Identification of immunostimulatory adjuvant(s) that will promote a Th17-type of immune response

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ABSTRACT

Immunostimulatory adjuvants are substances added to vaccines to promote and direct a robust Th1, Th2, or Th17 immune response. Murine Th17 cells are produced and differentiated from naïve T cells in the presence of transforming growth factor (TGF)-β and interleukin (IL)-6 and once differentiated, Th17 cells produce cytokines IL-17A, IL-17F, IL-21 and IL-22. We investigated how immunostimulatory molecules such as poly[di(sodium carboxylatoethylphenoxy)-phosphazene (PCEP), Alum, CpG oligodeoxynucleotide (CpG ODN), Curdlan, Leptin and Lipopolysaccharide (LPS), alone or in combination influenced differentiation and/or activation of Th17 type immune cells in mice. *In vitro* studies showed that murine splenocytes stimulated with CpG showed significantly induced production of IL-12, a cytokine important for induction of Th1 type immune cells and IL-12 is known to be inhibitory for differentiation of Th17-type immune cells. Curdlan + Leptin +/- PCEP and PCEP + Curdlan induced significant expression of TGF-β. No immunostimulant combination induced both IL-6 and TGF-β, which we anticipated would be required for Th17 cell differentiation. When we investigated the cytokines induced by the immunostimulants 48 hours after injection in muscle tissue, we determined that Curdlan + Leptin significantly induced production of IL-17, likely from activation of already differentiated T cells. TGF-β was significantly induced in response to Curdlan and Leptin, alone and in combination but they were poor inducers of IL-6. PCEP+/- CpG or LPS significantly induced expression of IL-6 but not TGF-β. Finally, we immunized mice via intramuscular (i.m.) route with OVA in the presence of the immunostimulatory adjuvants and assessed cytokine production from OVA-restimulated splenocytes 5 weeks later. ELISA results indicated that OVA-specific

IL-17 production was significantly induced in splenocytes from mice immunized with PCEP + OVA relative to the mice immunized with Curdlan + OVA, although it was insignificant with respect to the OVA immunization group presumably due to the highly variable responses. Using flow cytometric analysis, we observed that vaccination with PCEP + OVA and Curdlan + Leptin + OVA significantly induced the frequency of OVA-specific splenic $CD4^{+}IL\text{-}17^{+}$ cells. Curdlan + Leptin also significantly induced the frequency of OVA-specific $CD4^{+}Foxp3^{+}$ cells and $CD4^{+}IL\text{-}17^{+}Foxp3^{+}$ double positive cells. Thus, we conclude that *in vitro* studies are poorly predictive of the type of adaptive response that may be induced when immunostimulatory adjuvants were used in a vaccine. Furthermore, vaccines formulated with PCEP and Curdlan + Leptin adjuvants promote Th17 cell differentiation and should be investigated as a combinational adjuvant for bacteria or fungal based immunizations.

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DEDICATION

This thesis is dedicated to my parents and my best friend. Thank you for your unconditional love and support.

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LIST OF ABBREVIATIONS

° C – Degrees Celsius

ANOVA- Analysis of variance

B cell- B lymphocyte

BCR- B cell receptor

CFA- Complete Fruend's Adjuvant

CpG ODN- Cytosine-phosphate-guanosine oligodeoxynucleotides

CTLs- Cytotoxic T lymphocytes

ELISA- Enzyme-linked immunosorbant assay

FBS- Fetal Bovine Serum

FCM- Flow Cytometry

FMO- Fluorochrome minus one

IFA- Incomplete Fruend's Adjuvant

IFNγ- Interferon gamma

IL-1β- Interleukin 1β

IL-4- Interleukin 4

IL-6- Interleukin 6

IL-10- Interleukin 10

IL-12- Interleukin 12

IL-17- Interleukin 17

IL-21- Interleukin 21

IL-22- Interleukin 22

IL-23- Interleukin 23

IM- Intramuscular

L- Litre(s)

LN- Lymph Node

LPS- Lipopolysaccharide

MEM- Minimal Essential Media

mg- milligram

ml- millilitre

MPL- Monophosphoryl lipid A

ng- nanogram

PBS – Phosphate buffered saline

PCEP- Poly[di(sodiumcarboxylatoethyl-phenoxy)phosphazene]

pg- Picogram

T cell- T lymphocyte

TCR- T cell receptor

TF- Transcription factor

Th1- T helper cell 1

Th2- T helper cell 2

Th17- T helper cell 17

Treg- T regulatory cell

µg- Microgram

µl- Microlitre

CHAPTER 1 Introduction

Vaccines are highly important tools in public health that can protect against infectious diseases and even types of cancer [1-3]. Subunit vaccines are safe because they cannot revert to virulence but they often fail to promote a robust immune response. For more than 80 years, adjuvants have been included in vaccines to promote a more robust immune response to subunit vaccine antigens [4-6]. Different adjuvants mount different immune responses and therefore it is important to identify the mechanisms behind how each adjuvant works to select the adjuvant(s), which promotes the most appropriate immune response to combat each particular infection.

Although excessive activation may lead to induction of autoimmune diseases, Th17 type immune cells are effective in combating bacterial and fungal infections, especially at mucosal surfaces [7]. It is important to identify adjuvants that promote Th17-type immunity for use in future vaccines and immunotherapies against these pathogens.

I hypothesized that PCEP alone or in combination with other adjuvants, will promote an antigen-specific Th17-type of immune response when formulated as part of a vaccine. We were also interested to see whether *in vitro* assays may garner critical clues as to the type of adaptive immune response generated with adjuvants when formulated as part of a vaccine.

CHAPTER 2 Literature Review

2.1 Adjuvants

For more than 80 years, adjuvants have been included in vaccines to promote a more robust immune response to subunit vaccine antigens [4-6]. Adjuvants stimulate the innate immune response which educates and directs the adaptive immune responses so that it is more effective against individual pathogens. There is no universal mechanism of action by which adjuvants mediate their effects; some immunostimulatory adjuvants promote innate immune cells to secrete select cytokines which promotes T cell differentiation [8]. Other adjuvants promote antigen presentation and activation of antigen presenting cells (APCs), which are important to initiate the adaptive immune response [9]. Inclusion of adjuvants can reduce the dose of antigen required for an effective vaccine; a process known as dose-sparing which is an important economic factor in vaccine production [10]. Even with all of the current research and knowledge on the benefits of adjuvants in vaccines, there are still few adjuvants approved for use in human vaccines.

2.1.1 Adjuvant History

The word "adjuvant" was derived from a Latin word, "*adjuvare"* meaning "to aid" [11]. Adjuvants have been known as an immunologist's "dirty little secret" because although they have been used in vaccines for almost a century to trigger induction of robust immune responses, their mechanism of action remain poorly understood [12].

Vaccine development evolved from ingenious but risky experimental trials. In England, dairymaids were routinely exposed to cowpox during daily milking and they

experienced a mild immune response to cowpox, but they tended not to suffer from smallpox infection. To test whether cowpox infection could protect against small pox infection, country physician Edward Jenner in 1776 took fluid from a cowpox pustule from a dairymaid and vaccinated 8-year-old James Phipps [13, 14]. Later, he repeatedly attempted to infect Phipps with smallpox but the boy never developed the disease [13, 14]. Although it took many years, vaccination was eventually accepted by the medical establishment as an important means to prevent disease. From there, vaccines were created for different diseases such as polio (Jonas Salk, 1955; commercialized by Albert Sabin in 1961), measles (Dr. Peebles and Dr. Enders, 1963), diphtheria (Franklin Royer developed adequate doses of antitoxin, 1905; commercialized by Mulford Company of Philadelphia, 1920's), influenza (Ernest Goodpasture, Thomas Francis, Jonas Salk, Wilson Smith & Macfarlane Burnet, 1930s; US Military, 1940s) and many more [15].

Vaccines are important tools to protect domestic and livestock animals against infections. The first known use of an adjuvant in veterinary studies was by veterinarian Dr. Gaston Ramon who determined that substances added to a vaccine to produce an inflammatory response at the site of injection resulted in increased antibodies in sera [15]. In the 1930's, Alexander Glenny commercialized the use of aluminium salts (or alum) as an adjuvant in a human vaccine for pertussis and alum has since been commercialized in many human and animal vaccines [15]. Other early vaccine adjuvants include mineral oil-in-water emulsion known as Complete and Incomplete Fruend's Adjuvant (CFA and IFA) and Emulsigen, which promote strong antigen-specific humoral and cellular immune responses to vaccine antigens [15, 16]. However, CFA, IFA and Emulsigen have not been approved for commercial use in human vaccines in the United States of America

(USA) and Canada due to excessive reactogenicity. Recently, AS04 [a combinational adjuvant consisting of monophosphoryl lipid A (MPL) and alum] and MF59 [a squalene (a natural and easily metabolized oil), oil-in-water emulsion] adjuvants were approved for use in vaccines for the human papilloma virus and influenza, respectively [17, 18]. AS04 is not immunostimulatory as it is able to induce an immune response only when administered in combination with an antigen [9, 18]. MF59 has both high immunogenicity and low reactogenicity [9, 19]. Overall, there are fewer adjuvants licensed for use in human vaccines than veterinary vaccines as safety regulations are much more stringent for human administration. Licensed adjuvants approved for use in veterinary to human vaccines vary in different countries as not all nations have the same safety standards. For example, a liposomal adjuvant, which consists of lipid layers and encapsulate antigens, has been approved for use in a Hepatitis A vaccine in Europe but it has not yet been approved for commercial use in North America [20].

Research on adjuvants has evolved from using one adjuvant to promote an immune response to coadministering adjuvants in an attempt to mount coordinated or fine-tuned responses to pathogens [21, 22]. In 2011, an intradermal (i.d.) influenza vaccine containing adjuvant JVRS-100 was approved by the FDA [23-26]. JVRS-100 is a cationic liposome-DNA complex (CLDC) made up of cationic DOTIM/cholesterol liposomes and plasmid DNA, which has been shown to increase $CD4^+$ and $CD8^+$ T cell and antibody responses [27, 28]. Although select adjuvants are becoming commercialized and being used in vaccines today, there is still much research needed to be carried out to determine safety, efficacy and mechanisms of action.

2.1.2 Mechanisms of Action

Adjuvants have been classified by parameters such as their mechanisms of action, their source, or their physiochemical properties [29]. Edelman further refined the classification to these 3 categories: 1) immunostimulants which increased cytokine production and in turn leads to immune cell recruitment their immunomodulation, 2) carriers (immunogenic protein, virus vectors, outer membrane proteins, fatty acids, etc.) which provide T cell help through immunogenic proteins to increase antigen recognition and 3) vehicle adjuvants which create matrices for antigens to induce immune responses [30]. For example, water-in-oil emulsion adjuvants such as CFA (Complete Freund's Adjuvant) create a depot, which allows for the slow release of the antigen from the injection site [31]. This slow release ensures that there will be antigen present in the system for an extended period of time, creating more exposure, which can also lead to increased antibody titres [32]. Cytokines and chemokines are important components of any immune response that promote the recruitment of local immune cells. Alum, CpG and MF59 have been shown to activate "adjuvant core response genes", which in turn code for proteins that are known to increase cytokine-cytokine receptor interaction, host-pathogen interaction and can be characterized through the up-regulation of cytokines, chemokines and adhesion molecules, which all are associated to a healthy inflammatory response linked to vaccine adjuvanticity [33].

MF59 promotes recruitment of neutrophils, monocytes, macrophages and DCs at the site of injection in mice as well as increased antigen presentation on APCs [17]. Although it is not fully understood, antigen adsorbs onto Alum and interacts with lipids on the surface of the DCs through an abortive phagocytosis then the DCs internalize the

antigen leading them to initiate antigen presentation[17]. Alum does not directly affect APC maturation, it promotes APC activation by enhancing the surface expression of MHC class II and costimulatory molecules on the surfaces of APCs which is a requirement for T cell activation in immune responses [34, 35]. CpG and LPS induce maturation and activation of DCs through Toll-like receptors (TLRs), costimulatory molecules and a cluster of differentiation molecules [18, 36, 37]. CpG binds to TLR9 whereas LPS and AS04, which includes MPL (Monophosphoryl lipid A), bind TLR4; all of which activate signalling cascades [18, 38-40]. Adjuvant AS04 stimulates DC activation through TLR4 pathway signalling [18].

