance of cells obtained from the spleen facilitated comprehensive evaluation of immune populations by flow cytometry in response to treatment. Spleen

cell populations were assessed at two time points, on study day 25 (corresponding to 4 days post vaccination and mCPA treatment end) and study day 29 (corresponding to 8 days post vaccination and mCPA treatment end).

$CD8^+$ and $CD4^+$ T cells

On study day 25, mCPA treatment of tumour bearing mice had caused a significant decrease in $CD8^+$ T cells and $CD4^+$ T cells relative to non-mCPA groups. By study day 29 tumour bearing mice treated with mCPA monotherapy had increased all these populations to levels comparable to untreated mice. However, mice treated with mCPA in combination with vaccine still had low levels of $CD8^+$ T cells by day 29. This observation is consistent with that of the lymph node (Figure 4.7) where the total $CD8^+$ T cells were significantly reduced in the combination treated mice, but antigen-specific $CD8^+$ T cells were not. $CD4^+$ T cells were also low in the combination group, but this was not significant compared to vaccine only ($p=0.431$).

We detected no change in the splenic NK cell population (data not shown). Ghirenghelli *et al* reported that mCPA treatment of human cancer patients was associated with an increase in NK cells [459]. Doloff *et al* reported multiple rounds of mCPA treatment of tumour bearing *scid* mice resulted in increased tumour NK cells which corresponded with a decline in splenic NK cells [549]. Notably, in NOD-*scid*-IL2Rγ-null (NSG) mice which lack NK cells, mCPA therapy was less effective than in *scid* mice which have no adaptive immunity but still have NK cells. Therefore, in this study the lack of effect on NK cells may be because we only analysed spleens after a single round of treatment. Further, the RT-qPCR analysis of the tumour does indicate that NK cells may be elevated at the tumour site (Figure 4.15G).

B cells

Treatment with mCPA, alone or in combination with vaccine, resulted in a significant decrease in $CD19^+$ B cells by study day 25. By study day 29, B cell population rebounded in the mCPA only group, but remained low in tumour bearing mice treated with mCPA and DPX-R9F. The sensitivity of B cells towards various doses of cyclophosphamide treatment is well known, but the participation of B cells in anti-tumour immune responses induced by vaccination is not well defined [452, 460]. A suppressor subset of B cells, referred to as regulatory B cells or B10, has recently been

identified as $CD19^{+}CD5^{+}CD1d^{hi}$ and may play a role in tumour progression [228, 550]. B cells in general may play an underappreciated role in promoting tumour growth, as B cell deficiency results in slower tumour growth of other models [420, 551] Furthermore, the B cell depleting anti-CD20 monoclonal antibody has been shown to increase subsequent cellular responses to tumours in mice [552] and enhance T cell responses after vaccination [553]. Since treatment with combination therapy provided effective tumour control, it may be an indication that in this model the B cells are promoting tumour growth, and therefore reduction by mCPA contributes to immune enhancement. However, additional studies would need to be performed to investigate the role of B cells to C3 tumour growth.

Tregs

Low dose CPA is often associated with a selective decrease in the Treg population. This reduction is transient after a single bolus administration of CPA, typically reaching nadir 4 days after a single bolus administration of CPA and returning to normal 10-14 days later [434, 460]. In this study we found that Tregs were significantly reduced in combination treated tumour bearing mice on study day 25, but since the total levels of $CD4^{+}$ T cells were also reduced at this time point we could not conclude that the Treg reduction was selective. Total Tregs were comparable in all groups by study day 29. Others have also reported this lack of effect on Treg populations after low dose CPA treatment in both mouse models [467] and human clinical trials [477, 554]. However it has also been reported that instead of depleting Tregs, low dose CPA renders them non-functional, a finding that has not been addressed in this study [434]. Furthermore, others have reported that low-dose CPA selectively reduces levels of Tregs only in the tumour [467]. Another possibility could be that Tregs are not induced by this tumour type. The absolute number of Treg in the untreated tumour bearing mice in this study was not significantly higher than in the naïve mice by day 29 ($p=0.625$).

MDSCs

On study day 29, splenocytes of mice treated with mCPA only had a selective and significant increase in MDSCs. Increased $CD11b^{+}$/ $GR\text{-}1^{hi}$ MDSCs after low dose CPA treatment has been reported previously and it has been suggested that low dose CPA may be limited as a monotherapy because it leads to accumulation of MDSC that ultimately

results in the rebound of tumour growth [460, 555]. To compensate, low dose CPA treatment has been combined with drugs that specifically reduce MDSC levels such as gemcitabine or ATRA [556-558]. We found that the combination of mCPA plus DPX-R9F did not increase MDSC levels, negating the need for additional drugs. MDSCs are hematopoietic stem cells arrested in differentiation, presumably by the inflammatory cytokine milieu created by the tumour [393, 559]. The MDSC population was not increased in non-tumour bearing mice in response to mCPA treatment, which suggests that the presence of the tumour was promoting their selective expansion. We found that the MDSCs isolated from mice in each group retained suppressive functionality (Figure 4.13), which is in contrast to previous studies that found CPA-induced MDSCs had higher suppressive activity or no suppressive activity [464, 556]. Differences such as these may be a reflection of the type of tumour model used or assay conditions.

4.3.5 Proposed mechanism of mCPA enhancement of DepoVax peptide vaccine

Side-by-side assessment of immune cell populations in the spleens of tumour and non-tumour bearing mice identified several differences in response to treatment. The presence of the tumour influences the kinetics of the immune response to treatment, particularly in the context of mCPA which induces significant lymphodepletion (Figure 4.5B). These findings demonstrate why the study of immune modulators for cancer treatment must be performed in tumour bearing mice, and may help to explain why some of these observations differ from published studies. For example, the pivotal study by Lutsiak *et al* in which low dose CPA was first characterized for its selective depletion of Tregs was performed in non-tumour bearing mice [434].

From both sets of mice it is clear that mCPA treatment is causing temporary lymphodepletion, which creates an immunogenic "niche". In the absence of a tumour, the immune cells repopulate to normal levels 8-14 days after treatment has ended. However, in the presence of a tumour, the immune repopulation is influenced by the immunosuppressive nature of the tumour. This theory is exemplified in the finding of MDSC expansion in tumour bearing mice followed by mCPA treatment, which was not detected in non-tumour bearing mice. Treatment with mCPA works well with vaccination because it allows the immune response induced by the vaccine to develop with reduced

influence from tumour-induced immunosuppression. It appears that when the immune system repopulates after treatment with mCPA and vaccine, it is more influenced by the active immune response initiated by the vaccine rather than the tumour (Figure 4.16).

Besides the generation of an immunogenic niche, mCPA also has other effects, such as direct tumour toxicity, which may explain the partial efficacy of mCPA therapy. These effects may be important especially in the context of aggressive tumours, where the vaccine-induced immune response may benefit from the mCPA induced delay in tumour growth order to generate a robust response.

In a similar vein, low dose CPA has been used in the context of autologous T cell transfer therapy. In this case, pretreatment with low dose CPA increases the success of subsequent T cell transfer by augmenting proliferation and survival of activated, tumour-specific T cells generated *ex vivo* [560] in tumour bearing mice. The niche mechanism for low dose CPA has was also proposed by Salem *et al* following analysis of sbCPA in combination with peptide vaccine using OT-1 transgenic mice with no tumours [464]. Here they found that a single low dose of CPA caused significant lymphodepletion which resulted in more robust proliferation and survival of adoptively transferred OVA-specific T cells subsequent to vaccination. They were also able to identify an increase in the MDSC-like $CD11b^{+}Ly6G^{+}$ population in response to sbCPA treatment, but in their model this population had no suppressor activity and they proposed it may be helping the enhanced response to vaccination. At the time of the study, which was published in 2007, the MDSC population was not well defined which precluded investigation into the link between sbCPA and MDSCs by these authors. Also, since these mice were non-tumour bearing, the increase in this particular population of cells may not have had potent suppressive activity.

Metronomic CPA is increasingly being tested in clinical studies as monotherapy or in combination with other chemotherapies in an effort to increase anti-tumour immune responses [561, 562]. This is being done, in part, based on reports that mCPA can decrease tumour suppression mechanisms and enhance T cell activation. However, the results of this study would indicate that while mCPA may be able to provide some delay from tumour growth, it is ultimately ineffective at inducing long term protection from tumour growth. This study also suggests that providing mCPA to patients with pre-

existing tumours may result in aggressive rebounding of tumour growth since reconstitution of the immune system following treatment was influenced by the presence of the tumour in this mouse model. Only in the context of an active immune response, such as that induced by vaccination, could mCPA provide significant protection from tumour growth.

4.3.6 Summary of study findings

This study demonstrated that mCPA can be used in combination with a peptide-based vaccine to enhance anti-tumour immunity through the development of antigen-specific immune responses. Importantly, we determined that scheduling of mCPA and vaccine is flexible, and similar outcomes can be produced when the vaccine is given at the beginning or end of a week-long mCPA cycle. These findings can be used to design more effective clinical trials involving mCPA and vaccination. A major mechanism of mCPA-enhanced immune responses was speculated to be in the creation of an immunogenic niche which can only be repopulated with effective anti-tumour immune response when active immunity is induced by vaccination. This is in contrast to the mechanisms commonly attributed to mCPA which are anti-angiogenesis and selective reduction in Tregs [442]. Although these findings need to be confirmed in other models, they could help explain how mCPA can enhance immunogenicity vaccines without concomitant reductions in Tregs [471]. Metronomic CPA has been reported by others to induce an immunogenic niche, but the influence of the tumour on repopulation of the immune system has not been fully appreciated. Our findings would suggest that mCPA lymphodepletion should only be used in combination with vaccination so that active immune stimulation can properly guide the repopulation of the immune system. These findings have important implications on the use of monotherapy with mCPA in advanced cancer patients that could be explored in clinical settings.

4.3.7 Experiment limitations

Pre-clinical evaluation of mCPA and vaccination outlined in this thesis supports the use of mCPA to enhance the efficacy of cancer vaccines. However, such approaches in humans may provide varying results. sbCPA was also previously shown to enhance vaccines in pre-clinical models, but has not provided equivocal results in human clinical

studies. Translation of the sbCPA results into humans may have been impeded by the fact that humans have more complex immune systems and less defined tumour status than murine tumour models [180]. The advantage mCPA offers is that it appears to be less sensitive to timing of vaccination, but never-the-less, the approach may still need to be fine-tuned for human applications. Pre-clinical studies such as this one can provide insight into the mechanisms of mCPA-enhanced vaccine therapy which can assist in rationale designing of clinical trial shcedules.

Most of the studies performed in this project were done *ex vivo* using cells from treated animals. While this provided the most relevant information of how the immune system is affected by mCPA treatment, it did involve a large number of mice. Studying the effect of mCPA using *in vitro* systems was not possible for two reasons, first due to the multi-modal effects of mCPA we needed to assess its effect in the context of a complete immune system. Second, CPA is a pro-drug which cannot be directly applied to *in vitro* cultures. Its active metabolite, mafosfamide, can be purchased, but due to hydrolysis has a half-life of only 30 minutes after reconstitution, thus limiting its applicability for *in vitro* testing [563].

Previous studies investigating the immune modulating effects of sbCPA have described specific kinetics of effect, which reach nadir by day 4 and rebound by day 10 [434, 460]. However, it was difficult to evaluate mCPA in the same way in this study since treatment was continuous over a one week period. In this study we focused on day 8 post treatment as this day coincided with the peak immune response post vaccination. We also examined an earlier time point, day 4, to gain perspective of developing changes in the immune system. To fully understand the effects mCPA is having on the immune system would require a larger study evaluating the effects at several time points during and after treatment.

A snapshot of the immune microenvironment in the tumour was obtained using RT-qPCR. While this test could effectively compare the expression of many mRNA from a single small sample, it is limited by the fact that mRNA levels and protein levels may not always correlate. Factors affecting the correlation include post-translation modification of protein expression, and rates of protein degradation. RT-qPCR is also limited in that while several genes can be evaluated at once, there is no correlation

between them on per cell basis. In particular, this prevents analysis of MDSC population in the tumour by RT-qPCR because there is no one gene associated with their phenotype or function. The effects of treatment on tumour infiltrating leukocyte populations can be explored in greater detail using techniques such as immunohistochemistry and flow cytometry.