The inflammasome is an important complex for the innate immune response in myeloid cells [9, 41]. The inflammasome is a complex assembly of proteins that are activated by NOD-like receptor (NLRs) binding [9] or damage-associated molecular patterns (DAMPS) [42, 43]. Upon activation, the inflammasome activates caspase-1, which cleaves pro-forms of IL-1β, IL-18 and IL-33, which then activates the release of active forms of these cytokines. It is reasoned that adjuvants that activate the inflammasome will promote a more effective vaccine response [9].

2.1.3 Alum

Alum is the generic name for an adjuvant based on aluminum salts (aluminum hydroxide and magnesium hydroxide) and is the most common commercial adjuvant. . Alum promotes an increase in the recruitment of antigen presenting cells, including monocytes and macrophages to the site of injection as well as increased expression of cytokines and chemokines by these recruited cells [44-46]. When included in a vaccine,

alum has been shown to promote increased antigen-specific antibody production and Th2-type immune responses [15, 47, 48] as well as facilitation of antigen uptake into DCs through promoting antigen adsorption onto DC membrane lipids [49]. The DC then phagocytizes the antigen and antigen presentation is initiated [50]. *In vitro* experiments with murine and human DCs and macrophages suggest that Alum signals through NLRP3 to promotes activation of the of inflammasomes [51]. However, it is not definitively whether alum-induced inflammasome activation is a necessary for its adjuvant activity [44, 46, 51, 52].

2.1.4 LPS

Lipopolysaccharides (LPS) are one of the largest components of the Gram-negative bacterial cell wall and it is comprised of 3 major components: lipid A (endotoxin), core-oligosaccharide and O-antigen [53]. Endotoxin is crucial in maintaining bacterial outer membrane integrity, while the core-oligosaccharide is structural and the O antigens are variable, made up of repeating units of 3-5 highly versatile monosaccharides [53-55]. LPS induces a Th1 response through TLR4 signalling pathway and activates MyD88 to promote expression of IL-1β, IL-12, IL-18 and IFN-β as well as IFN-γ, through activation of transcription factor NF-κB [56-60]. LPS promotes DC maturation [61-63]. Because it is such as robust inducer of inflammation and fever, LPS can lead to deaths and has been deemed unsafe to use in a vaccine but it is still widely used for *in vitro* experimentation [64]. AS04, a combination of derivative of LPS known as Monophosphoryl lipid A (MPL, previously stated) and an aluminum salt, promotes equivalent immunomodulatory actions while being much less toxic than LPS

[65]. MPL is used in HPV vaccines and is also being investigated as a veterinary vaccine adjuvant [18, 66].

2.1.5 PCEP: Polyphosphazene

Polyphosphazenes have only been used as experimental vaccine adjuvants in the last 2 decades. They are water soluble polymers comprised of a phosphorous-nitrogen backbone with organic side-chains attached to the phosphorous molecules [67]. They induce expression of "adjuvant core response genes", which includes increased expression of chemokines, cytokines, innate receptors, interferon-induced genes and adhesion molecules [68]. Poly[di(carboxylatophenoxy)phosphazene] (PCPP) was one of the first polyphosphazenes to be studied as a vaccine adjuvant [21, 69]. It proved to have low reactivity at the site of injection and when administered intranasally to mice with influenza antigen, it induced a balanced Th1/Th2 response [70] as well as long-term memory B cells that produce antigen-specific IgG and SIgA antibodies in mucosal and systemic sites [70]. Another polyphosphazene, poly[di(sodiumcarboxyl-atoethylphenoxy)phosphazene (PCEP) was shown to induce a superior and predominantly Th1 type immune response relative to PCPP adjuvant and alum when formulated with bacterial and viral antigens in pigs and mice [71, 72]. PCEP is able to induce cytokines and chemokines secretion at the site of injections, to recruit T and B cells as well as induce expression of adjuvant core response genes IL-1β, IL-6, IL-18, IFNγ, TLR4, TLR9, CCL-2, CCL-12, Ltbr4 and NLRP3 genes [68, 73]. Andrianov et. al suggested that polyphosphazenes activate select TLRs but there is no evidence to support this theory [74]. Intramuscular injection of PCEP into mice led to increased recruitment

of neutrophils, macrophages and lymphocytes with more modest recruitment of monocytes and DCs. Flow cytometric analysis showed that the recruited myeloid cells may internalize PCEP, although how recruitment of distinct immune cells to the site of injection potentiates immune responses to antigens is not yet understood [73]. Research in the Mutwiri laboratory is being performed to refine our understanding of how PCEP mediates its mechanisms of action and how it can be used to promote effective vaccines.

2.1.6 CpG Oligodeoxynucleotide

CpG oligodeoxynucleotide (ODN) is a synthetic oligodeoxynucleotide which contains one or more unmethylated CpG dinucleotides of CpG motifs [75]. Bacterial DNA is unmethylated and activates immune cells [76, 77] whereas methylated vertebrate DNA does not [78]. Unmethylated CpG dinucleotides act as bacterial DNA mimetic and TLR9 agonists that mediate B-cell survival, activation and differentiation [39, 75, 79]. Further, upon recognition of the CpG molecule to TLR9 in the endosome, CpG induces signalling of TRAF6 and IRAK through a MyD88 signalling pathway, which induces production and nuclear translocation of transcription factor NF-κB and induced expression of IL-1, IL-6, IL-12, IL-18, IFNγ and TNF-α [9, 39, 40, 80-82]. Through this cascade of events, CpG induces a Th1-type of immune response. When CpG is co-administered with other adjuvants, such as a polyphosphazene, it promotes a more balanced Th1/Th2 response [21, 22, 79].

2.1.7 Curdlan

Curdlan is a water-insoluble β-glucan polysaccharide from the cell wall of soil

bacterium *Alcaligenes faecalis* [83-85]. It's a high molecular weight linear polymer consisting of β (1-3) linked glucose residues and has immunostimulatory qualities and is a Dectin-1 agonist [83-85]. Curdlan binds to Dectin-1, a C-type lectin receptor on DCs, that binds to pathogen-specific carbohydrate residues on select pathogens or yeast [86, 87], which in turn recruits Syk, a tyrosine kinase critical for cytokine production from cells [88]. Through this signaling cascade, Dectin-1 recognition of Curdlan on a DC, promotes secretion of proinflammatory cytokines from the DC: IL-6, TNFα and IL-23 which in turn instruct the differentiation of $CD4^+$ Th17-type cells in mice [83, 85, 86]. β – glucans like Curdlan can also NLRP3 inflammasome activation dependent on the Dectin-1-syk pathway, which is linked to the activation and secretion of IL-1β [84]. Lack of Dectin-1 or its loss of function can lead to an increased chance of pathogenic fungal infections in both humans and mice [83, 89, 90]. When T regulatory cells (Tregs) are present and secreting TGF-β and IL-6, signaling through Dectin-1-Syk can induce Th17 differentiation and therefore induction of a Th17-type immune response. In response to Curdlan, DCs produce IL-23 instead of IL-12 and IL-23 acts on differentiated Th17 cells to further support Th17 proliferation [86, 91]. In contrast, CpG triggers induction of IL-12 from DCs, which would trigger a shift from Th17 to Th1 type $CD4^+$ T cells.

2.1.8 Leptin

The protein Leptin is a member of the long-chain helical cytokine family (which includes IL-6 and IL-12) produced by adipose tissue (an adipokine) and circulates in plasma [92, 93]. Leptin serves to aid in sustaining energy in times of starvation by slowing down metabolism [94]. Injection of Leptin into an obese mouse can produce up

to 30 % weight loss which suggests that it plays a significant role in weight regulation [92]. The Leptin receptor is expressed on the surface of peripheral and bone marrow derived immune cells as well as in cells in major organs and muscle [95-98]. Leptin promotes the activation of natural killer cells (NK), neutrophils and the secretion of TNF-α, IL-6 and IL-12 from macrophages and granulocyte chemotaxis during an innate immune response [93, 99]. In the adaptive arm of the immune system, leptin inhibits Treg production and stimulates proliferation of naïve T cells into Th1 or Th17 cells [100, 101] through expression of transcription factor RORγt (ROR nuclear hormone receptor family) and STAT3, both regulators of Th17 differentiation [93]. Unfortunately, high serum leptin concentrations in mice has been associated with development of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE; similar to multiple sclerosis) so it may not be safe to use excessive quantities of leptin as a vaccine adjuvant to promote a Th17 type immune response [102].

2.2 Innate Immune Response

The immune system can be categorized into two groups: the innate immune system and the adaptive immune system. NK cells, mast cells, basophils, DCs, neutrophils and macrophages contribute to the innate response which is a non-specific first line of defence against pathogens [8]. Proteins in the blood, which include the complement system proteins among others, aid in initiating an inflammatory response, characterized by redness, heat, swelling and pain [8]. Activated mast cells release histamines which causes vasodilation of the nearby vessels while DCs (Langerhan's cells) and dermal macrophages release cytokines to induce high endothelial venules to

present adhesion molecule, cytokines or receptors in order to aid in diapedesis [8]. Leukocytes can also act as primary response cells that can kill the pathogens foreign cells [8]. Neutrophils are recruited through the blood vessel walls during inflammation via diapedesis or extravasation by chemoattractants and chemokines and they phagocytize the pathogen or antigen [8]. Once monocytes migrate out of the blood, they undergo maturation and differentiate into macrophages that process antigen and express cytokines to further augment inflammation in the local environment [103]. Neutrophils and then macrophages phagocytose the pathogen and release cytokines and chemokines to signal other innate immune cells and activate inflammation [8]. DCs are the major APC for T cell activation, aiding in the activation of adaptive immunity and upon antigen uptake, DCs undergo a maturation process, including the presentation of receptor CCR7 on their surface, which recognises chemokines CCL19 and CCL21 and causes them to migrate towards the lymph nodes (LN).

2.3 Adaptive Immune Response

Together the innate and adaptive arms of the immune system provide host defence. The adaptive immune response is activated by the innate immune response and it is much more specific toward select antigens rather than molecular patterns. Humoral or antibody-mediated immunity acts using antigen-specific antibodies from activated B cells and plasma cells that bind to the antigen/pathogen, attempt to neutralize the infection and/or eliminate the antibody-bound pathogen through effector mechanisms such as phagocytosis or cell lysis [8]. The cell-mediated immune response is mediated by $CD4^+$ and $CD8^+$ T cells, like Th1 and CTLs, respectively [104]. CTLs recognize antigens

through MHC class I on other infected cells and this recognition activates CTLs to kill infected cells matching the antigen at the site of infection through apoptosis by the secretion of perforin and granzymes or through Fas ligand attaching to Fas on the infected cells [8, 104]. CTLs, similar to Th1 cells, also secrete the cytokine IFNγ, which activates macrophages at the site of infection [8]. T helper cells are activated by APCs, mainly DCs, in the paracortex of the draining LN [8]. We will later discuss T helper cell differentiation of the cell-mediated immune response in more detail.

2.3.1 B cell activation

For a B lymphocyte to become activated, the surface immunoglobulin (antibody) known as the B cell receptor (BCR) must bind antigen that is most likely loose from infection in the lymph, migrating to the LN or is passed to it from follicular DCs in the draining LN [8, 105, 106]. Once a BCR binds its specific antigen, the antigen gets internalized then presented onto its MHCII on the cell surface. The B cell presents antigen on MHCII to an activated follicular T helper cell in the germinal centre of the LN (The immunological synapse between the B and T cells is explained below). Only the cognate activated follicular T helper cell can bind to the presented antigen but then it secretes cytokines to activate the B cell, which can then lead to class switching [8]. This class switching is dependent on the cytokines produced by the follicular T helper cell, and will cause the B cell to become either effector B cells (plasma cells) or memory B cells [8]. Plasma cells migrate to the bone marrow where they secrete antibodies that migrate through the blood vessels, the lymphatic system or to the site of infection for protection against the pathogen.