Finally, all the data obtained in this study are in a single tumour model, C3, which may or may not be reflective of other types of tumours in mice or humans. The advantage of this model was that the defined tumour-associated antigen, R9F, enabled us to assess how the antigen-specific response induced by a vaccine may be impacted by mCPA therapy. However, assessment of this combination therapy in other tumours would demonstrate the robustness of this anti-tumour effect and help define the limitations of this therapy.

4.3.8 Future directions

Confirmation of effects in alternate tumour models

To gain insight into the potential mechanisms of mCPA-induced vaccine enhancement, this system should be tested in other tumour models with different immune kinetics. For example, the 4T1 breast tumour model in BALB/c mice is known to preferentially induce high numbers of MDSCs [393]; therefore, it would be interesting to see if mCPA alone increases this population further, and whether combination treatment is still associated with significant decreases in MDSCs. The disadvantage with this model is that it is not often used to test peptide vaccines. There is no well-characterized MHC class I restricted tumour antigen commonly used in vaccination studies, typically tumour lysate is used to pulse DCs or a whole cell vaccine are used [564-566]. Tumor lysate could be prepared in DepoVax, but analysis of vaccine-induced immune responses to a defined tumour-associated antigen would be difficult. Another model that would be of interest is the B16 melanoma in C57B6 mice. B16 tumours are known to be only weakly immunogenic, and therefore difficult to treat with vaccination. B16 tumours express TAAs tyrosinase-related protein-2 (TRP-2) and gp100 for which murine MHC class I epitopes have been identified that could be used for peptide vaccination [567]. It would be interesting to see if mCPA could enhance tumour immunogenicity as well as

vaccine-induced immune response to provide better treatment. Both the 4T1 and B16 tumours generate metastatic tumours, the development of which would be interesting to monitor in response to combination therapy with mCPA and DepoVax vaccine [567, 568].

Role of B cells in anti-tumour immune response

Previous studies evaluating the immune modulatory effects of sbCPA have focused on the reported selective reduction in Tregs, an observation that has extended to investigations of mCPA [442]. However, this effect is not consistently observed in human clinical trials for either method of CPA administration [468, 470]. Other reported effects of mCPA are increased DC activation, T cell survival and NK activation [445, 459]. In this study, we show that mCPA can create an immunogenic niche by non-selectively depleting several types of immune cells, which may contribute to the enhanced immune responses to vaccination. Lymphodepletion by mCPA had a strong effect on $CD19^+$ B cells, which were significantly reduced by mCPA by day 4, and remained low by day 8 after treatment with mCPA in combination with vaccination. The contribution of the B cell reduction to the enhanced immune responses detected in the combination group remains unclear.

The role of B cells in immune responses towards solid tumours is not well understood. They could both enhance and suppress immune responses towards cancer vaccines [569]. To investigate this further, the possibility that B cells are generating anti-tumour antibodies could be monitored in tumour challenge studies. Mice would be bled at regular intervals and direct ELISA performed using C3 tumour lysate-coated ELISA plates. Antibodies specific to C3 tumour cells could also be evaluated in a flow cytometry based assay where serum is incubated with C3 cells which are then stained with fluorescent secondary antibodies towards mouse Ig. The relative MFI of C3 cells could be quantified as a measure of the antibody response. The possibility that B cells are acting as immune suppressors should also be investigated. Using the C3 treatment model developed in this project, B cells could be purified from untreated tumour bearing mice on day 21 and transferred into tumour bearing mice treated with mCPA and vaccination. Clearance of serum CPA can take up to 10 hours, therefore B cells transferred into recipient mice 12 hours after mCPA treatment should prevent B cell

depletion due to residual serum CPA [453, 454]. Control groups would receive B cells from naïve mice. Immune responses could be measured on day 29 by IFN-γ ELISPOT to see if the antigen-specific immune response differed in mice with reconstituted B cells. Also, tumour challenge or IFN-γ ELISPOT studies could be performed by combining vaccination with B cell depleting mAb (i.e. anti-CD20) instead of mCPA to see if this could enhance anti-tumour immune responses induced by vaccination.

Improving mCPA/ vaccine therapy with checkpoint blockade using monoclonal antibodies

Elevated tumoural expression of PD-1 and CTLA-4 mRNA coupled with increased infiltration with antigen-specific $CD8^{+}$T Cells in response to combination therapy is suggestive that mCPA/ DPX-R9F treatment may be enhanced with anti-PD1 or anti-CTLA4 mAb. Blockade of CTLA-4 and PD-1 with mAb has been associated with tumour regression in animal models with and without co-treatment with the vaccine [348, 349] and have shown promise in human clinical trials [570]. It would be interesting to add one or both of these therapies to the mCPA/ DPX-R9F regimen to see if therapy of advanced tumours could be enhanced by inhibiting this form of tumour-induced immune suppression.

CHAPTER 5: CONCLUDING REMARKS

5.1 *In vitro* to *in vivo* translational study of a novel vaccine adjuvant system

In this study we characterized the effect of poly I:C and Pam3CSK4 on the activation of B cells *in vitro*. These results document a previously unknown effect of dual TLR stimulation using poly I:C and Pam3CSK4 on B cell activation and function *in vitro*. Importantly, when used *in vivo* as an adjuvant system for two different protein vaccines, poly I:C and Pam3CSK4 enhanced antibody production, indicating that intrinsic TLR stimulation of B cells *in vivo* may be an important factor influencing the immune response towards vaccines.

Translation of *in vitro* studies to *in vivo* is often a very considerable challenge [152]. Correlating the dose used *in vitro* is influenced by multiple factors *in vivo*, such as enzymatic degradation, bioavailability, and interaction with multiple cell types. The doses of poly I:C and Pam3CSK4 used in this study were selected by performing dosing experiments in both the *in vitro* and *in vivo* systems. The *in vitro* testing system permitted us to evaluate multiple dose combinations of poly I:C and Pam3CSK4 to establish that enhanced B cell activation can occur with multiple doses of each agonist (data not shown). However, practical constraints posed by the *in vivo* system allowed testing of limited dose combinations.

Choosing the appropriate test system is critical, and often it is necessary to test in both murine and human *in vitro* cultures in order to demonstrate at an early stage that the effects are conserved between species. We did confirm that poly I:C and Pam3CSK4 could enhance the proliferation of human B cells (Appendix Figure B.4), but not surface expression of CD40. The differences observed are certainly a reflection of differences in the B cell maturation stage and TLR expression between murine splenic B cells and human circulating B cells. However, it does indicate that aspects of human B cell activation may be enhanced by the poly I:C/ Pam3CSK4 combination treatment and justifies further testing.

The most important follow up work to validate this adjuvant system using the mouse model will be to confirm that the antibodies induced to anthrax and influenza vaccines are functional, which requires toxin and hemagglutinin inhibition assays,

respectively. Once established, this adjuvant system may be a promising new development for these indications, as well as others.

5.2 *In vivo* development of a combinatorial immunotherapy for cancer

In this study we characterized an immune enhancing effect between mCPA and a cancer vaccine which resulted in better control of tumour growth in a preclinical mouse model of HPV16E7 induced cancer. These novel findings provided the framework for continued studies in human clinical trials.

Results from animal studies must be interpreted with caution since the results do not always predict how humans may respond to therapy [185]. However, they are useful for hypothesis testing and no other system exists in which the results of a vaccine can be tested in the context of a complete immune system. From this study we ascertained that the scheduling between mCPA treatment and vaccination is flexible and that antigen-specific immune responses may be gauged using IFN-γ ELISPOT. These observations were considered by Immunovaccine in designing a phase I clinical study in ovarian cancer patients to test treatment with a DepoVax based peptide vaccine, DPX-Survivac, and mCPA. In one arm of the study patients were vaccinated with DPX-Survivac every three weeks, and in the second arm patients were treated with mCPA for one week on/ one week off, as well as receiving vaccine every three weeks. Immune responses were measured by IFN-γ ELISPOT using patient PMBCs. As shown in Figure 5.1, the ELISPOT results in vaccinated patients with mCPA treatment were higher than those of patients who received vaccination only, mirroring the results obtained in the mouse model. These clinical results justified our hypothesis that mCPA can be used to augment immune responses to vaccination in cancer patients.

The current trend in cancer vaccine research is the combining of multiple modes of immune therapies in order to boost the immune response to the tumour as well as decrease the immune suppression induced by the tumour. As these treatments become more complex, using mouse models to establish timing and potential biomarkers will become more important. Our model was useful in evaluating mCPA and vaccine, and our follow-up work will examine the inclusion of checkpoint blockade mAb, such as anti-PD1 or anti-CTLA4, on the ability of the immune system to overcome more advanced

tumours. Established mouse mAb are available for testing PD-1 or CTLA-4 blockade in mice, but as immune therapies advance it would be critical to develop humanized mouse models that express human target proteins and antigens.

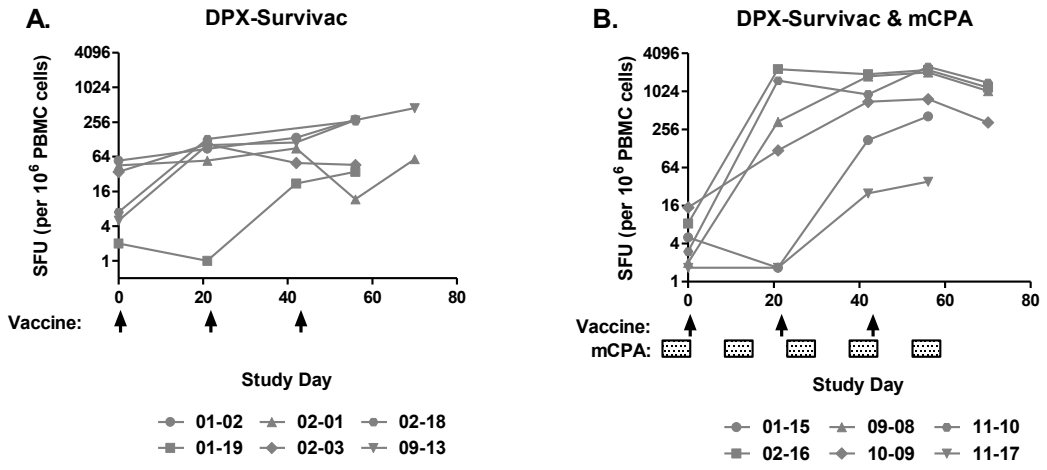

Figure 5.1: Metronomic cyclophosphamide enhances immunogenicity of DPX-Survivac in ovarian cancer patients in a phase I clinical trial. Ovarian cancer patients were vaccinated with DPX-Survivac alone **(A)** or in combination with mCPA (50 mg/day, oral BID, one week on/ one week off) treatment **(B)**. At various times during the study, patient blood samples were collected and peripheral blood mononuclear cells (PBMCs) were stimulated overnight with survivin peptides in an IFN-γ ELISPOT assay. Arrows indicate vaccination schedule and boxes the mCPA treatment periods. Data shown as the number of spot forming units (SFU) per million PBMC from individual patients at different time points. Results from study NCT01416038, manuscript submitted for publication (Berinstein NL, Karkada M, Oza AM, Odunsi K, Villella JA, Nemunaitis JJ, Morse M, Pejovic T, Bentley J, Buyse M, Nigam R, Weir GM, MacDonald L, Sharp K, Penwell A, Sammatur L, Stanford MM, Burzykowski T, Mansour M. Survivin targeted immunotherapy drives robust polyfunctional T cell generation and differentiation in advanced ovarian cancer patients).

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APPENDIX A: REVIEW ARTICLE

IMMUNE MODULATION BY CHEMOTHERAPY OR IMMUNOTHERAPY TO ENHANCE CANCER VACCINES

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Review

Immune Modulation by Chemotherapy or Immunotherapy to Enhance Cancer Vaccines

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Abstract:

Chemotherapy has been a mainstay in cancer treatment for many years. Despite some success, the cure rate with chemotherapy is unsatisfactory in some types of cancers, and severe side effects from these treatments remain a concern. Recently, understanding of the dynamic interplay between the tumor and immune system has led to the development of novel immunotherapies, including cancer vaccines. Cancer vaccines have many advantageous features, but their use has been hampered by poor immunogenicity. Many developments have increased their potency in pre-clinical models, but cancer vaccines continue to have a poor clinical track record. In part, this could be

due to an inability to effectively overcome tumor-induced immune suppression. It had been generally assumed that immune-stimulatory cancer vaccines could not be used in combination with immunosuppressive chemotherapies, but recent evidence has challenged this dogma. Chemotherapies could be used to condition the immune system and tumor to create an environment where cancer vaccines have a better chance of success. Other types of immunotherapies could also be used to modulate the immune system. This review will discuss how immune modulation by chemotherapy or immunotherapy could be used to bolster the effects of cancer vaccines and discuss the advantages and disadvantages of these treatments.

Keywords: Cancer, vaccine, chemotherapy, immunotherapy, immune-modulation

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Abbreviations: myeloid-derived suppressor cells (MDSC), antigen-presenting cell (APC), regulatory T cell (Treg), dendritic cells (DC), major histocompatibility complex (MHC), cyclophosphamide (CPA), doxorubicin (DX), gemcitabine (GEM), paclitaxel (PX), monoclonal antibody (mAb),

1. Introduction

Literally, chemotherapy is the use of chemicals to treat cancer. The first chemotherapeutic agents were actually derived from mustard gas in the 1940's after the discovery that those exposed during war had reduced white blood cell counts [1]. Given intravenously, this treatment provided a remarkable benefit to lymphoma patients. Over the last 70 years the number of chemicals that can be used for cancer treatment has grown substantially. The most common types of chemotherapies in use today are summarized in Table 1.

Table 1. Common Chemotherapy Agents and Their Classification (adapted from [2])

Type	Mechanism	Examples
Alkylating Agents	Modification of nucleic acid functional groups	Cyclophosphamide, dacarbazine
Antimetabolites	Nucleoside analogs, perturb RNA and DNA synthesis	5-fluorouracil, gemcitabine
Taxanes	Disruption of microtubule formation, stop cell division	Paclitaxel, docetaxel
Anthracylines	Interfere with DNA replication machinery, inhibit RNA and DNA synthesis	Doxorubicin
Platinum based	Cross link DNA	Cisplatin, carboplatin, oxaliplatin

In general, the mechanisms of chemotherapy result in the death of all rapidly dividing cells, tumor and healthy alike. Most tumors have a fast growth rate and are therefore targeted, but not without damage to by-standing healthy cells. Some of the most rapidly dividing healthy cells are leukocytes and bone marrow precursors, therefore chemotherapies are generally considered to be immunosuppressive. The crudeness of chemotherapy is both a benefit and a disadvantage. One advantage is that it is difficult for tumors to resist the widespread effects of chemotherapy, but the major detriment is that chemotherapy causes damage to healthy cells. Chemotherapy is a fine balance between tumor toxicity and general toxicity, and dosages must be carefully monitored to ensure the scales are not tipped toward the latter.

Chemotherapies are not equally effective in all patients. Slow growing tumors, or tumors arrested in growth by chemotherapy, are difficult to treat because chemotherapies target rapidly dividing cells. Patients with advanced disease may first undergo debulking surgery because the drugs are not able to penetrate large tumors. Frequently, tumors develop resistance and are no longer affected by a regiment that was previously effective [3, 4]. When chemotherapy is successful, there is a risk of developing secondary malignancies caused by the chemotherapy treatment itself, particularly in younger patients [5, 6]. Chemotherapy has had significant success in extending patient survival, but frequently at the price of quality of life. For a long time there were no other options for cancer treatment.

2. Tumor-Immune System Dynamics

Historically, a healthy immune system was deemed irrelevant for treating cancer in the context of chemotherapy [2]. However, the importance of the immune system and how it interacts with the tumor has been realized. The immune system is fully capable of killing tumor cells, but it has trouble recognizing them due to tumor-induced immune suppression [7]. Tumors have developed sophisticated mechanisms of avoidance and escape. Tumor evolution proceeds on two fronts: 1) conditioning the immune system through induced immunosuppression; and 2) adaptation to immune recognition by altering expression of surface markers. Far from being independent, the tumor and immune system evolve symbiotically, and recognition of this is the defining feature of immunotherapies.

2.1 Tumor Influence on the Immune System

One important mechanism tumors use to escape immune detection is by engaging the immune system's natural mechanisms to avoid self-recognition. Regulatory immune cells are a diverse group found in adaptive and innate immune cell subsets that prevent autoimmunity by suppressing self-recognizing T cells. Tumors hijack this natural mechanism to escape immune detection by secreting particular cytokines into its microenvironment to promote differentiation of many types of regulatory cells [7]. Tumor-induced immune suppression is the consequence of increased proportion of regulatory cells and coinciding reduction in the activity of effector T cells targeted

towards the tumor [8]. The two main types of regulatory cells now known to be associated with this process are the $CD4^{+}CD25^{hi}FoxP3^{+}$ T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) [9, 10].

TGF-β, produced in abundance by many types of tumor cells, promotes differentiation of naïve CD4+ T cells into Tregs [11]. Increased Treg frequency is correlated with poor outcome and in several animal models were Tregs were selectively depleted, tumor regression was enhanced [8, 12-14]. Tregs can inhibit antigen presenting cells (APCs) by inducing upregulation of inhibitory B7-H4 molecules or directly killing them through perforin and granzyme release. They engage CD80/86 on APCs with cytotoxic T lymphocyte antigen 4 (CTLA-4), leading to T cell anergy and death. Finally, they secrete immunosuppressive cytokines IL-10 and TGF-β to preserve and spread immunosuppression within the tumor microenvironment [15].

MDSC are a heterogeneous population of precursor myeloid cells that have the ability to cause immune suppression. In healthy individuals, the MDSC population is low as myeloid progenitors differentiate normally into mature myeloid cells, but under some pathological conditions maturation is arrested at various stages and the cells take on a suppressive capacity [16, 17]. Tumor-derived factors, such as pro-inflammatory cytokines IL-6 and IL-1β, promote the formation of MDSCs resulting in their accumulation in the blood, lymphoid organs and tumor [18, 19]. In cancer patients, the ratio of mature DCs to immature myeloid cells in the blood is inversely proportional to the stage of disease [20, 21]. . In mice, MDSCs express CD11b and Gr-1, for which there is no human homolog [9]. MDSCs can be divided into two groups based on morphology, the granulocytic MDSC are polymorphonuclear whereas the monocytic are mononuclear. These two subsets may have different functions in cancer [22]. MDSCs express various other surface markers including ICAM-1, CD80 and CD15, and exhibit great variability between individuals depending on the type of tumor.

MDSCs represent a significant hurdle to therapy because of their diverse immune suppression effects, both direct and indirect. They are able to directly inhibit $CD8^{+}$ and $CD4^{+}$ T cells in a cell-contact dependent manner through arginine and cysteine depletion, both amino acids are essential to T cell activation [23, 24]. They can also inhibit T cell function though reactive oxygen species production [25]. Monocytic MDSC elevate

iNOS, which may play a role in antigen-specific T cell suppression by increasing nitrosylation of MHC [25, 26]. MDSC may also inhibit through antigen-independent mechanisms, it was recently shown that they reduce expression of L-selectin on naïve T cells, preventing their circulation through lymph nodes and tumors, thereby reducing the number of active T cells [27]. MDSC also indirectly cause suppression by inducing Tregs [28]. Interestingly, Treg induction may occur through CD40 expressed on MDSCs, and it was shown before this mechanism was discovered that blocking this interaction leads to reversal of $CD4^+$ T cell anergy [29, 30].

Suppressive subsets of many immune cell types have been found within the tumor microenvironment, including $CD8^+$ T cells, NK cells and macrophages [31-34]. This diversity alludes to the intensity of suppression maintained within tumors, and the obstacles in raising an effective immune response for tumor elimination.

2.2 Tumor Immune Evasion

Besides inducing immune suppression, tumors have evolved other mechanisms to avoid immune detection. Firstly, tumors down-regulate expression of MHC class I and other proteins involved in antigen presentation [35-37]. Tumors can also decrease, or shed, expression of proteins that are recognized by the immune system, this concept is called immunoediting since it describes how the immune system directly impacts tumor malignancy [38, 39]. Thirdly, tumors can by-pass death mechanisms by elevating expression levels of survival factors, such as anti-apoptotic proteins (survivin, BCL-X_L), metastatic proteins (VEGF, MMPs) and proliferation factors (EGFR, c-Myc). The transcription factor STAT3 is upregulated in a number of tumors and controls expression of some of these genes [40].

Tumors contain a heterogeneous population of cancer cells that are at various states of development, allowing it to evolve quickly in response to new stresses. Tumor cells adapt to immune recognition by downregulating expression of antigens, and can also adapt to chemotherapy by increasing expression of adenosine-triphosphate binding cassette (ABC) pumps to actively secrete intracellular drugs [41]. Ironically, a successful chemotherapy regiment can also increase the chance of reoccurrence since there is potential for a few highly resistant cells to survive treatment and seed a secondary

malignancy. These cells are referred to as cancer stem cells, and have been identified as a phenotypically distinct subset in some human cancers, such as AML [42].

3. Cancer Vaccines

The goal of cancer vaccines is to initiate an active immune response towards a tumor. There are several types of cancer vaccines in development: adenoviral, dendritic cell, tumor cell, adoptive T cell transfer and peptide (rev. in [43]). Many types of cancer vaccines have been tested in clinical trials and some do elicit de novo antigen-specific immune responses, but so far few have demonstrated significant efficacy. It had long been assumed that if only cancer vaccines could elicit a strong enough immune response they could overcome tumor induced immune suppression, but after poor clinical results of so many promising vaccines it is now being realized that immunogenicity is not enough. In addition to a strong vaccine, tumor-induced immunosuppression must be actively reduced, and this may be achieved through combination with the arsenal of chemotherapy agents already in use.

4. Chemo-Induced Immune Modulation

It has long been understood that chemotherapies induce immunosuppression, yet it has only been of late that the specificity through which they induce suppression has been appreciated. In 2005, cyclophosphamide was the first chemotherapeutic agent that was shown to selectively deplete a regulatory immune cell population at some doses, and has inspired research into the potential immunomodulation of other chemotherapies [44]. Chemotherapies have the potential to enhance cancer vaccine-induced immune responses by lowering the defenses of the tumor [2]. There are three mechanisms through which chemotherapies may work to do this: (1) targeting the immune system to reduce tumor-induced immune suppressive cells; (2) targeting the tumor to increase immunogenicity (increase MHC or antigen expression); (3) directly stimulating effector response by activating T cells. Any one of these effects would enhance the tumor specific immune response elicited by a vaccine, and some chemotherapies may even work through multiple mechanisms.

4.1 Cyclophosphamide

It was first recognized in the 1980's that low doses of cyclophosphamide (CPA) specifically inhibit a population of suppressor $CD4^+$ T cells and enhance immune responses against antigens [45]. It was not until 2005 that Lutsiak *et al.* showed that CPA treatment specifically affects the $CD4^+CD25^+$ T cells (Tregs) [44]. They found that mice given a low dose of CPA had a reduced Treg population with attenuated suppressor function. The Tregs were shown to undergo apoptosis, but effector $CD4^+CD25^-$ and $CD8^+$ T cell populations were not compromised. The effect was transitory, maximal Treg reduction was observed 4 days after treatment but returned to normal levels by day 10. This landmark study prompted investigation into the combined use of low dose CPA with peptide vaccines. Several reports of CPA combination therapy with various cancer vaccines have demonstrated the feasibility of this treatment in murine models [46-48]. Some have demonstrated that besides reducing Treg cells, CPA therapy can also enhance $CD8^+$ T cell activation and memory development through induction of type 1 interferons [49, 50].

In humans, low dose CPA treatment also selectively reduces the Treg population, but reports of its augmentation of cancer vaccines have been conflicting [51-53]. In fact, investigation into the effects of CPA and other chemotherapy treatments on the immune system has emphasized the inadequacy of murine models for cancer. Human cancers are heterogeneous in nature and are characterized by a high degree of immunosuppression. In contrast, the majority of murine tumor models rely on use of implanted cell lines that are clonotypic and after years of culture *in vitro*, have lost some of their initial immunosuppressive capabilities [54]. There are some models of spontaneously arising tumors, but the advantage to using implanted cell lines is their predictability and control. Therefore, while testing cancer immunotherapies in mice does provide some indication of their efficacy, but translation into humans is difficult.