2.4 Naïve T Cells

Naïve T cells originate in the bone marrow and migrate to the thymus where they develop from lymphoid precursor cells with the help of thymic epithelial cells [107]. Thymic epithelial cells express both MHC molecules and the lymphocyte will become either a $CD4^+$ naïve T cell (MHC class II) or a $CD8^+$ naïve T cell (MHC class I), based on which receptor binds to the thymic epithelial cell [8]. Cells just arriving to the thymus are called double negative thymocytes as they currently do not express a TCR, CD4 or CD8 molecule [8]. However, express a precursor receptor called TCR-β, which allows the cell to continue to proliferation and avoid early apoptosis [8]. Once in the thymus, Rag-1 and Rag-2 protein expression on the thymocytes will cause rearrangement of TCR genes in these cells, later allowing for the thymocytes to present both CD4 and CD8 TCRs on its surface [8]. Until a naïve thymocyte's interaction with the thymic epithelial cells, they are double positive for both CD4 and CD8 molecules [8]. Epithelial cells expressing self peptides on both MHC class I and class II loosely bind to the double positive TCR of thymocytes causing them to become either CD4 or CD8 linage [8]. It is currently unknown if this is due to random processes or through specific unknown signals in the thymus [8]. The process of positive selection allows healthy T cells that recognize self- antigen or MHC molecules to survive and again avoid apoptosis, while another process known as negative selection aids in the destruction and apoptosis of those cells binding strongly and reacting to self-antigen [8]. These mechanisms are important for proper T cell development and immunity, avoiding pathogenesis. Mature but naive $CD4^+$ and $CD8^+$ T cells migrate from the thymus and circulate in the blood stream and lymphatics.

Naive T cells interact with DCs in the paracortex of the LN [8]. Extensive experimentation has revealed that there is a high degree of diversity in T-cell–DC contact dynamics which may be responsible for fine-tuning the T-cell activation process [108, 109]. At first, a CD4 molecule on the T cell binds to the MHC class II complex on the DC (or alternatively CD8 molecules of $CD8^+$ cells bind to MHCI on the DC). The T cell receptor (TCR) recognizes antigen being presented on the MHCII complex and then binds to the complex [8]. Next, the CD40 ligand on the T cell binds with CD40 on the DC causing B7 molecules to be expressed on the DC cell surface, which in turn bind CD28 on the T cell and ICAM-1 on the DC, respectively which promotes stability during activation [8]. After binding with the naïve T cell, the DC expresses cytokines to aid in the differentiation of the naïve T cell into a CTL (in the case of CD8 T cells or T helper cell or T regulatory cell in the case of $CD4^+$ T cells. The type of cytokines produced by the DCs influence into which specific T cell subset the naïve $CD4^+$ T cell will differentiate [8]. Following these steps, the DC begins to secrete IL-2 which prevents T cell apoptosis and the T cell begins to undergo activation of the T helper lineage [8]. Multiple signals from the DC, many of which are not yet fully understood, educate the T cell about where they need to migrate in the body to combat the pathogen and what type of T cell response would be most appropriate to combat the infection [110-112]. Each T helper subtype and regulatory T cells have transcription factors that act as master regulators to promote their differentiation. Over time, cytotoxic T-lymphocyte antigen 4 (CTLA4) surface expression is increased which down-modulates T-cell activation following antigen recognition [111].

It wasn't until the 1980's that different subsets of $CD4^+$ T cells were characterized [113]. $CD4^+$ naïve T cells can differentiate into Th1, Th2 and Th17 helper cells as well as Tregs. Upon activation, effector and memory lymphocytes combat pathogens and reduce the response time upon reinfection, respectively [8]. Memory lymphocytes have a much longer life and migrate through the body until they recognize their cognate antigen and mount a much quicker response than do naïve T cells [8]. T regulatory cells contribute by maintaining homeostasis through self-tolerance as well as oral tolerance, maternal tolerance to fetus and feedback control of responses produced by the T helper cells [114-117]. Figure 1-1 portrays the cytokine and transcription factors required for naïve T cells to differentiate into T helper (Th) or T regulatory cells (see blue arrows). The two key factors contributing to the differentiation of Th cells from naïve T cells are the cytokine environment and the activation of Th cell specific transcription factors [113].

2.4.1 Th1 cells

Th1 and Th2 cells were characterized in 1986 by scientists Coffman and Mosmann as each subset produced different cytokines upon activation [118]. Th1-type cells are $CD4^+$ effector T lymphocytes characterized by the expression of IFNγ, IL-2 and TNFα, while Th1's transcription factors (master regulators) are T-bet and STAT4 [113]. STAT4 as well as STAT1 are induced by the production of IL-12 and IFNγ, respectively and aid in the binding of T-bet to the T cell [119, 120]. Th1-type cells are associated with a cell-mediated type of response as they activate macrophages through increased IFNγ production [113, 121]. Th1 cells recognize antigens through MHC class II on infected macrophages and further activate macrophages to destroy the antigen, while, upon

activation, these T cells produce cytokines which induce antigen-specific production of cytokines by effector and memory T cells [8]. Th1 contributes to the development and maintenance of memory $CD8^+$ T cells but are not required for $CD8^+$ T cell activation [122]. Pathogenesis due to excessive Th1 production can lead to organ-specific autoimmune diseases [113].

2.4.2 Th2 cells

Th2 cells differentiation requires the APC to express IL-4 while Th2 cells themselves produce a combination of IL-4, IL-5, IL-13 and IL-25 with IL-2, IL-7 (Figure 1-1) as well as TSLP and the master regulator GATA3 and STAT5. STAT5 is induced by the production of IL-4 and aids in the binding of GATA3 to the T cell [123]. Th2-type cells are associated with the humoral immune response as they promote antibody production of IgE, eosinophil and mast cell recruitment and activation and extracellular parasite clearance through the production of cytokines and neutralizing antibodies IgG [8, 113, 124]. Pathogenesis can occur due to excessive Th2 production, which can cause diseases such as asthma and other allergic reactions [113].

2.4.3 T regulatory cells

Tregs, also known as suppressor T cells, attempt to restore homeostasis to a system by suppressing an activated immune response, which is critical to minimize potential pathogenesis [8, 125]. Differentiation of Tregs from naïve T cells occurs in response to transformation growth factor-β (TGF-β) or IL-2 exposure (Figure 1-1), which leads to activation of the Treg's master regulator Foxp3 alongside STAT5 [126]. STAT5

is induced by the production of IL-2 and aids in the expression of Foxp3 in the Treg cell [127]. Tregs produce cytokines IL-10, IL-35 and TGF-β and can be identified by measuring IL-10 or Foxp3 in a $CD4^+$ T cell, for Treg development and proliferation [125, 126].

2.4.4 Th17 cells

In 2005, scientists were exploring a homolog of the IL-12 family, p19 (now referred to as IL-23) as it formed a heterodimer with the p40 chain of IL-12. They conducted an experiment with IL-12 and IL-23 knockout mice, which determined that there was indeed another subset of T helper cell induced by IL-23 and that this $CD4^+$ effector T lymphocytes was characterized by the expression of IL-17A, IL-17F, IL-21 and IL-22 (Figure 1-1) [128-130]. Th17 cells are differentiated from naïve T cells in the presence of both TGF-β and IL-6, produced by stromal cells and DCs, respectively, and are further upregulated in the presence of IL-1β and IL-23, produced by DCs and macrophages at the site of infection [131-133]. DC will produce IL-6 and IL-1, while many other stromal cells will produce the TGF-β needed for Th17 cell differentiation [8, 132]. Under circumstances where there is TGF-β but limited IL-6, IL-21 will also promote Th17 differentiation [7, 132]. The IL-6 from the DC and TGF-β from other cells allow for ideal Th17 cell differentiation conditions [8, 132]. IL-6 and IL-1 promote transcription factor RORγt expression in the Th17 cell and IL-6 from the DC also promotes STAT3, the master regulators activated to aid in development and proliferation of Th17 cells and expression of its cytokines [8, 113, 132]. STAT3 is induced by the production of IL-6, IL-21 and IL-23 and aids in the expression of RORγt in the T cell

[134]. STAT 3 is also able to down-regulate T-bet, GATA3 and Foxp3 expression, alluding that STAT3 is able to supress a Th1, Th2 or Treg-type of immune response, allowing a Th17-type of immune response to be produced [134]. Th17 cells present CCR6 on its surface, which recognizes and binds to chemokine CCL20, produced by tissue cells and macrophages at the site of some bacterial and fungal infections [8, 132].

Th17 cells are most commonly found in the mucosa and secretion of IL-17 activates epithelial cells to recruit leukocytes to fight bacterial and fungal infections. Through cytokine expression, Th17 cells are able to induce inflammation and recruit leukocytes, mainly neutrophils as well as some monocytes to the site of infection [8, 132]. Some research has shown that an imbalance of Treg to Th17 cells increases the chance of disease inflammatory and autoimmune diseases onset [135-137]. However, Th17 cells have a high plasticity and are unstable effector cells, meaning they may convert into another T helper cell or even a T regulatory cell under certain conditions.

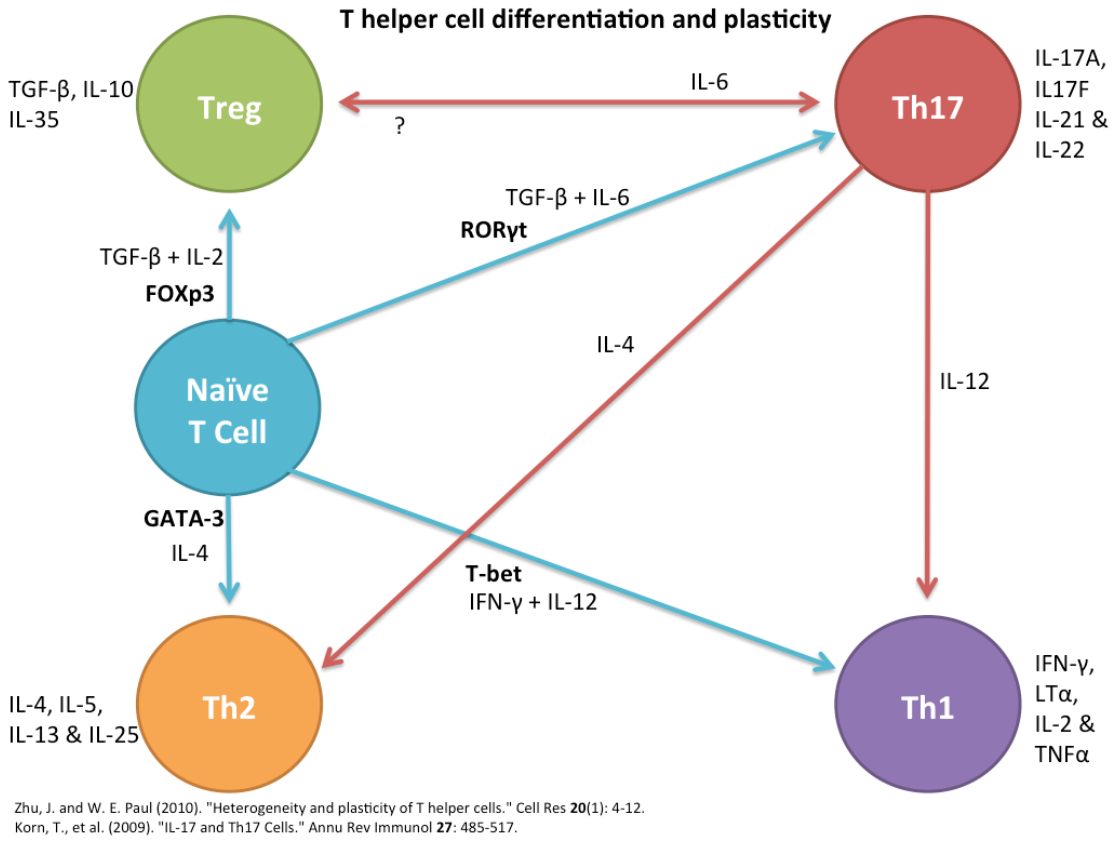

Figure 1-1. Differentiation of naïve T cells. Naïve T cells differentiate into T helper and T regulatory cells after binding with APCs carrying antigen. The APC in turn expresses cytokines that bind with the naïve T cells inducing expression of transcription factors inside the cells causing production of cytokines associated with the T helper and regulatory cells [113, 132].