A recent report by Tongu *et al.* looked at the combination of low dose CPA plus the anthracyline doxorubicin (DR) to therapeutically treat murine CT-26 colon carcinomas [55]. The combination of CPA (i.p.) + DR (i.t.) synergistically reduced tumor growth without vaccine therapy. The effect was shown to be T-cell dependent, since no effect was seen in nude mice, and tumor specific, it could not protect from a second challenge with a different tumor. The authors speculated that CPA treatment removed

Treg suppression, enhanced $CD8^+$ T cell function and that in combination with DR, which is known to induce immunogenic cell death, the tumors became immunogenic. CPA and DR were combined with a GM-CSF-secreting breast tumor cell vaccine in a small clinical study [56]. Both agents were delivered intravenously and various dose combinations were tested. In twenty-two patients who received CPA + DR and vaccination, serum levels of GM-CSF remained elevated and levels of HER2 antibodies were augmented. Clinical responses were not evaluated, but these results are promising and demonstrate how two chemotherapies with slightly different mechanisms can be combined for enhanced tumor rejection. One important caveat was the effect of CPA treatment was found to be highly dependent on dose, above 200mg/m2 it was immunosuppressive. This highlights the importance of dose selection when considering the immunomodulatory effects of chemotherapy.

Recently, metronomic dosing of CPA has emerged as a promising application of this drug for immune modulation. Continuous low dose CPA treatment was initially investigated for its anti-angiogenic effect since the rapidly dividing vascular intratumor endothelium are most susceptible to treatment [57, 58]. It was then demonstrated that a continual low dose schedule of CPA (50-100 mg/day, p.o.) can also specifically reduce Tregs, as well as restore effector T cell and NK cell function [51]. An attractive feature of this approach is the convenience and low toxicity, which increases patient compliance.

Besides reducing Tregs, CPA treatment can also deplete B cells, augment activation and function of DCs, and skew the development of $CD4^+$ T cells towards Th1 and Th17 during recovery after CPA induced lymphodepletion (rev. in [59]). Interestingly, when Liu *et al* evaluated the effects on the tumor infiltrating cell population in mice bearing tumors and treated with low dose CPA, they found a concurrent increase in the levels of myeloid derived suppressor cells (MDSCs) with the decreased levels of Tregs [60]. This work could suggest that the desirable effects of CPA treatment on the Treg population may be offset if they actually increase the level of an alternative suppressor cell, MDSCs. However, as MDSCs are loosely defined as a heterogeneous population of progenitor myeloid cells, this could merely be a reflection of enhanced lympho-proliferation following depletion.

A recent study has provided a hypothesis as to the preferential effects of low dose CPA treatment. Zhao *et al* found that cells, such as Tregs, which have low levels of intracellular ATP have reduced capacity to detoxify internalized CPA [61]. Defining the mechanism through which CPA can selectively effect a particular population of cells will help in designing best chemotherapy-immunotherapy dosing schedules.

4.2 Nucleoside Analogs: Gemcitabine & 5-Fluorouracil

A study by Liu *et al.* evaluated the tumor infiltrating cell populations in mice bearing large or small tumors after low-dose CPA treatment [60]. They did confirm that CPA reduced the $CD4^+CD25^+$ population of Tregs, and also found an increased level of $Gr\text{-}1^+CD11b^+$ MDSCs, suggesting that in advanced tumors CPA treatment may enhance other suppressive cells. Gemcitabine (GEM) is a nucleoside analog that reportedly suppresses MDSCs specifically and has been used to reduce tumor growth in several murine models [62, 63]. Like low dose CPA, GEM treatment is also transient [64]. In murine models, GEM combination with vaccine therapy significantly reduces regulatory T cells and enhances $CD8^+$ T cell activation [65, 66]. Knowing that MDSC can promote Treg differentiation, GEM could potentially reduce multiple suppressor cell types with a tumor both directly and indirectly.

5-fluorouracil (5-FU), another nucleoside analog, has also been reported to specifically suppress MDSCs. A comprehensive study by Vincent *et al.* evaluated several types of chemotherapies (GEM, CPA, DR, 5-FU, paclitaxel, oxaliplatin) on MDSC in EL4 thymoma tumor bearing mice [64]. They found that 5-FU specially induced apoptosis of GR^+CD11b^+ MDSC, both granulcytic and monocytic subsets were equally affected. 5-FU was more potent than GEM, and in combination with CPA significantly repressed tumor growth in a T-cell dependent manner.

5-FU and GEM have also been reported to increase immunological visibility of tumors by increasing expression of TAA on their surface. 5-FU or GEM were able to synergistically enhance antibody dependent cell-mediated cytotoxic (ADCC) mediated killing of colon cancer cell lines by cetuximab (a monoclonal antibody targeting epidermal growth receptor, EGFR) by increasing expression of EGFR on tumors [67]. Similar findings have been reported in other cancer models [68, 69].

4.3 Paclitaxel

Paclitaxel (PX) therapy is common in most standard of care regimens used today because it is efficacious in many different types of cancer [70]. PX arrests cells in mitosis by preventing microtubule formation ultimately resulting in apoptosis. Recently, PX has also been shown to have stimulatory effects on the immune system, especially at lower doses than typically used for chemotherapy [71]. Conversely, standard dose PX treatment is broadly immunosuppressive and inhibits a number of cell types involved in tumor rejection: macrophages, effector T cells and NK cells [70]. The disparity between low and high dose effects has been noted with other chemotherapeutic drugs as well [72]. Interestingly, PX has been shown to be a ligand for TLR4 on murine DCs, which may be indicative of a direct effect on the immune system [73]. PX has also been shown to enhance activation of human DCs, but independently of TLR4 binding, and this effect is partially responsible for its immune-enhancing effect [74]. Investigations by the Gabrilovich group have discovered that PX treatment of cancer cells causes up-regulation of cation-independent mannose-6-phosphate receptor on the surface of tumor cells, which increases the efficiency of Granzyme B mediated cytotoxic killing (reviewed in [75]).

Low dose PX treatment has been combined with a number of vaccine types in murine models to effectively reduce tumor growth [76-78]. Used as metronomic therapy (continuous), low dose PX is a potent inhibitor of angiogenesis and specifically down-regulates expression of VEGF-receptor 2 on endothelial cells in a murine 4T1 breast cancer model [79]. In the clinic, low dose PX has not been tested in combination with cancer vaccines, but the anti-angiogenic effects of metronomic therapy have been confirmed [80-82].

4.4 Platinum Based Drugs: Cisplatin and Carboplatin

The platinum based drugs, cisplatin and its less toxic analog carboplatin, are often co-administered with PX in standard chemotherapy treatments. Many clinical studies have consistently shown synergism between cisplatin or carboplatin and PX treatment (rev. in [83, 84]). The mechanisms contributing to the synergistic effect are unknown, but addition of a third drug (e.g. GEM or epirubicin) provides no additional benefit and may in fact interfere with primary treatment [85, 86]. The mechanism underlying this

combinatorial effect may have to do with the unique pathways used by platinum based drugs for import and export at the cellular level, due to the presence of the heavy metal atom [41]. It is less likely that tumors can simultaneously adapt to resisting two completely different drugs.

Carboplatin on its own has little reported evidence of an immunomodulatory effect, but recently an interesting study evaluated the effect of paclitaxel/carboplatin treatment on tumors and the immune system [87]. Preliminary studies *in vitro* showed the induction of apoptosis in SKOV3 ovarian cell lines by PX/carboplatin treatment. Treated cells were also more likely to be phagocytosed by dendritic cells which acquired activated phenotype (increase MHC II, CD80/86) and were subsequently able to prime $CD8^+$ T cells *in vitro,* indicating the treatment induced immunologic death of the tumors. In the same study, blood samples were collected from 13 patients with ovarian cancer receiving primary therapy with PX/carboplatin before treatment then at regular intervals afterwards. Monitoring the levels of $CD4^+$ T cell, $CD8^+$ T cell and NK subsets revealed that prior to treatment patients were immunocompromised as evidenced by increased Tregs and decreased Th1, Tc1 and NK cells. A single course of PX/carboplatin treatment reversed the immunosuppression, peaking around 2 weeks after treatment before returning to pre-treatment levels. Therefore, it was suggested that 2 weeks following chemotherapy treatment would be the optimal time for secondary immunotherapy treatment, however this was not studied. This systematic study of the temporal effects on the immune system show how sensitive the timing of combination therapies can be, and how they could be planned for optimal efficacy.

PX/cisplatin treatment has been tested in combination with immunotherapy in a mouse study [88]. Lewis-lung carcinoma tumor bearing mice were treated with a standard course of PX/cisplatin followed by adoptive cell therapy with cytokine-induced killer cells (CIKs). The chemotherapy pre-conditioning resulted in enhanced tumor rejection which was accompanied by reduced intratumoral Tregs and increased homing of the CIKs to the tumor and spleen. Therefore, even at standard doses this chemotherapy regiment has the potential to enhance immunotherapy.

5. Considerations for Chemotherapy-Vaccine Combinations

Chemotherapies exert various effects on the immune system that could be exploited to enhance the efficacy of cancer vaccines. However, there are several pitfalls to consider. Chemotherapeutic regiments are not universally applied, meaning that significant differences in approach are taken depending upon the type of cancer, the stage, and patient characteristics. Adding cancer vaccines into the program introduces another layer of complexity. Indeed, several studies looking at vaccine-chemotherapy combinations highlighted the fact that chemotherapies must be carefully dosed and delivered at particular times in relation to the vaccine for optimal effect [55, 56]. When using chemotherapies at doses considered suboptimal for primary treatment, unforeseen effects on tumor growth may occur. For example, it is possible that low dose chemotherapy could allow tumors more time to adapt and thus become more resistant to treatment.

Although there has been significant research combining chemotherapies and vaccines in mouse models, information from human studies is sparse. Mouse models do not accurately mimic human disease, but given the success in these models more research is justified in humans. Preliminary studies, such as the one performed by Wu *et al.* [87] in ovarian cancer patients, to characterize the effects of chemotherapy alone on human patients immunity would provide valuable information for designing chemo-vaccine combination trials.

The most attractive feature of cancer vaccines is their safety, and it must be acknowledged that combining vaccines with known toxic immunosuppressants may compromise this beneficial property. Few studies have so far reported increased adverse events associated with combined treatments, but these have been mostly performed on mice. Along this line, the potential for long lasting effects of previous chemotherapy treatments should also be examined before one considers using cancer vaccines in the clinical setting. This may be especially relevant for first-in-man studies of new cancer vaccines that are typically performed in a compassionate use setting in patients with advanced cancer who have been heavily pre-treated with multiple therapies. Owing to the active role the immune system plays in tumor clearance, it is likely that the benefits of cancer vaccines will be best observed in patients with early, untreated disease.

5.1 Clinical Experience with Chemotherapy-Vaccine Combinations

All types of cancer vaccines stand to benefit from chemotherapy combinations, and many have already been tested in clinical studies. Due to the complexity of these combinations (scheduling and dosing of both components, as well as cancer indication and stage), rarely are two studies the same which makes comparisons difficult. Table 2 summarizes the results of some relevant studies published recently. Gemcitabine, cyclophosphamide and dacarbazine (or temozolomide, which is metabolized to dacarbazine in vivo [89]) in particular have been used. Most trials do not include control arms and instead rely on historical controls. Outcomes have been varied, from no effect whatsoever [52, 90] to indication of increase PFS or OS (compared to historical controls) [89, 91, 92]. Same have noted changes to immune response profile in terms of increased diversity in epitope recognition by T cells (i.e. epitope spreading) [93] or increased cellular and humoral responses [92, 94]. Importantly, no studies have reported increased safety risks due to vaccine combinations with chemotherapy.