2.4.4.1 Differentiation Characteristics and Plasticity

Even under the optimal conditions for Th17 cell development, Th17 cells can be induced to differentiate into another T helper cell subset, as T helper cells are highly plastic. Th1 and Th2 are able to convert into the other classes of cells at an early stage of differentiation, most often seen as a switch from Th1 to Th2 or visa versa [113]. Treg and Th17 cells can be influenced to convert into other T cells at both early and late stages of differentiation [113]. For example, Curdlan, one of the adjuvants in this study has been observed to cause a switch from $CD4^+Foxp3^+$ Tregs to $CD4^+IL\text{-}17^+$ Th17 cells [85]. Research has shown that Th17 cells when exposed to IL-12 can produce IL-4 or IFNγ, often while also producing IL-17, which shows us there is a conversion or even a mixed cell response [113]. This plasticity makes it difficult to pinpoint conditions for induction of a Th17-type of response.

2.4.4.2 Protection

Th17 are large contributors to protection against bacterial and fungal infections due to their presence in the mucosa, production of antimicrobial peptides like human β-defensins, mediated by the production of IL-17 and IL-22 and recruitment of neutrophils [7, 138-140]. Th17 cells express chemokine receptor CCR6 and cytokines GM-CSF (granulocyte macrophage colony stimulating factor), which aid in the recruitment of neutrophils at the infection site [7, 138]. Another study showed that Th17 cells can activate CTLs [141, 142].

There is evidence for a protective role against tumors [141]. Th17's role in protection against tumors comes from the production of $CD8^+$ CTLs and chemokines like

CXCL-8 and CXCL-2 as well as CCL20, which promotes DC recruitment within a tumor leading to activation of $CD8^+$ T cells [142, 143]. Th17 cells within the tumor also stimulate the tumor to express CCL20, recruiting the $CD8^+$ T cells to their location, expressing perforin/granzyme B to kill the tumor cells [141-145].

2.4.4.3 Side-effects and implications of Th17-type cell activation

Th17 cells are a recent discovery with respect to T helper cell immunity. Research for immunotherapies has been limited, as it was believed that Th17 cells and the Th17-type of immune response mostly aided in pathogenesis and autoimmune diseases. When Th17 cells are increased and there is an imbalance with Treg cells, the result can be chronic and severe inflammation, which may lead to diseases such as cystic fibrosis and multiple sclerosis [138, 146]. These negative Th17-type observations have impeded beneficial research for vaccines and immunotherapies linked to a positive and balanced Th17-type of immune response.

In summary, Th17 cell's high degree of plasticity and low stability may make it difficult to identify adjuvants that can lead to the establishment of a safe, protective and effective Th17-type immune response. Thus, research towards identifying adjuvants that can promote a balanced Th17-type response may prove to be challenging but rewarding for future vaccine candidates against bacteria and fungi.

CHAPTER 3 HYPOTHESIS AND OBJECTIVES

3.1 HYPOTHESIS

PCEP, or another adjuvant, alone or in combination with other adjuvants, will promote a Th17-type of immune response.

There were 3 main objectives to validate this hypothesis.

3.2 OBJECTIVES

Objective 1

Perform murine *in vitro* studies to identify immunostimulatory adjuvants that induce production of cytokines IL-1β, IL-6, IL-12, IL-17, IL-23 and TGF-β that directly or indirectly influence Th17 cell differentiation.

Objective 2

Perform murine *in vivo* studies with adjuvants from Objective 1 wherein adjuvants are injected into muscle and then quantify local production of IL-6, IL-12, IL-17 and TGF-β, which contribute to Th17 cell differentiation.

Objective 3

Immunize mice with Th17-inducing adjuvants, selected from Objectives 1 and 2, with an antigen to determine whether the adjuvant combination promotes an antigen-specific Th17-type of immune response.

CHAPTER 4 MATERIALS AND METHODS

4.1 Animal Experiments

All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals as indicated by the Canadian Council on Animal Care and was approved by the Animal Care Committee of the University of Saskatchewan. In objective 1, fifteen BALB/c mice (Charles River) were acclimatized for one week, mice were euthanized and then spleens from all fifteen mice were excised. The spleens were pooled in sets of three to make a sample size of five (n = 5) and splenocytes were isolated (see below).

In objective 2, eighty-four BALB/c mice (n = 6 per group) were injected intramuscularly with 25 μL into both semi-membranous muscles with an immunostimulatory adjuvant or a combination of adjuvants. Blood and muscle tissue from the site of injection were harvested from all mice in every group at 48 hours post-injection.

In objective 3, one hundred and twenty BALB/c mice (n = 8 per group) were randomly allocated into eight groups with sixteen mice per group. On Day 0, eight naïve mice were euthanized and their spleens harvested. The remaining mice were immunized with OVA experimental antigen and the various immunostimulatory adjuvants. Mice were bled on day 0, 14, 21 and 35. On day 21, 8 mice from each group were euthanized, and had their spleen harvested. The 8 remaining mice from each group received a booster immunization on day 21 and were euthanized on day 35 and their spleens were collected.

4.2 Adjuvants

The adjuvants used in this study were Alum, CpG ODN 1826, Curdlan, Leptin, Lipopolysacchride (LPS), PCEP (poly[di(sodiumcarboxylatoethyl-phenoxy)phosphazene]). A list of adjuvants used and their sources are listed on Table 4-1. PCEP was synthesized by Idaho National Laboratory (Idaho Falls, ID, USA) using methods described previously (Andrianov et al., 2004; Mutwiri et al., 2007a) and, prior to use, its endotoxin levels were determined to be less than 0.034 ng/ml as assessed by the Limulus Amebocyte Lysate assay (Biowhittaker, Walkersville, MD, USA). The adjuvant CpG ODN 1826 was class B, (5'-TCCATGACGTTCCTGACGTT-3') and contains a full phosphorothioate backbone [Merial (Lyon, France)].

4.3 Spleen Cell Isolation

Spleens were placed into Minimal Essential Media (MEM) (Sigma Life Science) containing 10 mM HEPES (Lifetech, ThermoFisher Scientific, Waltham, Massachusetts, USA) and 50 U/mL Penicillin/Streptomycin (Gibco, ThermoFisher) and kept on ice, then minced and pushed through a 0.2 μm cell strainer using the end of a sterile glass syringe. Splenocytes were centrifuged at 350 x g for 10 minutes at 10 $^{\circ}$C. Supernatant was discarded, pellet disrupted by knocking tubes together, 1 mL Gey's solution (Red blood cell lysis buffer; ($CaCl_2$ (0.220 g), KCl (0.370 g), KH_2PO_4 (0.03 g), $MgCl_2$ (0.210 g, $MgSO_4$ (0.070 g), NaCl (8.000 g), $NaHCO_3$ (0.227 g), Na_2HPO_4 (0.120 g), D-glucose (1.000 g) in 1 L distilled water) (Sigma-Aldrich)) was added to the cells and incubated at room temperature (RT) for 10 minutes [147]. Cells were centrifuged at 350 x g for 10 minutes at 10 $^{\circ}$C after the volume was adjusted to 10 mL with PBS. Supernatant was

discarded and AIM V (Gibco) containing 10 % Fetal Bovine Serum (FBS) (Gibco), 50 U/mL Penicillin/Streptomycin (Gibco) and 0.1 μM β-mercaptoethanol (Gibco) was added to the cells, centrifuged at 350 x g for 10 minutes at 10 °C and resuspended in AIM V 10 % media again. Cells were counted using hemocytometer under standard protocol by diluting 1:4 in Trypan Blue (Gibco).

4.4 Cell culture and stimulation

Splenocyte numbers were adjusted to a concentration of 1.0 $x10^6$ cells/well ($1.0x10^7$ cells/mL) and introduced into 96 well tissue culture plates (Thermofisher) at 100 μL. They were incubated in a 37 °C, 5 % CO_2 incubator for 1 hour to stabilize. Concentrations for immunostimulatory adjuvants for the *in vitro* and *in vivo* work are detailed in Table 4-1. Adjuvants in AIM V 10 % were added at 100 μL as a single, double or triple combination and incubated for 48 hours [68] before supernatants were collected and frozen at - 20 °C until used (Figures 5-1, -2 and -3 of the results section).

Table 4-1. Information for adjuvants, their manufacturer and the concentrations used of these adjuvants in the *in vitro*, injection and immunization studies.

Adjuvant	Manufacturer	*in vitro/ex vivo* (μg/mL)	Immunizations (μg/mL)
Alum	Imject Alum, Thermo Scientific	40	40
CpG-ODN 1826	Coley Pharmaceuticals Group (MA, USA)	1, 5	10
Curdlan	Curdlan, Wako Pure Chemical Industry, Ltd. Japan	1	10
Leptin	Sigma Life Science	1	10
LPS (E55:B5)	Sigma Life Science	1, 5	10
PCEP	Idaho National Laboratories	5, 25, 50, 125	50

4.5 Adjuvant Injection Study

Six to eight week old female BALB/c mice were ordered one week prior to injection study so that there were six mice per injection group. Mice were randomly placed into cages by Animal Care where they were allowed to acclimatize for a week prior to the study. No antigen was used in this study, only adjuvants diluted in PBS at pH 7.4 (Gibco). Injections were administered to BALB/c mice per cocktail group at 25 μL per semi-membranosus muscle of each mouse for a total of 50 μL. Mice were euthanized 48 hours after injections were administered (Figure 5-4 of the results section).

4.6 Muscle Tissue Homogenization

Forty-eight hours post-injection, the muscle tissue from the two sites of injection were excised from each mouse and placed into a 2 mL micro tube (VWR, North America) with 2.4 mm zirconia beads (Biospec Products, Inc.). The cells were homogenized in a MINI Bead Beater (Biospec Products, Inc.) at 48 (4800 osc/sec) for 10

seconds to disrupt the tissue and lyse cells. The 2 mL centrifuge tubes and all contents were frozen at - 20 °C such that when they are thawed in the future, we anticipate that the remaining intact cells may be lysed. At this time, the tubes were centrifuged (Sorvall Legent RT, Mandel) at 10, 000 x g for 5 minutes and the supernatant was removed and placed into PCR microplates on ice (Axygen Scientific) for ELISA analysis (Figure 5-4 of the results section).

4.7 Immunization Study

Six to eight week old BALB/c mice were randomly assigned to an immunization group (n = 8 per group). The vaccines were comprised of 10 μg/mL Lyophilized Ovalbumin (Endograde, Hyglos), the antigen, with immunostimulatory adjuvants (concentrations are detailed in Table 4-2). Mice were vaccinated on Day 0 with 25 μL into both semi-membranosus muscles of each mouse for a total of 50 μL. An i.m. booster was administered into the semi-membranosus muscles with the same quantity as the primary immunization on Day 21 and mice were euthanized on Day 35 to harvest spleens. Blood sera were collected into SST tubes (BD Biosciences) from tail bleeds for further analysis on Day 0, 14, 21 and 35. SST tubes were centrifuged at 2000 x g for 20 minutes then the supernatant was removed and placed into a 96 well PCR plates and frozen at -20 °C until needed (Figure 5-5, -6 and -7 of the results section).

Table 4-2. Immunization conditions for each treatment group. Antigen and adjuvant concentrations, which were diluted into 2 mL PBS (Gibco) and administered at 25 μL per semi-membranosus muscle for a total of 50 μL per mouse**.**

Group	Immunization Dose
A	Naïve
B	OVA 10 μg/mL
C	OVA 10 μg/mL + 50 μg/mL PCEP
D	OVA 10 μg/mL + 10 μg/mL Curdlan
E	OVA 10 μg/mL + 10 μg/mL Leptin
F	OVA 10 μg/mL + 50 μg/mL PCEP + 10 μg/mL Curdlan
G	OVA 10 μg/mL + 10 μg/mL Curdlan + 10 μg/mL Leptin

4.8 Cytokine Detection in Mice (ELISA)

ELISA- R&D Systems Duoset kits (Fisher Scientific): IL-1β, IL-4, IL-6, IL-10, IL-17, IL-21 and IL-23, IFNγ and TGF-β were used throughout this study as per protocol provided by R&D Systems, Fisher Scientific. IL-12 was purchased from BD Biosciences and used exactly as protocol from the R&D Duoset kits. All assays were performed on Immulon II, 96 well microtiter plates (VWR), quantified with a Spectramax Plus384 (Molecular Devices) and analyzed on Softmax Pro 5.2 (Molecular Devices). ELISA values were calculated with respect to the ELISA threshold limit of detection for each individual cytokine assay, which can be seen in Table 4-3.

Table 4-3. Threshold of detection for cytokines in ELISAs based on the cytokines in each figure.

Figures	Cytokines	Threshold of detection in pg/mL
Figure 5-1	IL-1β	310
	IL-6	160
	IL-12	390
	IL-17	470
	IL-23	390
	TGF-β	310
Figure 5-2	IL-1β	310
	IL-6	160
	IL-12	390
	IL-17	470
	IL-23	390
	TGF-β	310
Figure 5-3	IL-6	150
	IL-12	780
	IL-17	370
	TGF-β	310
Figure 5-4	IL-6	150
	IL-12	780
	IL-17	370
	TGF-β	310
Figure 5-5	IFNγ	310
	IL-4	310
	IL-10	625
	IL-17	500

4.9 Mouse T Cell Phenotyping through intracellular flow cytometry

After completing mouse spleen cell isolation on immunized mice, 100 μL of $1.0x10^6$ cells/well were added to 96-well tissue culture plates and then stimulated with OVA at 1 μg/mL or mock stimulated with 100 μL AIM V 10 % media alone. Restimulated cells were incubated at 37 $^{\circ}$C in a 5 % CO_2 incubator for 48 hours, at which point 20 μL of Monensin (BD Bioscience), a Golgi blocker, was pipetted into each well

for 8-12 hours. Cells were centrifuged at 500 x g for 4 minutes at RT, the supernatant was removed by flicking the plates then 200 µL flow cytometry buffer (FCM Buffer; FA COLA or PBSG; 1.5 g Sodium Azide and 10.0 g Gelatin into 5 L PBS pH 7.3) was added to wash the cells. The cells were centrifuged 500 x g for 4 minutes at RT and this wash was repeated twice. A volume of 100 µL of Fix/Perm Solution (BD Bioscience) was added to each well and plates were placed at 4 $^{\circ}$C for a minimum of 30 minutes to permeablize the splenocytes. Cells were centrifuged at 500 x g for 4 minutes at RT and washed twice with Perm Wash (BD Bioscience). Supernatants were removed by flicking, then 10 µL of pre-diluted antibody (seen in Table 4-3) was added to each well and the plates were nutated and placed in the dark at RT for 10 minutes. Along with the cocktail stains used in this study, single, fluorochrome minus one (FMO), isotype, negative and positive control stains were used in this analysis and were added to wells at the same time as the cocktail stains. This study used 3 different cocktails to observe different T cells or mixed responses, the cocktails can be seen in Table 4-4 and individual antibodies in Table 4-5. After 10 minutes in the dark, cells were washed with 200 µL of Perm Wash and centrifuged at 500 x g for 4 minutes at RT and resuspended in 200 µL FCM buffer. Cells were measured on FacsCalibur (BD Biosciences Bio-Rad Laboratories) and CellQuestPro by acquiring 100,000 cells/sample. All data from FCM was analyzed on Kaluza (Beckman Coulter Life Sciences) and Graphpad prism with an example of the gating strategy shown in Figure 4-1.

Table 4-4. Flow cytometry fluorochrome cocktails. All Antibodies were Rat, anti-mouse antibodies directly conjugated to fluorochromes (BD Pharmingen).

Cocktail	*Fluorochrome conjugated antibodies
Cocktail 1 (C1)	CD4-Cy5.5, IL-4-Alexa Fluor 647, IL-17-PE and IFNγ-FITC
Cocktail 2 (C2)	CD4-Cy5.5, IL-17-PE and Foxp3-Alexa Fluor 647
Cocktail 3 (C3)	CD4-Cy5.5, CD8-PE and IFNγ-FITC

* All fluorochrome conjugated antibodies were purchased from BD Pharmingen.

Table 4-5. Antibody fluorochrome chart for intracellular FCM performed on mouse spleen cells from immunization study (BD Pharmingen). Concentrations were made from stock to be administered per well or sample.

Antibodies specific for:	Fluorescence Channel	Name	Isotype	Concentration of antibodies used per well
CD4	FL3	PerCP-Cy™5.5 Rat Anti-Mouse CD4 Clone RM4-5	Rat (DA) IgG2a, κ	0.0625 μg/well
CD8	FL2	PE Rat Anti-Mouse CD8b.2 Clone 53-5.8	Rat (LOU) IgG1, κ	0.25 μg/well
Foxp3	FL4	Alexa Fluor® 647 Rat Anti-mouse Foxp3 Clone MF23	Rat IgG2a, κ	0.25 μg/well
IL-4	FL4	APC Rat Anti-Mouse IL-4 Clone 11B11	Rat IgG1	0.25 μg/well
IL-17	FL2	PE Rat Anti-Mouse IL-17A ClonTC11-18H10	Rat IgG1 , κ	0.25 μg/well
IFNγ	FL1	FITC Rat Anti-Mouse IFNγ Clone XMG1.2	Rat IgG1, κ	0.25 μg/well

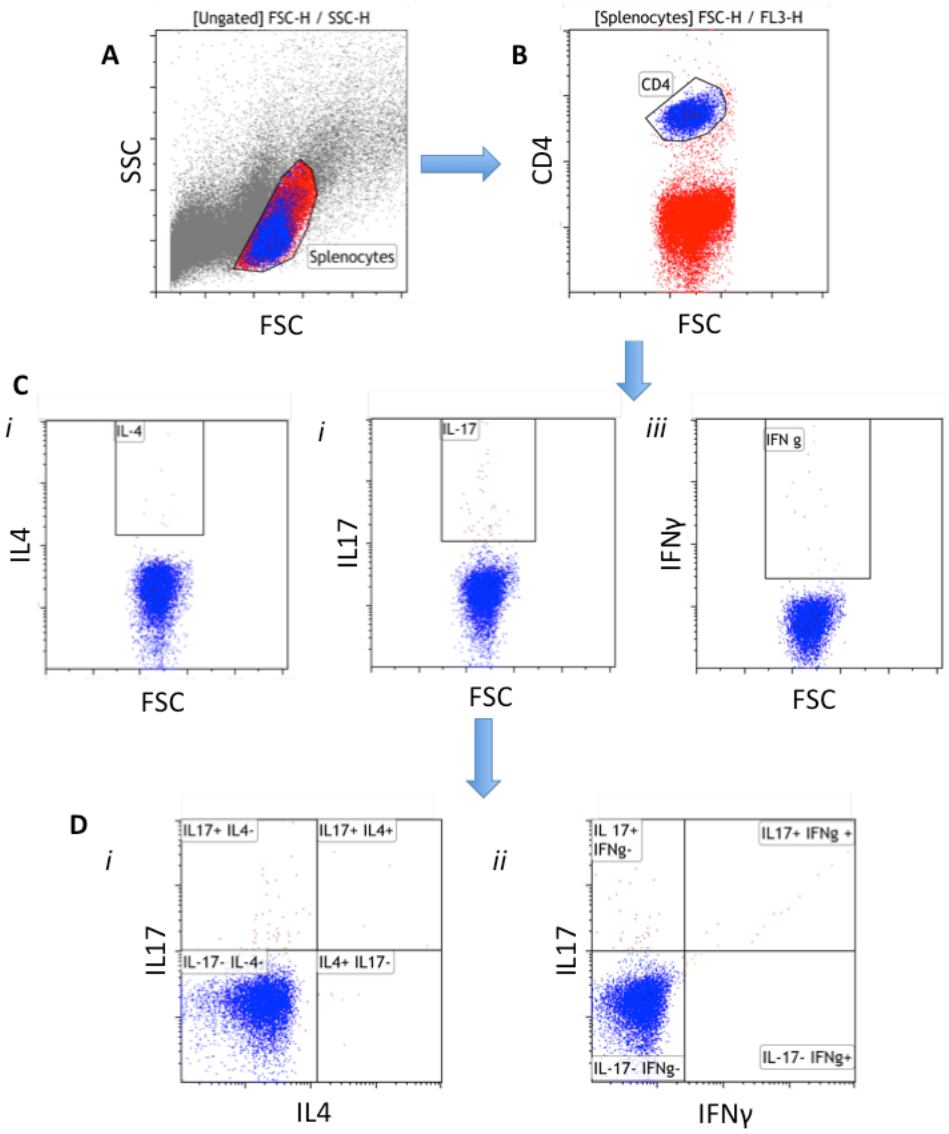

Figure 4-1. Intracellular FCM gating strategy example of cocktail 1. FCM was used to identify quantities of different T cell subsets within a spleen cell population from immunized BALB/c after a 48 hour incubation with OVA. **A** portrays the live primary splenocyte gating from total population where cells are presented as side scatter (SSC) representing cell granularity and forward scatter (FSC) representing the size of cells. **B** portrays the gating of $CD4^+$ cells from within the splenocyte gate. **C** portrays gating of intracellular cytokines i: IL-4, ii: IL-17 and iii IFNγ with the $CD4^+$ gate. **D** gating was used to identify any $CD4^+$ cells that may be producing multiple cytokines, which may give evidence of a mixed response or mixed T cell subset (i.e. Th1/Th17) i: $CD4^+$ IL-17 and IL-4 and ii: $CD4^+$ IL-17 and IFNγ, these results are based on the CD4 gate.

4.9.1 Statistical Analysis

Statistical analyses were carried out using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences in the ELISA cytokine production were identified using a non-parametric Kruskal-Wallis ANOVA test where Dunn's multiple comparisons test was used post-hoc to identify statistically significant differences in cytokine production (Figure 5-1, 5-2, 5-3, 5-4 and 5-5 in the results section). Differences in the FCM cytokine expression from $CD4^+$ and $CD8^+$ T cells were identified using a non-parametric Kruskal-Wallis ANOVA test where Dunn's multiple comparisons test was used post-hoc to identify significant differences in OVA restimulated splenocytes expressing cytokine production as well as a Wilcoxon method t test between OVA restimulated and unstimulated (media) splenocytes within the immunization group (Figure 5-6 and 5-7 in the results section). Differences were presented with an asteryx (*) or through a letter system where all groups with the same letter are not significant from one another and those groups containing different letters are significant. Differences were considered statistically different if p value <0.05.

CHAPTER 5 Results

5.1 Cytokine expression after *in vitro* stimulation of murine spleen cells with adjuvants

We sought to identify which adjuvant(s) (Alum, CpG, Curdlan, Leptin, LPS and PCEP; Table 4-1) alone or in combination would stimulate production of IL-1β, IL-6, IL-17, IL-23 and TGF-β cytokines that may promote Th17-type immunity or the cytokine IL-12, that may inhibit or decrease Th17 cell stability and differentiation in murine splenocytes. Multiple experiments were performed on different groups of adjuvants to determine optimal cytokine expression on treated mouse splenocytes *in vitro*.

In figure 5-1, splenocytes from naïve mice were stimulated with varying doses (5, 25, 50 and 125 μg/mL) of PCEP or media (mock-stimulation). ELISA cytokine analysis of IL-1β, IL-6, IL-12, IL-17, IL-23 and TGF-β expression (Figure 5-1) from mice splenocytes stimulated PCEP were performed on the supernatants after 48 hours. IL-1β (Figure 5-1A) was expressed at significantly higher levels by splenocytes cultured with media alone compared to the different PCEP concentrations, which suggests that PCEP inhibits IL-1β cytokine expression. Similar to IL-1β, IL-12 (Figure 5-1C) was expressed at higher levels in splenocytes with media alone compared to the PCEP treatments, which was significant with relation to PCEP 5 and 125 μg/mL. No concentrations of PCEP were able to significantly induce expression of cytokines, IL-6, IL-17, IL-23 and TGF-β, associated with Th17-type differentiation. Therefore, PCEP at the concentrations tested, does not induce significant expression of cytokines associated with a Th17-type cellular differentiation.