Somewhat counterintuitive are results from recent clinical studies showing that chemotherapy *after* vaccination may be a better treatment schedule than chemotherapy pre-treatment or concurrent treatment. Results of a clinical study published by Antonia *et al* indicated that patients with extensive stage small cell lung cancer were actually more responsive to second-line chemotherapy treatment after vaccination with dendritic cells transduced with wild-type p53 via adenoviral vector [95]. More recently, the TG4010 viral vector encoding MUC1 and interleukin-2 was tested in a Phase II study in NSCLC patients [96]. The two arm study compared chemotherapy (cisplatin + vinorelbine) administered concurrently with vaccination or administered after vaccination. The results of the study indicated a positive outcome for both treatment arms, but number of evaluable patients was too low to conclude a preference for either schedule. For some types of cancer vaccines, this dosing schedule may be optimal because it primes the immune system before insult with chemotherapy. However, it may not be optimal for all treatment types or indications. Leffers *et al* reported no benefits to secondary chemotherapy in ovarian cancer patients that had previously received a p53-synthetic long peptide (SLP)® vaccine, despite observing a significant benefit to NSCLC patients [97].

Table 2. Clinical Reports of Peptide-Vaccination in Combination with Chemotherpay

Vaccine	Chemotherapy	Indication	Outcome	Ref.
Personalized peptide vaccine (once/ week for 8 weeks)	Gemcitabine (1000 mg/m2, i.v.; once/ week for 3 weeks, one week off, then repeat)	Advanced pancreatic cancer	Phase II study, single arm. Response rate of 67%, both cellular and humoral responses detected	[94]
WT-1 peptide vaccine (day 8, 22)	Gemcitabine (100 mg/m2 on day 1, 8, 15)	Pancreatic and biliary tract	Phase I study, single arm study. Combination safe. GEM treatment increases numbers of monocytes and DCs.	[98]
Melan-A + gp100 peptide vaccine + IFN-a (day 1, 8, then every 21 days for 5 courses)	Dacarbazine (800 mg/mq i.v.; one day before each vaccination)	Melanoma	Phase I study, single arm. Dacarbazine treatment resulted in increased diversification of TCR repertoire	[93]
GV1001 (3 injections during week 2, 2 injections during week 3, single injection on weeks 6, 7 and 11)	Temozolomide (200 mg/m2, p.o.; 5 consecutive days every 28 days)	Advanced melanoma	Proof-of-concept study, single arm. Safe. Increased OS compared to predicted survival. Development of polyfunctional cytokine profile. Durable GV1001-specific T cell responses.	[89]
EGFRvIII vaccine (day 21 of each 28 day cycle)	Temozolomide (a) 200 mg/m2 for first 5 days in each cycle; b) 100 mg/m2 for first 21 days in each cycle)	Newly diagnosed glioblastoma	Phase II study, 2 arm, historical controls. Compared two different dose schedules of chemotherapy. Both groups resulted in better OS than historical control. Interestingly, longer treatment (b) caused more profound and persistent lymphopenia with an increase in Tregs, yet still mounted potent cellular and humoral immunity.	[92]
GV1001 (days 1, 3, 5, 8, 15, 22, 36	Cyclophosphamide (300 mg/m2 i.v.;	Advanced HCC	Phase II study, single arm. No significant effects on immune	[52]

followed by 4 weekly injections)	single pre-treatment 3 days before vaccination)		response or tumor growth observed.	
MELITAC – containing 12 melanoma CTL epitopes (days 1, 8, 15, 29, 36, 43 then month 3, 6, 9, 12)	Cyclophosphamide (300 mg/m2 i.v.; single pre-treatment)	Resected stage IIB to IV melanoma	Phase I/II study, 4 arms testing two vaccines with or without CPA. "Cyclophosphamide provided no detectable improvement in CD4 or CD8 T-cell responses or in clinical outcome."	[90]
BLP25 – MUC1 peptide delivered in liposome formulation (weekly vaccinations for 6 weeks)	Cyclophosphamide (300 mg/m2; single pre-treated 3 days before vaccination)	Unresectable Stage III NSCLC	Phase I/II study, single arm. Safe	[99]
EGF vaccine (day 1, 14 then monthly after completion of Cis/Vin chemotherapy)	Cyclophosphamide (200 mg/m2 3 days before first vaccination and before monthly vaccination) Cisplatin (100 mg/m2) + vinblastine (6 mg/m2) once every 21 days for 4-6 cycles	Advanced NSCLC	Phase I study, single arm. Safe. Median survival better than previous reports.	[91]
Personalized peptide vaccine (once/ week)	estramustine phosphate (280 mg/day, p.o.; continuous)	Castration resistant prostate cancer	Phase II study, 2 arms comparing vaccine + low dose chemo to standard dose chemo. Median PFS in chemo/vaccine combo group was significantly longer than standard dose chemo alone	[100]
TG4010: rec. viral vaccine expressing MUC1 and IL-2 (once per week for 6 weeks, then once	Cisplatin (100 mg/m2 on day 1) + vinorelbine (25 mg/m2 on day 1 and 8; up to 6 cycles) – chemo given	Advanced NSCLC	Phase II study, 2 arms, historical control. Patients that developed CD8+ T cell response to MUC1 correlated with better survival;	[96]

every 3 weeks)	during or after vaccine therapy			
DC-CAP-1 peptide vaccine (days 4, 10, 17 – first cycle only)	8 cycles of: Capecitabine (2000 mg/m2 PO per day days 1-14) + oxaliplatin (130 mg/m2 on day 1)	Stage III colon cancer	Phase I study, single arm. Evidence of increased T cell proliferation.	[101]

6. Strategies for Selecting Optimal Chemo-Vaccine Combinations

To overpower tumor immune evasion and suppression strategies, a successful treatment should attack the tumor from multiple angles, targeting different mechanisms quickly to minimize the chance of adaptation. To accomplish this, a targeted approach like cancer vaccines should be combined with one or more chemotherapies to help lower tumor defenses and boost the immune system. The best chemotherapies to combine with cancer vaccines would work on two levels: (1) increasing tumor visibility to the immune system through increased expression of MHC class I and unique surface antigens; (2) decreasing tumor-induced immune suppression. A third mechanism that could be exploited is the ability of some chemotherapies to increase T cell stimulation, however careful consideration must be made when combining these treatments with vaccines since this could lead to overstimulation and anergy. How these three mechanisms could work to enhance vaccine efficacy is depicted in Figure 1: vaccine-induced tumor specific T cell response could be enhanced by chemotherapies that increase T cell stimulation. Other chemotherapies can increase tumor immunogenicity, for example by increasing expression of tumor-associated antigens or MHC expression. Chemotherapies can also condition the immune system to reduce tumor-induced immune suppression, thereby allowing the vaccine-induced immune response to prevail. Examples of chemotherapies that can mediate each mechanism are given in Table 3.

Figure 1. Combined effect of Chemotherapy and Vaccine Therapy on Tumor Immunity. Chemotherapy can enhance cancer vaccines in three ways: (1) Reducing tumor induced immune suppression; (2) Increasing tumor immunogenicity; (3) Directly stimulating the immune system to enhance effector T cells. Chemotherapy could condition both the

immune system and the tumor so that cancer vaccines have the best chance of success. Cancer vaccines focus the immune response towards the cancer and will be most effective when tumor defenses are lowered.

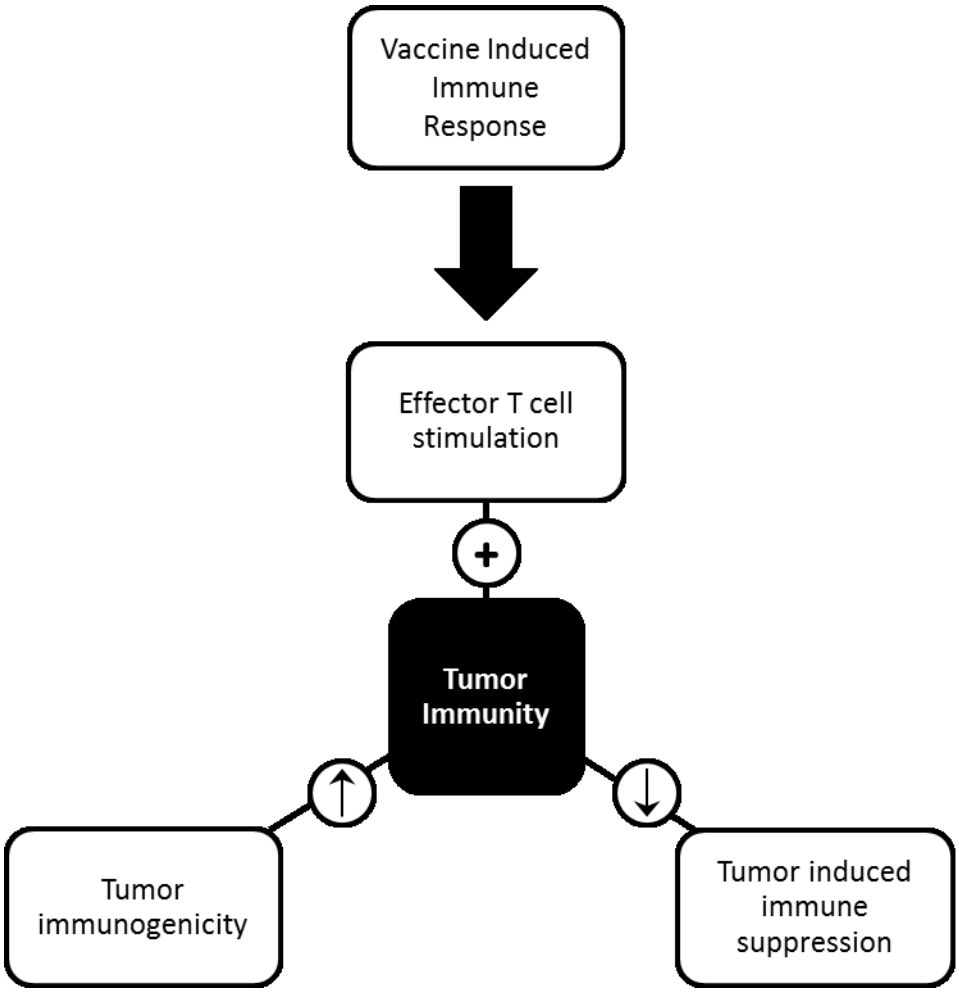

Table 3. Mechanisms of Chemotherapeies That Could be Used with Cancer Vaccines

Mechanism	Chemotherapy	Ref
Increase Effector T cell Stimulation	Cyclophosphamide	[78]
	Paclitaxel	[49]
Increase Tumor Immunogenicity	Doxorubicin	[102]
	5-Fluorouracil	[103]
	Cisplatin	

Decrease Tumor Induced Immune Suppression	5-Fluorouracil	[64]
	Cyclophosphamide	[44]
	Gemcitabine	[62]
	Paxlitaxel/ carboplatin	[87]

CPA and PX were shown to increase T effect stimulation via shifting the immune response towards Th1 after vaccination with a GM-CSF-secreting whole-cell vaccine [78]. Tumor immunogenicity can be increased in several ways, DR is an example of a chemotherapy that can induce the immunologic death of tumor cells [102]. Another way to increase tumor immunogenicity is by causing upregulation of tumor-specific markers, for example 5-FU and cisplatin were shown to cause increase in tumor-antigen expression in cancer lines in vitro, leading to increased recognition and killing by antigen-specific CD8 T cell lines [103]. CPA and GEM are prominent types of chemotherapies that have a direct effect on discrete components of the immune system, Tregs and MDSCs respectively, and were discussed in detail in preceding sections.

Some chemotherapies can work through multiple mechanisms, for example CPA can not only reduce Tregs [44], but also increase effector T cell function [78]. Combining multiple chemotherapies is another approach to targeting different anti-tumor mechanisms, for example one study described above has demonstrated that CPA + DR is a viable combination that could potentially synergize with vaccination [55]. However, some chemotherapy combinations may not work well together, for example mitomycin C did not synergize with DR like CPA can [55]. More research should also be conducted to discover the mechanisms through which these chemicals work, and how they are selective for these pathways. For example, why does GEM only target MDSC? It is possible that GEM is in fact a growth promoter that can facilitate MDSC differentiation into a mature myeloid cell. In which case, GEM would be an optimal candidate for combination with vaccine therapy as the vaccine could guide the activation of the newly differentiated myeloid cells.

In addition to their immune modulating effects when used concurrently with immunotherapy, chemotherapies can also be utilized to increase the sensitivity of tumors to subsequent immunotherapy treatments. In this scenario, chemotherapy is used to

destroy the most susceptible tumor cells and reduce tumor burden, potentially leaving behind residual cancer cells not susceptible to treatment, i.e. the cancer stem cells. At this point, with low tumor burden and fairly uniform cancer cell population, the patient could be treated with a cancer vaccine targeting specific proteins essential to the stem cell survival. Alternatively, chemotherapy can actually increase expression of some tumor associated antigen on the tumor surface, making it more susceptible to immune-mediated killing. 5-FU, cisplatin and paclitaxel have all been reported to do this [103, 104].

Another consideration in chemo/vaccine combinations could be the molecular target of the vaccine. For example, survivin is an anti-apoptotic protein that is upregulated by many types of cancers to such an extent that it has been proposed as a "universal" cancer target [105]. Several pre-clinical and clinical studies have evaluated survivin-based peptide vaccines and demonstrated variable efficacy. In addition to its role in preventing cell death, survivin is also an essential regulator of the cell cycle that binds to and stabilizes the mitotic spindle [106]. As described above, the mechanism through which PX induces tumor apoptosis is through arresting cells undergoing mitosis. Therefore, PX treatment could be complementary to a survivin-targeted vaccine since not only does it induce immunologic death of tumors, but by freezing cells in this state it could increase the expression of the vaccine target.

7. Antibody-Induced Immune Modulation

Chemotherapy has been the mainstay of cancer treatment for many years, but the latest breakthrough in the field is the development of monoclonal antibodies (mAb). Treatments with mAb were initially designed to target tumor cells directly and subsequently induce tumor destruction through several different mechanisms. There are in fact nine mAb of this type that have been approved for various cancer indications since 1997 [107]. Avastin, developed by Genentech/Roche, has a slightly different mechanism in that it binds to vascular endothelial growth receptor (VEGF) which is over-produced by tumor cells to stimulate angiogenesis by endothelial cells. The approved mAb can be broadly divided into two groups, non-conjugated and immunoconjugated. The latter are used to deliver a toxic payload in the form of a chemical agent or radioactive particle directly to the tumor.

Basic mechanisms through which monoclonal antibodies work include blockade of growth receptors or activation receptors, antibody-dependent cell-mediated cytotoxicity and complement mediated cytotoxicity [107]. Antibodies can also enhance tumor cell phagocytosis and tumor antigen processing by linking to Fc receptors on antigen presenting cells (APCs), thereby serving a link to induction of cellular immunity. A study by Rafiq *et al.* first demonstrated that administration of tumor-targeted antibodies not only induces T cell immunity towards the targeted epitope but also others through epitope spreading [108].

Unlike chemotherapies that have dose-dependent toxicity and are crudely tumor-selective, mAb have a relatively good safety profile and defined targets. Although effective, the main limitation of mAb therapy is applicability; they can only be used to treat cancers that express the target, and even then are generally only effective in about 30% of patients [109]. For example, trastuzumab is only applicable for breast cancer patients positive for Her2/*neu* expression, about 15-20%. Furthermore, tumors can develop resistance through the shedding of the mAb target (immunoediting).

Monoclonal antibodies can also be used for immune modulation. This type of mAb actually targets components of the immune system to enhance or block effect. For example, antibodies targeting the suppressive co-stimulatory receptors CTLA-4 or PD-1 on T cells block inhibitory signals typically transmitted through these receptors and prolong the life of activated T cells. Several mAb that target the immune system are in various stages of clinical development, summarized in Table 4. Importantly, mAb that target immune system are less likely to be rendered unusable since the immune system cannot shed the targets as tumors can. The mechanisms of mAb immunotherapy are, in theory, easier to predict than chemotherapy since the target is known, yet in practice has proven difficult due to the redundancy of the immune system and our lack of complete understanding.

Table 4. Immune Modulatory Monoclonal Antibodies in Development for Humans (adapted from [110])

Target	Expression	Human Antibodies Available	Type	Development Stage
CTLA4	Activated T cells	Ipilimumab (Bristol-Myers	Fully human	Phase III

		Squibb)	IgG1	complete
		Tremelimumab (Pfizer)	Fully human IgG2	Development halted after Phase III
CD25	Tregs, activated T cells	Daclizumab (Hoffmann-La Roche)	Humanized IgG1	Phase III
PD-1	Activated T cells	CT-011 (CureTech)	Humanized IgG1	Phase II
		MDX-1106 (Bristol-Myers Squibb)	Fully human IgG4	Phase II
CD137	Activated T cells, Tregs, NK cells, NKT cells, DCs, neutrophils and monocytes	BMS-663513 (Bristol-Myers Squibb)	Fully human IgG4	Phase II
GITR	Tregs	TRX518 (Tolerx Inc.)	Humanized IgG1	Phase I
CD40	DCs, B cells, monocytes, macrophages	Dacetuzumab (Seattle Genetics, Inc.)	Humanized IgG1	Phase I

7.1 Anti-CTLA-4 Therapy

The most developed mAb of this type target the T cell surface protein CTLA-4. CTLA-4 is a negative regulator of effector T cell activity and is induced upon activation. CTLA-4 out-competes the co-stimulation molecule CD28 for binding B7 molecules on antigen presenting cells and instead delivers an inhibitory signal [111]. Therefore, CTLA-4 is used as a braking mechanism to control T cell responses. It is also used by Tregs for immune suppression; Tregs constitutively express CTLA-4 and induce suppression to DCs when binding through B7 [112]. The DCs in turn induce apoptosis and anergy in T cells [15]. Two fully human antibodies have been developed that target CTLA-4: tremelimumab (by Pfizer) and ipilimumab (by Bristol-Myers Squibb). Potentially, these antibodies could work on two fronts, first by blocking effector T cell CTLA-4 and thereby extending their survival, and second by blocking Treg CTLA-4 to prevent this mechanism of suppression. However, studies have demonstrated that in humans anti-CTLA-4 treatment targets effector T cells only [113, 114]. Ipilimumab was recently

approved by the FDA for second line treatment of advanced melanoma, but both have been tested in a number of clinical trials targeting various indications, such as melanoma, and have provided positive benefit [115]. Despite being able to induce tumor regression in 10% of patients, Pfizer halted the development of tremelimumab based on a dismal increase of overall survival of only 1 year in a recent phase III trial [116].

The results of a phase III clinical trial of ipilimumab, which supported FDA approval for this mAb, were presented at the American Society of Clinical Oncology (ASCO) meeting in 2010 [117]. The 1:1:3 randomized study containing 750 patients compared ipilimumab treatment alone to vaccination with GVAX (peptide vaccine targeting the melanoma TAA gp100) and to combination treatment with both ipilimumab and GVAX. Patients who received ipilimumab alone or in combination with GVAX were not significantly different and experienced a 10% increase in 2-year survival rates and increased overall survival compared to patients who received GVAX alone. Although these results were used to approve ipilimumab treatment in advanced melanoma patients, they are somewhat controversial because GVAX alone was used as the control arm, and not the common dacarbazine treatment used for advanced melanoma patients [118]. From a vaccine perspective the results are discouraging. Other peptide vaccines targeting gp100 have shown immunogenicity in other small clinical trials, demonstrating that it is possible to break tolerance towards this TAA, yet in this study no effect was attributed to GVAX treatment [119, 120]. Pre-clinical research had also indicated that murine anti-CTLA-4 could in fact synergize with peptide cancer vaccines in mice [121-123]. The advanced stage of the patients in the ipilimumab study may have been detrimental to vaccine efficacy, and could show that although ipilimumab does provide some benefit to these patients, it cannot synergize with peptide vaccines in this cohort. Notably, the authors did not report if gp100-specific T cells were raised in any group so it is unclear if the patients immune systems responded at all to vaccination [124]. It is also possible that ipilimumab cannot synergize with cancer vaccines due to the isotype of this antibody. Ipilimumab, like the majority of mAb developed to date, is IgG1 isotype, which induces moderate complement activation and strongly induces phagocytosis by binding to Fc receptors. Although this isotype is ideal for mAb targeting tumor cells for destruction, ipilimumab targeting activated T cells may inadvertently enhance their elimination. In contrast,

tremelimumab is IgG2 isotype, which is a poor activator of complement and weak binder of Fc making it an ideal subclass for blocking interactions. It would be interesting to compare both anti-CLTA4 mAb in combination with vaccination to see if tremelimumab can induce a greater synergistic effect than was observed with ipilimumab. Indeed, a better understanding of which antibody isotypes synergize best with vaccines is needed for rational design of future clinical trial protocols involving these two emerging immunotherapies for cancer.

7.2 Anti-PD-1 Therapy

PD-1 (programmed death 1) is a member of the CD28 superfamily, like CTLA-4, and is upregulated on T cells upon activation [112]. PD-1 is a suppressive regulator of T cell activity, ligation with its receptor results in inactivation and apoptosis. The receptors for PD-1, PD-1L and PD-2L, are normally expressed on self-cells to prevent autoimmunity, however PD-1L is upregulated by a number of tumors to quell anti-tumor T cell responses [125-127]. Accordingly, tumor infiltrating $CD8^+$ and $CD4^+$ T cells have been shown to have increased expression of PD-1 and are anergic [128, 129]. Combined treatment of anti-PD-1 treatment and a GM-CSF secreting whole cell vaccine significantly prolonged mice challenged with B16 melanoma or with CT26 colon cancer, whereas monotherapy with either treatment had no effect [130]. The combined treatment was associated with increased antigen-specific $CD8^+$ T cell infiltration of the tumor. Another study by Mongsbo *et al.* also found that monotherapy with anti-PD-1 is not as effective as anti-CTLA-4 monotherapy, but together may have an additive effect in prevention of MB49 murine bladder cancer [131]. The combined blockade of both PD-1 and CTLA-4 was found to synergize with a vaccine in treating of B16-B6 melanoma tumors [132]. The synergistic effect on tumor growth was mirrored with increased tumor infiltration of $CD8^+$T cells expressing CTLA-4 and PD-1, presumably without treatment these cells would have been anergized. Dual blockade of PD-1 and CTLA-4 signaling eliminates two T cell suppressive mechanisms, therefore this is a logical combination that should increase longevity of T cells. A human PD-1 antibody (MDX-1106) was recently tested in a clinical trial in patients with several types of advanced cancer [133]. In the small phase I study, 39 patients were treated with antibody monotherapy and levels of PD-1 on circulating PBMCs as well as levels of PD-L1 on tumor cells were monitored.

They found that tumor expression of PD-L1 may be indicative of responsiveness to MDX-1106 treatment, but overall clinical responses were low.

An alternate, or perhaps additional, mechanism for the synergistic effect of combined PD-1 and CTLA-4 blockade is by inhibition of MDSC suppression. One group reported that MDSCs isolated from mice bearing I8D ovarian tumors had elevated levels of both PD-1 and CTLA-4 [134]. When blocking antibodies were administered *in vitro*, the MDSCs had reduced arginase I activity; arginase I is a mechanism through which MDSCs attenuate T cell activation. *In vivo* treatment of tumor bearing mice reduced tumor burden and increased survival.