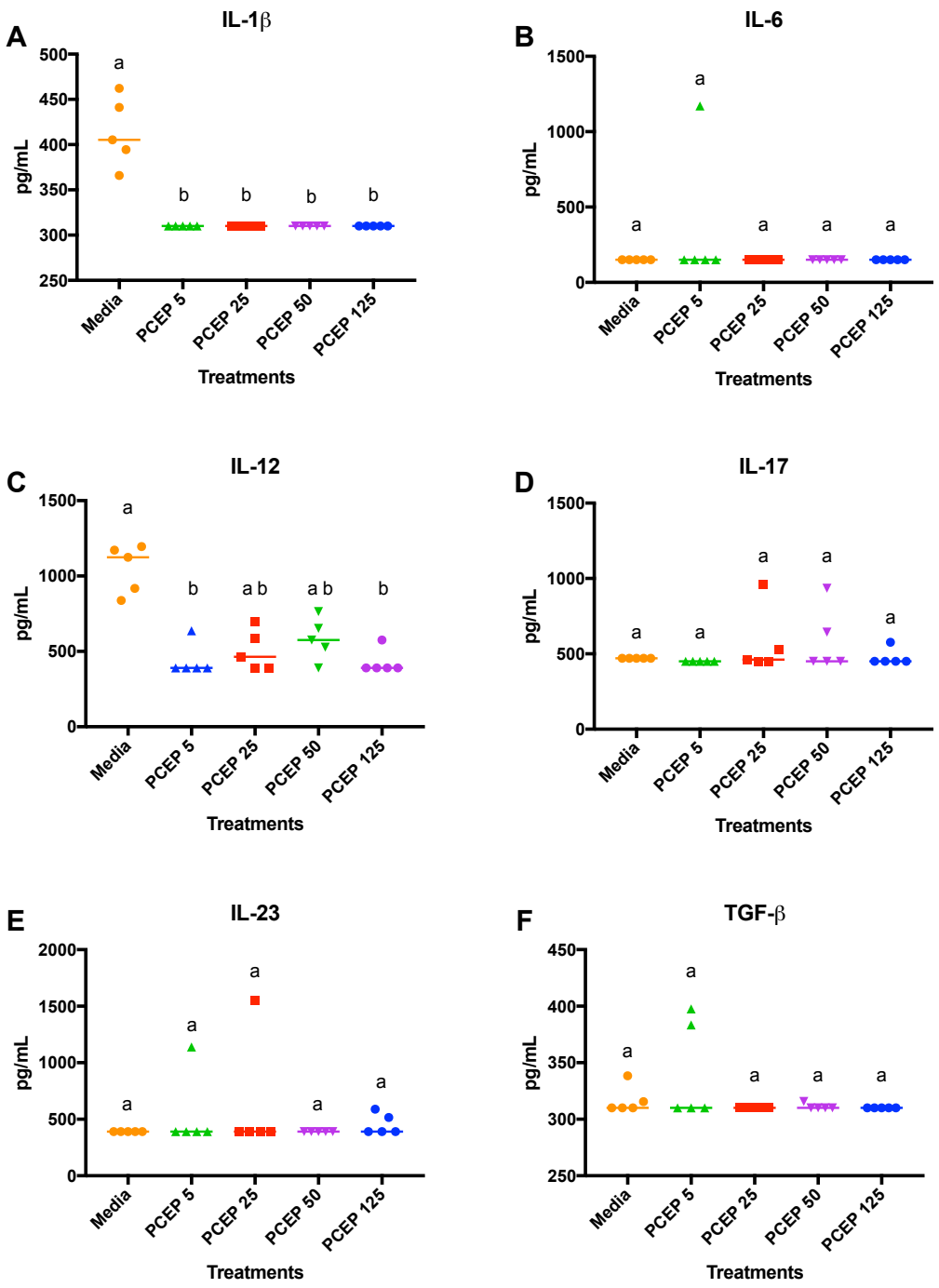

Figure 5-1. Titration of PCEP for optimal induction of IL-1β, IL-6, IL-12, IL-17, IL-23 and TGF-β cytokine expression. Spleen were harvested from BALB/c mice (n = 5) and splenocytes isolated and incubated at 1.0 x 10^6 cells/well for *in vitro* incubation with PCEP at concentrations of 5, 25, 50 and 125 µg/mL. Supernatant was collected 48 hours after treatment and analyzed through ELISA with cytokine concetrations displayed as pg/mL.

Figure 5-2 displays an ELISA analysis of IL-1β, IL-6, IL-12, IL-17, IL-23 and TGF-β expression in mice splenocytes treated with CpG, PCEP, Alum, LPS, PCEP + CpG, PCEP + Alum, PCEP + LPS, PCEP + LPS + Alum, and PCEP + CpG + LPS or mock-stimulated cells for 48 hours. PCEP, PCEP + LPS + Alum and PCEP + CpG + LPS, did not induce significant IL-1β expression (Figure 5-2A) relative to splenocytes mock-stimulated with media. LPS and PCEP + LPS treatments induced significant IL-1β expression in splenocytes relative to splenocytes stimulated with PCEP, PCEP + LPS + Alum and PCEP + CpG + LPS. IL-6 (Figure 5-2B) was significantly expressed by splenocytes treated with PCEP + CpG and PCEP + CpG + LPS relative to splenocytes mock-stimulated with media as well as PCEP, PCEP + Alum and PCEP + LPS + Alum. Furthermore, PCEP + CpG + LPS treatment induced significant IL-6 expression relative to Alum. PCEP + CpG + LPS did not induce significant IL-6 expression relative to PCEP + CpG suggesting that the triple combination was not more effective than the double adjuvant combination, PCEP + CpG relative to untreated cells in inducing IL-6 expression in splenocytes (cells in media alone). In short, PCEP + CpG were just as effective in inducing IL-6 expression as PCEP + CpG + LPS. Adjuvants and adjuvant combinations that were able to significantly induce IL-12 (Figure 5-2C) expression in splenocytes were CpG, PCEP + CpG and PCEP + CpG + LPS relative to PCEP and PCEP + LPS + Alum. Although CpG was not able to induce significant expression of IL-12 relative to media, PCEP + CpG or PCEP + LPS + Alum, it had a much higher concentration than all treatment groups, which suggests that CpG alone may be sufficient for inducing splenocytes to express IL-12 *in vitro*. IL-17 (Fig 5-2D) was not significantly expressed by cells treated with any of the adjuvants alone or in combination. However,

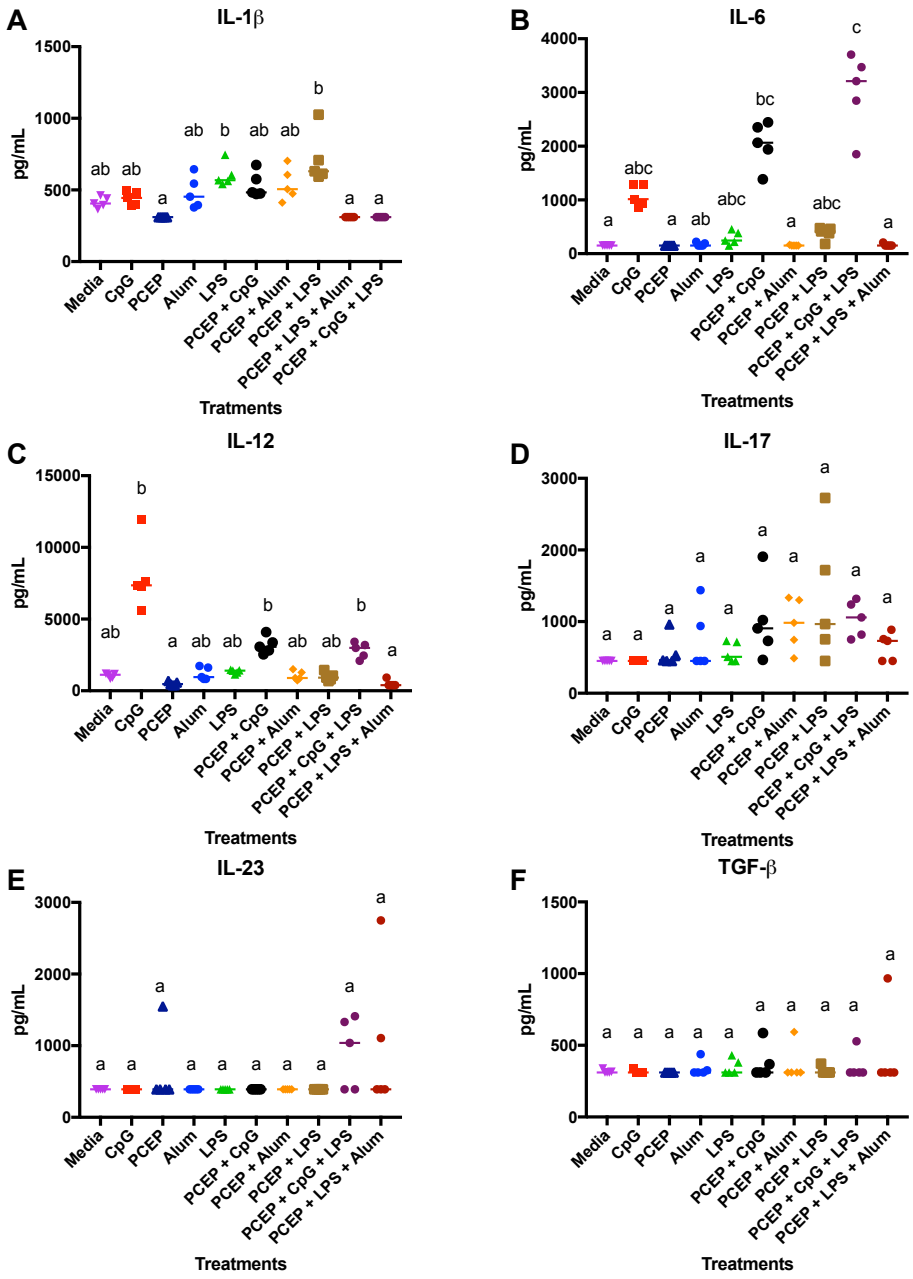

Figure 5-2. Expression of IL-1β, IL-6, IL-12, IL-17, IL-23 and TGF-β under single, double and triple adjuvant treatment conditions. Spleens were harvested from BALB/c mice (n = 5 minimum) and splenocytes isolated and incubated at 1.0 x 10^6 cells/well for *in vitro* culture with CpG (5 µg/mL), PCEP (25 µg/mL), Alum, (40 µg/mL) LPS (5 µg/mL), PCEP + CpG (25 + 5 µg/mL, respectively), PCEP + Alum (25 + 40 µg/mL, respectively), PCEP + LPS (25 + 5 µg/mL, respectively), PCEP + CpG + Alum (25 + 5 + 40 µg/mL, respectively) and PCEP + CpG + LPS (25 + 5 + 5 µg/mL, respectively). Supernatant was collected 48 hours after treatment and analyzed through ELISA displayed as pg/mL.

there were multiple high responders within the PCEP + CpG, PCEP + Alum, PCEP + LPS, PCEP + CpG + LPS and PCEP + LPS + Alum treatment groups. IL-23 and TGF-β (Figure 5-2E and 5-2F, respectively) were not significantly expressed by splenocytes from any of the treatment groups and there were very few individuals/samples responding to the different treatments. Therefore, all of the adjuvants and their combinations tested in this study did not induce cytokines associated with the development of a Th17-type of immune response.

As the previous adjuvants did not induce a cytokine expression profile that promotes a Th17-type of immune responses, we added Curdlan, Leptin and their combinations because these two immunostimulants have been observed to induce signals for inducing Th17 cell differentiation through TLRs and syk-CARD9 [86, 93]. We also eliminated the IL-1β and IL-23 ELISAs as they only indirectly influence Th17 cell differentiation.

Figure 5-3 displays ELISA analysis of murine splenocytes stimulated with CpG, PCEP, Leptin, Curdlan, PCEP + Leptin, PCEP + Curdlan, PCEP + CpG + Leptin, PCEP + Curdlan + Leptin, CpG + Curdlan + Leptin as well as mock-stimulated for 48 hours and then IL-6, IL-12, IL-17 and TGF-β cytokines were quantified.

IL-6 (Figure 5-3A) was significantly expressed by splenocytes stimulated with PCEP + CpG + Leptin relative to splenocytes in media. CpG, PCEP + CpG + Leptin and CpG + Curdlan + Leptin were able to induce splenocytes to express significant IL-6, relative to PCEP + Curdlan + Leptin. CpG and PCEP + CpG + Leptin also induced splenocytes to express significantly more IL-6 relative to PCEP, PCEP + Leptin, PCEP + Curdlan and Curdlan + Leptin.

IL-12 (Figure 5-3B) was significantly expressed by splenocytes treated with CpG and CpG + Curdlan + Leptin relative to splenocytes in media. CpG and CpG + Curdlan + Leptin were also able to induce splenocytes to significantly express IL-12 relative to splenocytes stimulated with PCEP, PCEP + Leptin, PCEP + CpG + Leptin, PCEP + Curdlan and PCEP + Curdlan + Leptin. Splenocytes treated with Curdlan were also able to induce IL-12 expression relative to PCEP and PCEP + Leptin. CpG + Curdlan + Leptin was not able to induce splenocytes to significantly express IL-12 relative to CpG, concluding that CpG alone is just as effective as the triple combination in inducing IL-12 expression. IL-12 has the ability to switch from Th17 cells into Th1 or Th1/Th17 cells so an adjuvant combination that does not induced IL-12 secretion may be desirable when inducing a Th17-type immune response.

IL-17 (Figure 5-3C) was significantly expressed by splenocytes treated with Curdlan + Leptin as well as PCEP + Curdlan + Leptin relative to splenocytes in media alone. Curdlan + Leptin and PCEP + Curdlan + Leptin were also able to induce significant IL-17 expression relative to splenocytes stimulated with CpG, PCEP + CpG + Leptin, Curdlan, PCEP + Curdlan and CpG + Curdlan + Leptin. The triple combination, PCEP + Curdlan + Leptin, did not induce significant expression of IL-17 relative to Curdlan + Leptin, indicating that the double adjuvant combination is just as effective in IL-17 expression as the triple combination.

Lastly, Curdlan, PCEP + Curdlan and CpG + Curdlan + Leptin were able to induce splenocytes to significantly induce expression of TGF-β (Figure 5-3D) relative to media alone. Curdlan and PCEP + Curdlan treated splenocytes were also able to induce TGF-β expression relative to PCEP + Leptin and PCEP + CpG + Leptin. CpG + Curdlan

+ Leptin was also able to induce significant TGF-β expression relative to CpG, PCEP, Leptin, PCEP + Leptin and PCEP + CpG + Leptin.

It was expected that IL-6 and TGF-β would be expressed by similar adjuvants that promote Th17 cell differentiation characteristics. However, we observed that IL-6 and IL-12 were both produced by CpG alone or in combination with other adjuvants. This is undesirable, as IL-12 is known to promote a switch in differentiation from Th17 to Th1. Curdlan + Leptin in the presence or absence of PCEP were shown to induce expression of IL-17 although no adjuvant combination induced expression of both IL-6 and TGF-β.

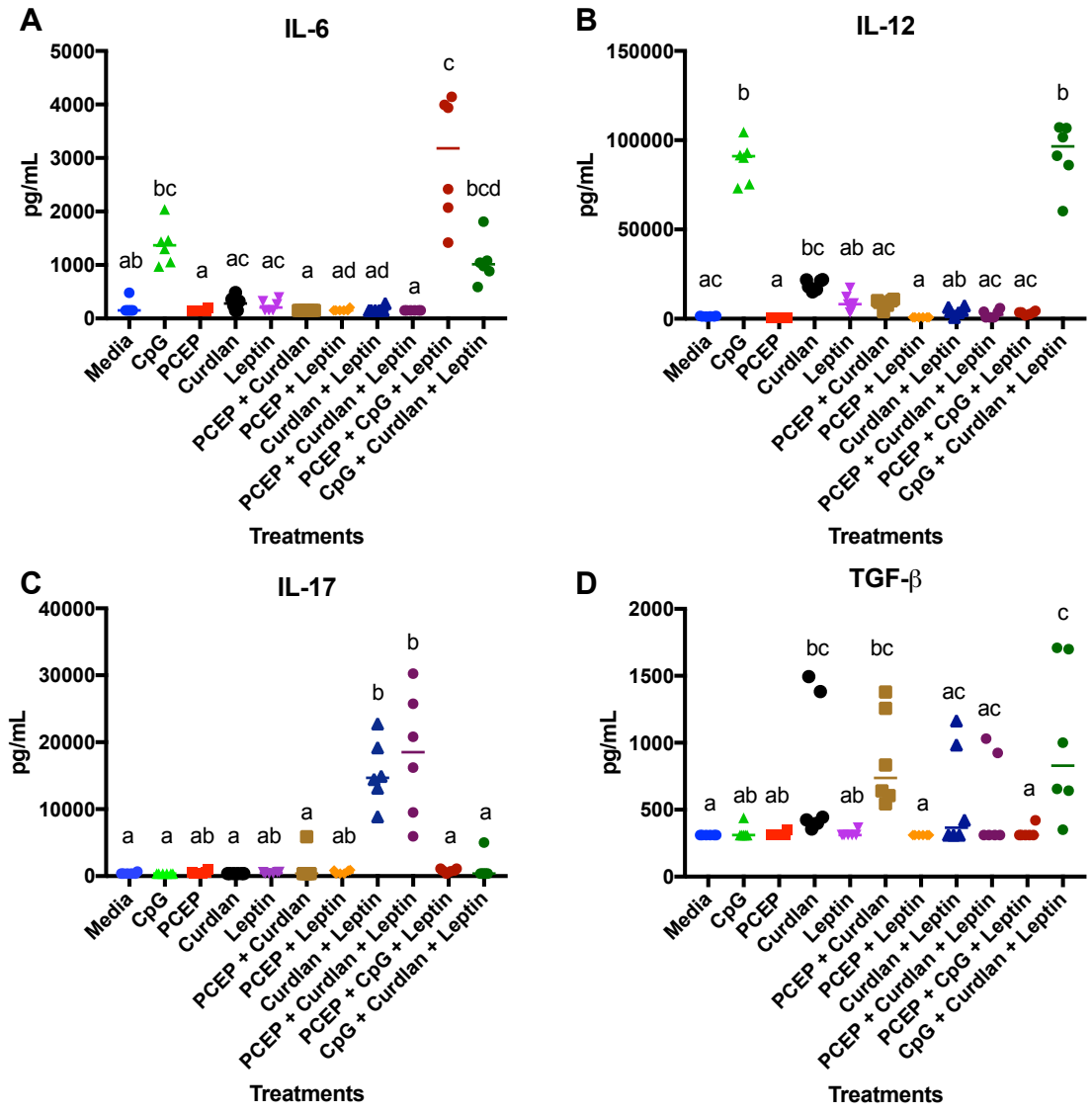

Figure 5-3. Expression of IL-6, IL-12, IL-17 and TGF-β under single, double and triple adjuvant treatment conditions. Spleen were harvested from BALB/c mice (n = 6) and splenocytes isolated and incubated at 1.0 x 10^6 cells/well for *in vitro* treatment with CpG (5 µg/mL), PCEP (25 µg/mL), Leptin (1 µg/mL), PCEP + Leptin (25 + 1 µg/mL, respectively), PCEP + CpG + Leptin (25 + 5 + 1 µg/mL, respectively), Curdlan (1 µg/mL), PCEP + Curdlan (25 + 1 µg/mL, respectively), Curdlan + Leptin (1 + 1 µg/mL, respectively), PCEP + Curdlan + Leptin (25 + 1 + 1 µg/mL, respectively), CpG + Curdlan + Leptin (5 + 1 + 1 µg/mL, respectively). Supernatant was collected 48 hours after treatment and analyzed through ELISA displayed as pg/mL.

5.2 Local cytokine production in a murine model after *ex vivo* stimulation of intramuscular injected adjuvants

Next, using adjuvants in Objective 1, we investigated what cytokines would be locally expressed in murine muscle tissues after injection with CpG, PCEP, LPS, Alum, Curdlan, Leptin, PCEP + CpG, PCEP + LPS, PCEP + Alum, PCEP + Curdlan, PCEP + Curdlan + Leptin, CpG + Curdlan + Leptin or PBS (See Table 4-1 for concentrations).

IL-6 (Figure 5-4A) expression was significantly induced at the site of injection by PCEP, CpG, PCEP + CpG and PCEP + LPS injections relative to injections of PBS alone. IL-6 expression was also significantly induced by the PCEP, CpG, PCEP + CpG and PCEP + LPS injections relative to Curdlan, Leptin and Curdlan + Leptin. PCEP + CpG were not able to induce significant IL-6 expression at the site of injection relative to the single PCEP and CpG adjuvant injections, showing us that single adjuvants PCEP and CpG are just as effective as the double combination at inducing IL-6 expression.

IL-12 (Figure 5-4B) expression was significantly induced at the site of injection with Curdlan + Leptin relative to the PBS injection group. IL-12 was also significantly expressed by Curdlan + Leptin relative to the PCEP, LPS, PCEP + LPS, Alum and PCEP + Curdlan injection groups which may mean these adjuvants may promote Th1-type cell differentiation over Th17-type cell differentiation.

IL-17 (Figure 5-4C) expression was induced by an injection of Curdlan + Leptin relative to the PBS injection as well as to the CpG, PCEP + CpG, LPS, PCEP + LPS, Alum and PCEP + Alum injection groups. Injections of Curdlan, PCEP + Curdlan, Leptin, PCEP + Curdlan + Leptin and CpG + Curdlan + Leptin were able to induce

significant IL-17 expression at the site of injection relative to CpG, LPS, PCEP + LPS and Alum.

Lastly, TGF-β (Figure 5-4D) expression was significantly induced at the site of injection in response to Curdlan + Leptin relative to the mice injected with PBS as well as LPS.

As with the *in vitro* data, we observed that the combination of Curdlan + Leptin was able to induce significant expression of IL-17 at the site of injection, which may indicate Th17 cell differentiation but due to the short time period it is likely that IL-17 expression represents activation of effector Th17 or other cells like monocytes/macrophages, which also have the ability to express IL-17 and are present in the muscle [148]. From here, we moved onto an immunization study to determine if there was an antigen-specific Th17-type of immune response using adjuvants previously observed to promote IL-17 expression.