7.3 Anti-GITR Therapy

Complementary to T cell boosting strategies with anti-CTLA-4 or anti-PD-1 would be Treg inhibition using Treg-specific antibodies. Initially, antibodies towards the relatively non-specific CD25 surface marker found on Tregs were used in an attempt to target this T cell subset. However, anti-CD25 mAb clinical trials (daclizumab by Hoffman-LaRoche) have experienced mixed results; although this antibody does deplete Tregs, it also has an effect on activated effector T cells, which also upregulate CD25 [135]. The result is too devastating on the developing anti-tumor immune response unless timed correctly, which could present technical limitations for heterogenous human patients [135, 136]. A new target is GITR (glucocorticoid induced TNF receptor), a co-receptor expressed in constitutively high amounts by Tregs and also increased on activated T effectors. Interestingly, while co-stimulation of CD3 and GITR results in proliferation of both Tregs and effector T cells, the expanded Tregs become functionally unresponsive while the effector T cells gain functional activity [137]. A single administration of the murine anti-GITR antibody DTA-1 eradicates or reduces tumor growth in different mouse models [138-140]. Mice challenged with B16 tumors and treated with DTA-1 developed strong antigen-specific T cell responses, and when combined with a melanoma vaccine, DTA-1 treatment enhanced primary and recall $CD8^+$ T cell responses [141, 142]. The mechanisms underlying DTA-1 treatment are truly two-fold, they can both enhance effector T cells and reduce Tregs. The mechanism through which they reduce Treg function is not clear, in one study Tregs isolated from tumors of

DTA-1 treated mice did not have impaired suppressive function and yet relative numbers of Tregs were reduced compared to CD4 or CD8 T cells, suggesting depletion [138]. However, some studies have found no change in the absolute number of $CD4^+$ T cells after DTA-1 treatment, and no death observed *in vitro*. Instead, it has been proposed that DTA-1 treatment reduces the lineage stability of Tregs through loss of FoxP3 expression [139]. This could mean that Tregs are converted to Th17 cells, these cells are known to be reciprocally regulated and instances of Treg conversion into Th17 have been documented [143]. It would be interesting to see if this was the case with DTA-1. In any case, the combined blockade of CTLA-4 and GITR with mAb was recently shown to synergistically reduce tumor formulation in two different murine tumor models, demonstrating that their respective effects on Tregs and effector T cells, in the end, work together [144].

8. Considerations for Antibody-Vaccine Combinations

Antibody therapies for immune modulation are an exciting new area of discovery in immunotherapy research. As an alternative to chemotherapy, immune modulation they offer a defined mechanism of action since the target is known. However, due to the redundancy of the immune system and the fact that we still do not fully comprehend its complexity, antibody therapies still carry the risk of off-target side effects. Further, since immuno-modulatory doses of chemotherapies are often low and non-toxic, antibody therapies may loose their safety-edge since they still must be used at standard doses. Obtaining relevant pre-clinical data for mAb therapy is also difficult since the human antibodies cannot be tested in common strains of mice, so we must rely on translation in models that use murine homologs of the antibodies. Several clinical trials are currently evaluating these antibody therapies in conjugation with vaccine therapy. As the results of these trials emerge, and our understanding the of the immune system increases, antibody therapies may emerge to become the standard complementary treatment to vaccines in the future of immunotherapy.

9. Closing Remarks

Since the proposal of a “magic bullet” for cancer treatment, researchers have been looking for the one cure that will stop all cancers. With each new development – surgery, radiotherapy and then chemotherapy – it has become increasingly obvious that the best

course of treatment utilizes multiple methods. Immunotherapy is the next step in cancer care, and may also have best results when used in combination with other therapies. Different immunotherapy approaches have different strengths, vaccines elicit and guide an immune response and antibodies or chemotherapies can reverse tumor-induced immune suppression. The future of cancer therapy lies in combining these treatments effectively, which hinges on our understanding of the role of the immune system in tumor rejection. It is for this reason that cancer immunotherapy is evolving alongside our understanding of the immune system.

Whatever the approach, it is increasingly becoming apparent that the most promising cancer therapies cannot work alone. Cancer vaccines, chemotherapies and immunotherapies must be combined effectively to attack the tumor from multiple sides to quickly and thoroughly eliminate cancer.

Conflict of Interest

The authors declare no conflict of interest.

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APPENDIX B: SUPPLEMENTARY FIGURES

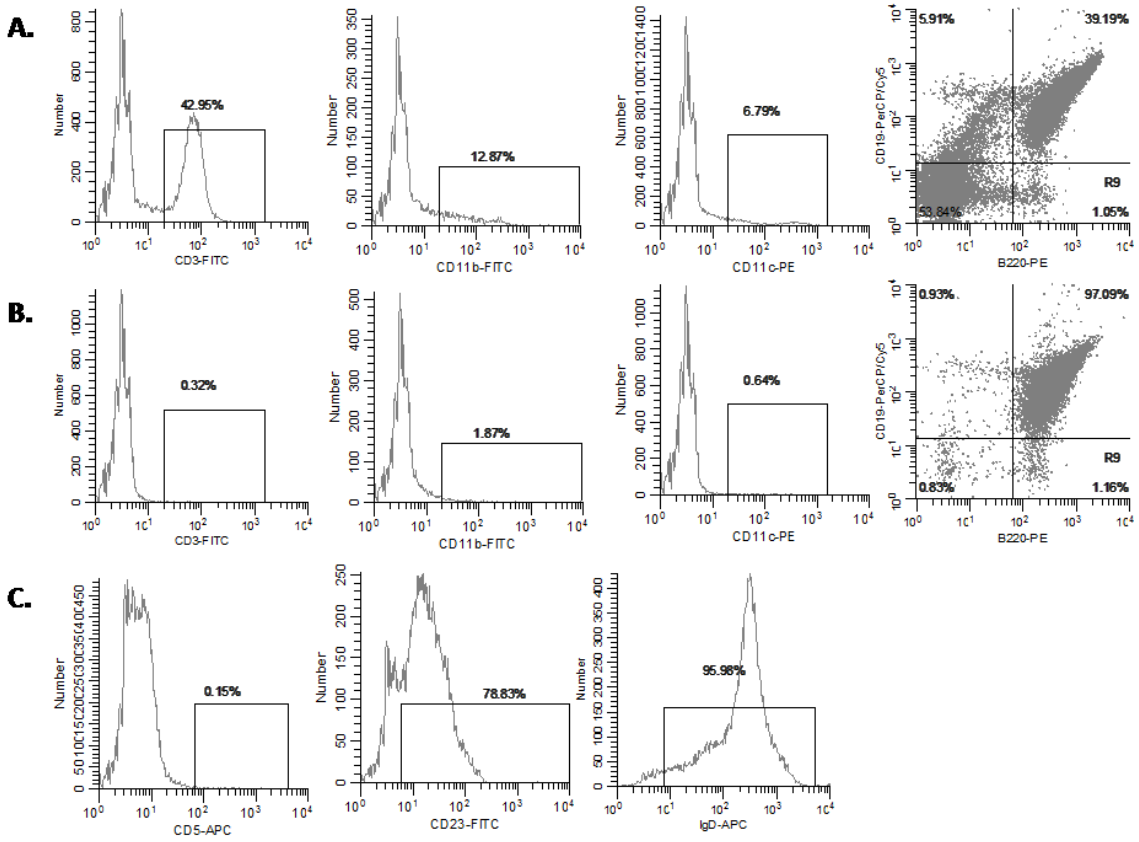

Figure B.1: Phenotype of B cells purified from the spleen. B cells were isolated from C57BL/6 mice using negative selection with magnetic beads. Phenotype of cells before **(A)** and after **(B)** isolation. **(C)** Phenotype of purified $CD19^{+}$ B cells.

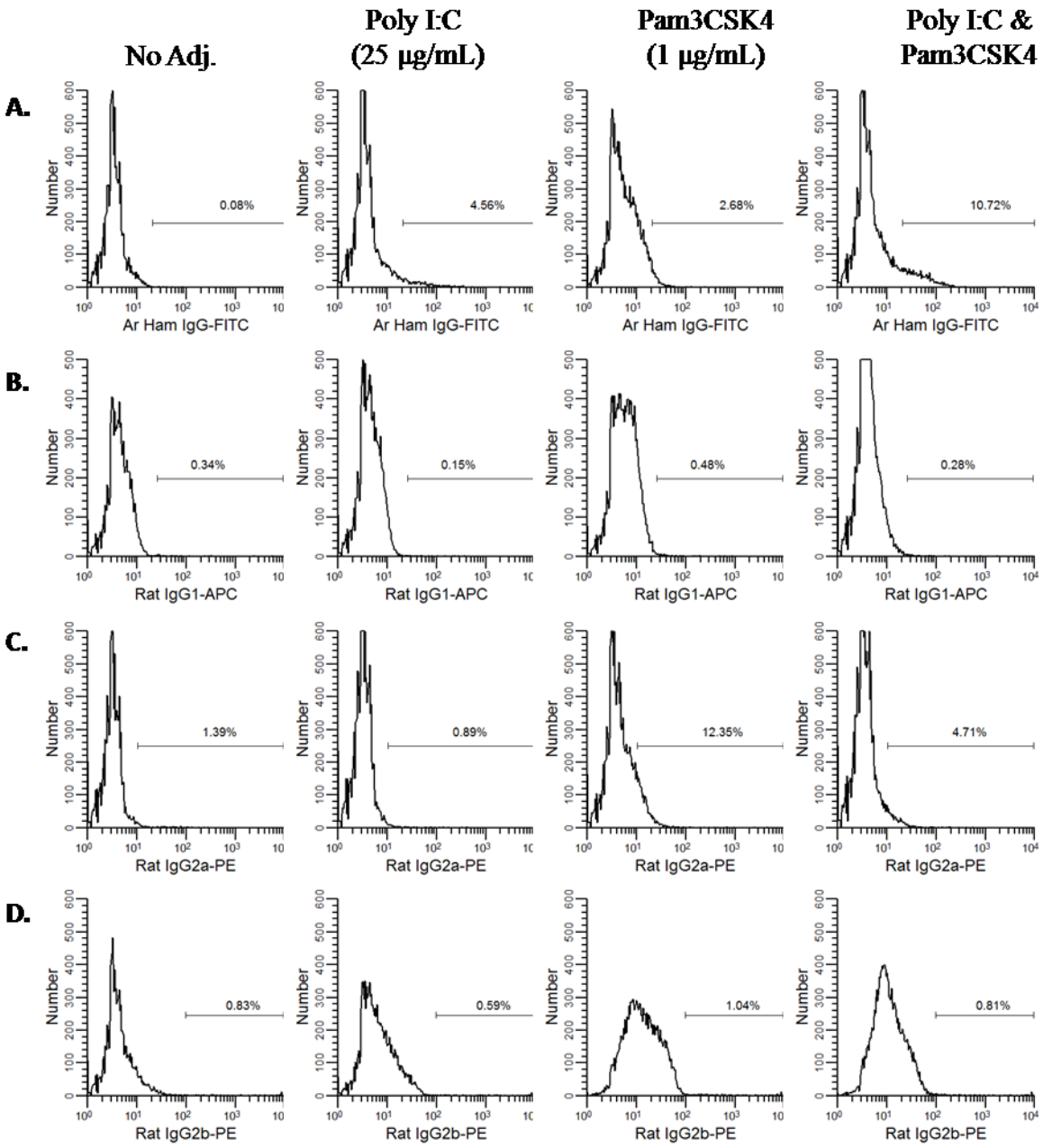

Figure B.2: Stimulated B cells stained with isotype controls. (A) Armenian Hamster IgG-FITC, **(B)** Rat IgG1-APC, **(C)** Rat IgG2a-PE, **(D)** Rat IgG2b-PE.

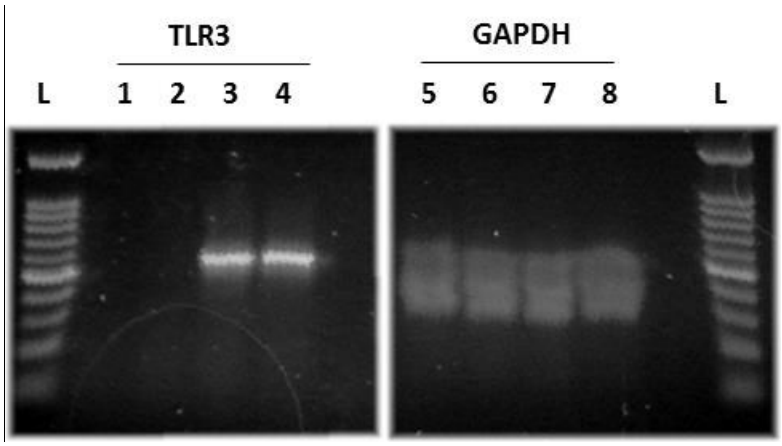

Figure B.3: RT-PCR confirmation of TLR3 wild-type and knockout mice phenotype. Expression of TLR3 in 2 wild type (lanes 3,4,7,8) and 2 TLR3 knockout (lanes 1,2,5,6) mice was determined by reverse transcription PCR using spleen sample from each. Primers were designed to span deleted exon. "L" = 100bp ladder. Data obtained by O. Hrytsenko.

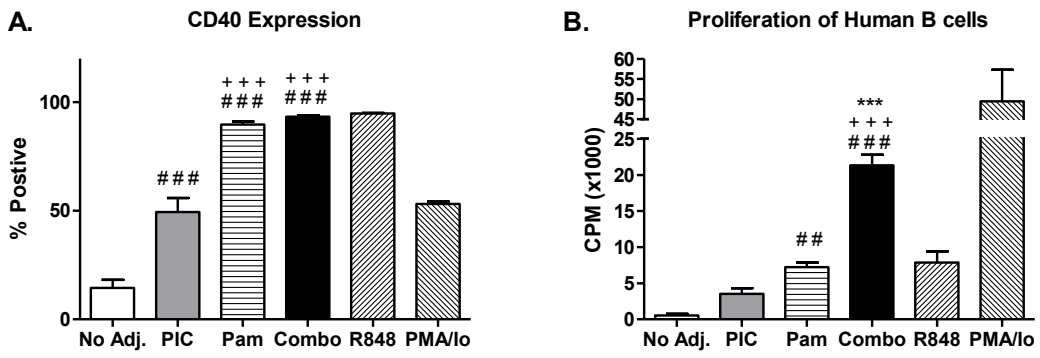

Figure B.4: Effects of Poly I:C and Pam3CSK4 stimulation of human B cells. Human B cells were purified from healthy donor PBMC (n=3). B cells were stimulated with no adjuvant, poly I:C (25 μg/mL), Pam3CSK4 (1 μg/mL), the combination of poly I:C and Pam3CSK4, R848 (2.5 μg/mL) or PMA/ Io cocktail. **(A)** CD40 (clone 2D10.4) expression by flow cytometry after 24 hours, data shown are mean ± SEM; **(B)** Proliferation after 4 days measured by [^{3}H]-TdR uptake. Data shown are mean of three replicates ± standard deviation. Statistics by ANOVA with Tukey multiple comparisons post-test: "#" compared to no adjuvant, "+" compared to poly I:C; "*" compared to Pam3CSK4.

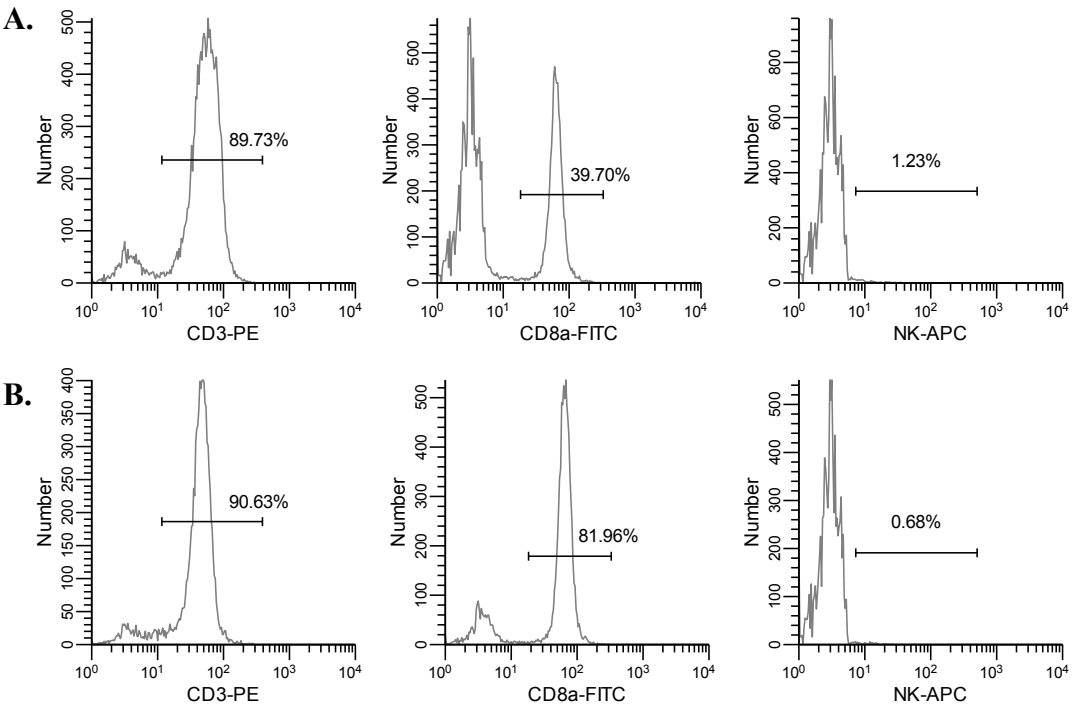

Figure B.5: Purity of T cells after immunomagnetic purification. **(A)** T cell purity after CD3-negative selection using Stemcell Technologies kit. **(B)** CD8 T cell purity after CD8α-negative selection using Stemcell Technologies kit.

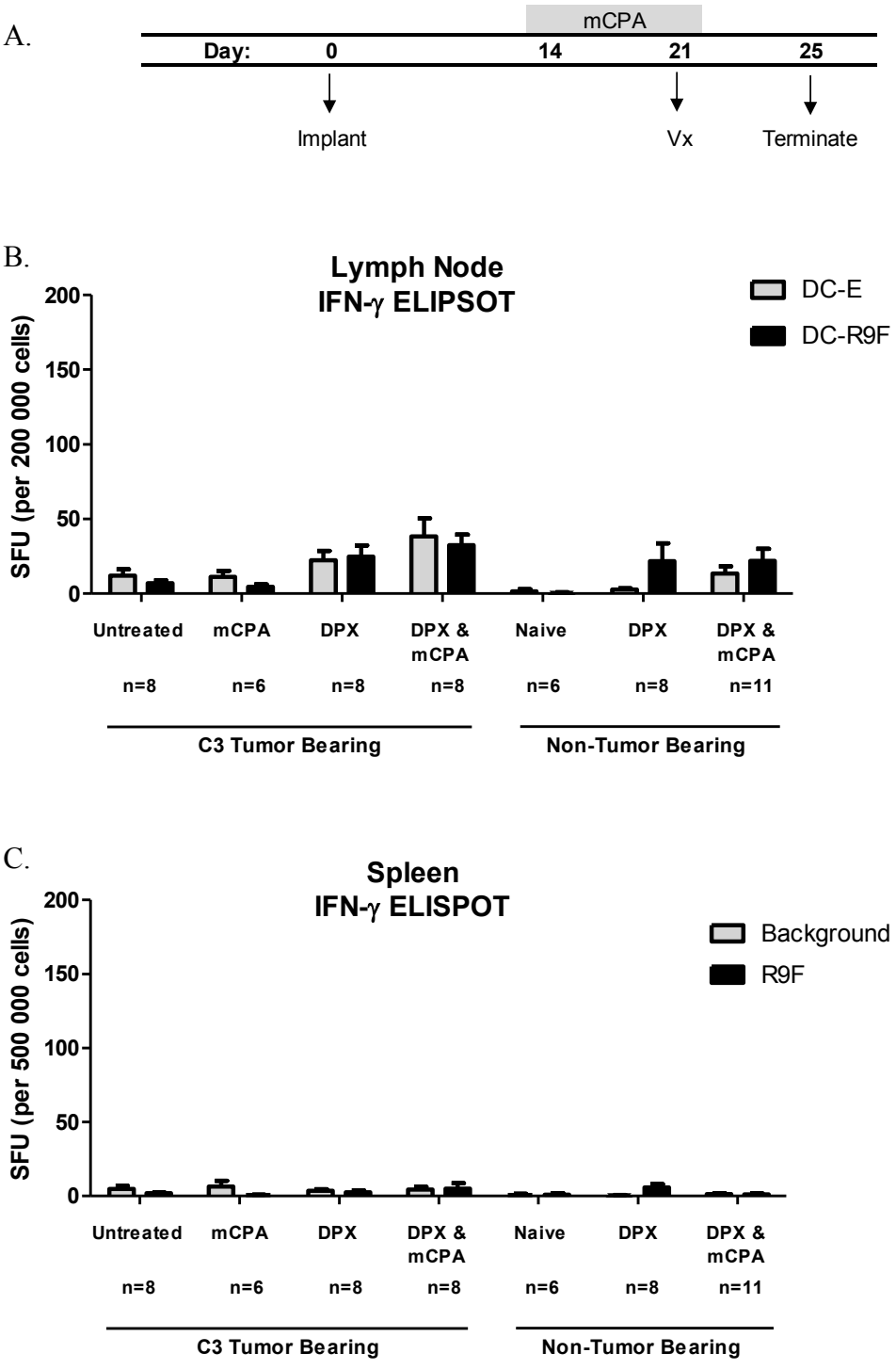

Figure B.6: IFN-γ ELISPOT performed on four days after mCPA/ DPX-R9F treatment. (A) Mice were implanted with C3 tumors on day 0. On days 14-21 mice were treated with mCPA (20 mg/kg/day, PO) and vaccinated with DPX-R9F on day 21. Non tumor bearing mice were treated in parallel. Mice were terminated on day 25. IFN-γ ELISPOT was performed with **(B)** lymph node cells and **(C)** splenocytes.

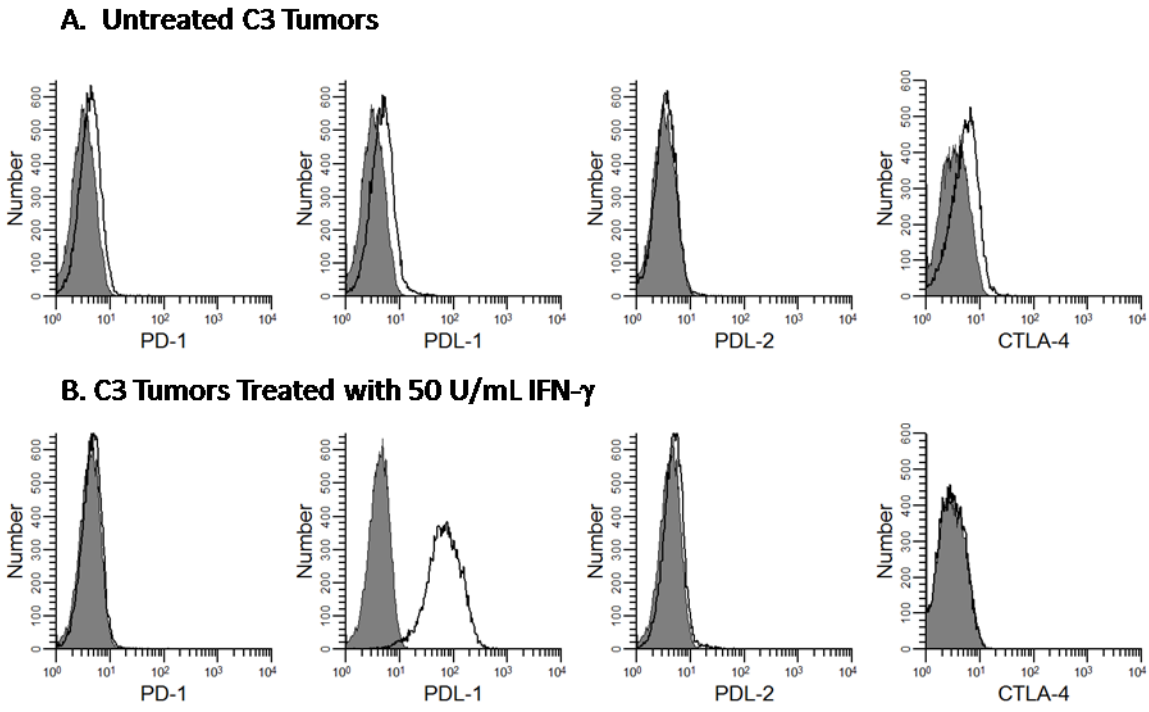

Figure B.7: Expression of surface receptors on C3 cells grown *in vitro* and exposed to IFN-γ. C3 cells were seeded at 2×10^5 cells in a 6-well plate and allowed to adhere overnight. Next day, 50 U/mL of purified IFN-γ was added to one well. After two more days, cells were removed from wells using 0.5% trypsin and stained with fluorochrome-labeled antibodies to detect PD-1 (J43), PDL-1 (M1H5), PDL-2 (TY25) or CTLA-4 (UC10-4B9). Grey histograms indicate staining of isotype control (rat IgG2a).