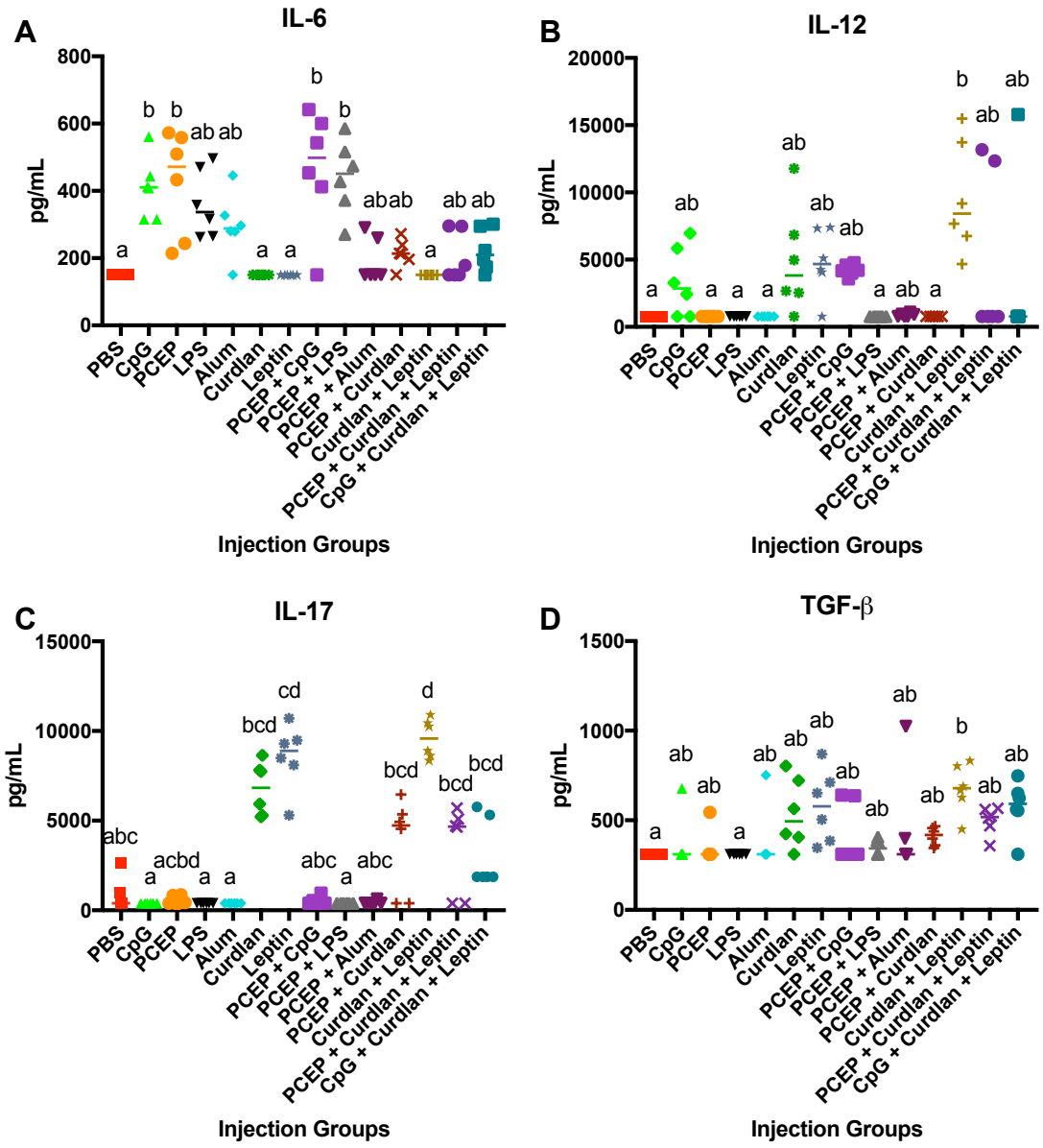

Figure 5-4. Expression of IL-6, IL-12, IL-17 and TGF-β at the site of injection. BALB/c mice (n = 6) were injected with PBS alone and adjuvants diluted in PBS: PCEP (50 µg/mL), CpG (10 µg/mL), LPS (10 µg/mL), Alum (40 µg/mL), Curdlan (10 µg/mL), Leptin (10 µg/mL), PCEP + CpG (50 + 10 µg/mL, respectively), PCEP + LPS (50 + 10 µg/mL, respectively), PCEP + Alum (50 + 40 µg/mL, respectively), PCEP + Curdlan (50 + 10 µg/mL, respectively), Curdlan + Leptin (10 + 10 µg/mL, respectively), PCEP + Curdlan + Leptin (50 + 10 + 10 µg/mL, respectively) and CpG + Curdlan + Leptin (10 + 10 + 10 µg/mL, respectively). Muscle tissue was excised 48 hours after injection and subjected to ELISA analysis to identify cytokine expression at the site of injection displayed as pg/mL.

5.3 Murine cytokine expression post-immunization with Ovalbumin and single or combinational adjuvants

After quantifying cytokine expression *in vitro* and at the site of injection, we concluded that Curdlan + Leptin and PCEP + Curdlan + Leptin were able to induce IL-17 expression *in vitro* and Curdlan + Leptin was able to induce IL-17 expression at the site of injection. Thus, we decided to use PCEP, Curdlan, Leptin alone and in combinations in our immunization study to assess Th17-type cell differentiation.

We immunized mice with vaccines formulated with OVA alone, PCEP + OVA, Curdlan + OVA, Leptin + OVA, PCEP + Curdlan + OVA, Curdlan + Leptin + OVA or left mice unimmunized (naïve). Mice received identical booster immunizations on Day 21 and we harvested spleens on Day 35. ELISA results from the splenocytes, which were mock-stimulated or restimulated with OVA from all immunization groups are shown in Figure 5-5 for induction of IFNγ, IL-4, IL-10 and IL-17, which are related to the presence of Th1, Th2, Tregs and Th17 cells, respectively. Flow cytometry data from splenocytes, mock-stimulated or restimulated with OVA in Figure 5-6, shows the frequency of $CD4^+$ T cells that express IFNγ, IL-4, IL-10 or IL-17, indicative of Th1, Th2, Tregs and Th17-type cell subsets, respectively. In Figure 5-7, splenocytes were gated for $CD4^+$ or $CD8^+$ cells expressing IFNγ to identify whether IFNγ was produced primarily from Th1 cells or CTLs.

Cytokine ELISA results from the immunization trial showed that no vaccine, even with adjuvant combinations, induced significant OVA-specific induction of IFNγ (Figure 5-5A), IL-4 (Figure 5-5B) or IL-10 (Figure 5-5C). IL-17 (Figure 5-5D) expression was

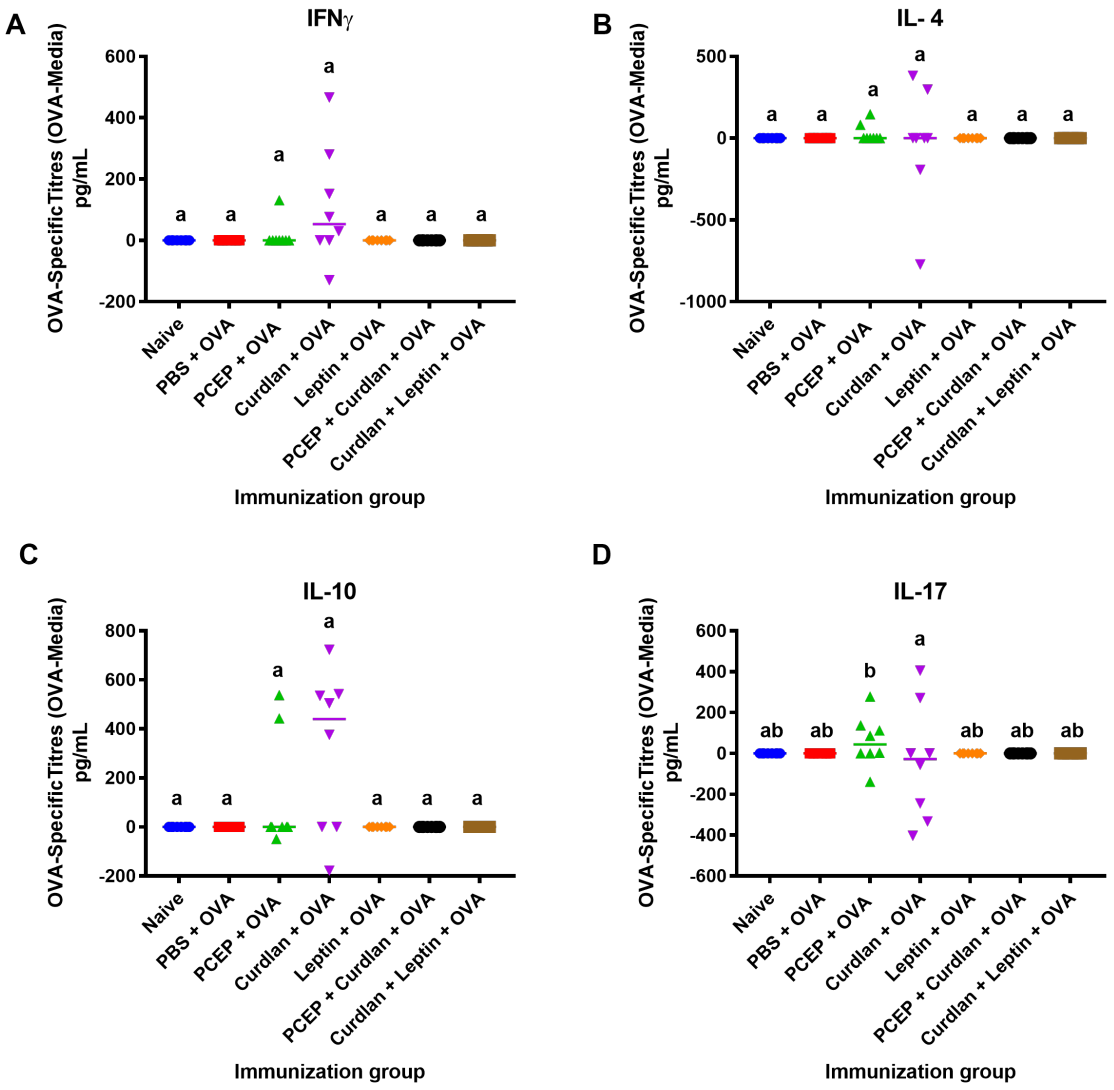

Figure 5-5. Expression of IFNγ, IL-4 IL-10 and IL-17 after OVA restimulation in immunization groups. BALB/c spleen were harvested (n= 8) and splenocytes isolated from all mice, naïve and immunized with OVA and OVA + Adjuvants: Naïve (no immunization), PBS + OVA (10 µg/mL), PCEP + OVA (50 + 10 µg/mL, respectively), Curdlan + OVA (10 + 10 µg/mL, respectively), Leptin + OVA (10 + 10 µg/mL, respectively), PCEP + Curdlan + OVA (50 + 10 + 10 µg/mL, respectively) and Curdlan + Leptin + OVA (10 + 10 + 10 µg/mL, respectively). Cells from these mice were mock-stimulated with media alone as well as restimulated with OVA (1 µg/mL), incubated at 1.0 x 10^6 cells/well for 48 hours and analyzed through ELISA in pg/mL. OVA-specific titres were calculated by subtracting titres from unstimulated splenocytes from the OVA restimulated splenocytes.

significantly induced in splenocytes from mice immunized with PCEP + OVA relative to the mice immunized with Curdlan + OVA, although it was insignificant with respect to the OVA immunization group presumably due to the highly variable responses.

Using flow cytometry analysis, we quantified the percentage of $CD4^+$ splenocytes that expressed IFNγ, IL-4, IL-17 or Foxp3 in response to OVA restimulation (Figure 5-6). Neither IFNγ nor IL-4 (Figure 5-6A and Figure 5-6B, respectively) were expressed by $CD4^+$ splenocytes in response to OVA from any of the vaccine formulations.

Immunization with PCEP + OVA induced a significant increase in the frequency of OVA-specific $CD4^+IL17^+$ splenocytes (Figure 5-6C) relative to mice immunized with OVA alone, and PCEP + Curdlan + OVA. Immunization with Curdlan + Leptin + OVA induced significantly more OVA-specific $CD4^+IL17^+$ splenocytes relative to mice immunized with PCEP + Curdlan + OVA. Splenocytes from mice immunized with PCEP + OVA and Curdlan + Leptin + OVA restimulated with OVA for 48 hours also had significant expression of IL-17 on $CD4^+$ T cells relative to the unstimulated splenocytes. The frequency of $CD4^+$ splenocytes expressing Foxp3 (Figure 5-6D) showed no significant difference between the immunization groups restimulated with OVA. However, there was a significance difference between the unstimulated and OVA restimulated splenocytes from the Curdlan + OVA, Leptin + OVA and Curdlan + Leptin + OVA immunization groups. Mice immunized with Leptin + OVA showed a significant increase in the frequency of $CD4^+$ splenocytes expressing both IL-17 & IFNγ (Figure 5-6E) relative to Curdlan + OVA and PCEP + Curdlan + OVA immunization groups. Mice immunized with OVA and Leptin + OVA showed a significantly higher frequency of $CD4^+$ splenocytes expressing both IL-17 & IL-4 (Figure 5-6F) relative to Curdlan + OVA

immunization group. Mice immunized with Curdlan + Leptin + OVA showed a significant increase in the frequency of OVA-specific $CD4^{+}IL\text{-}17^{+}$ T cells, $CD4^{+}Foxp3^{+}$ T cells and $CD4^{+}IL\text{-}17^{+}Foxp3^{+}$ T cells (Figure 5-6 C, D and G).

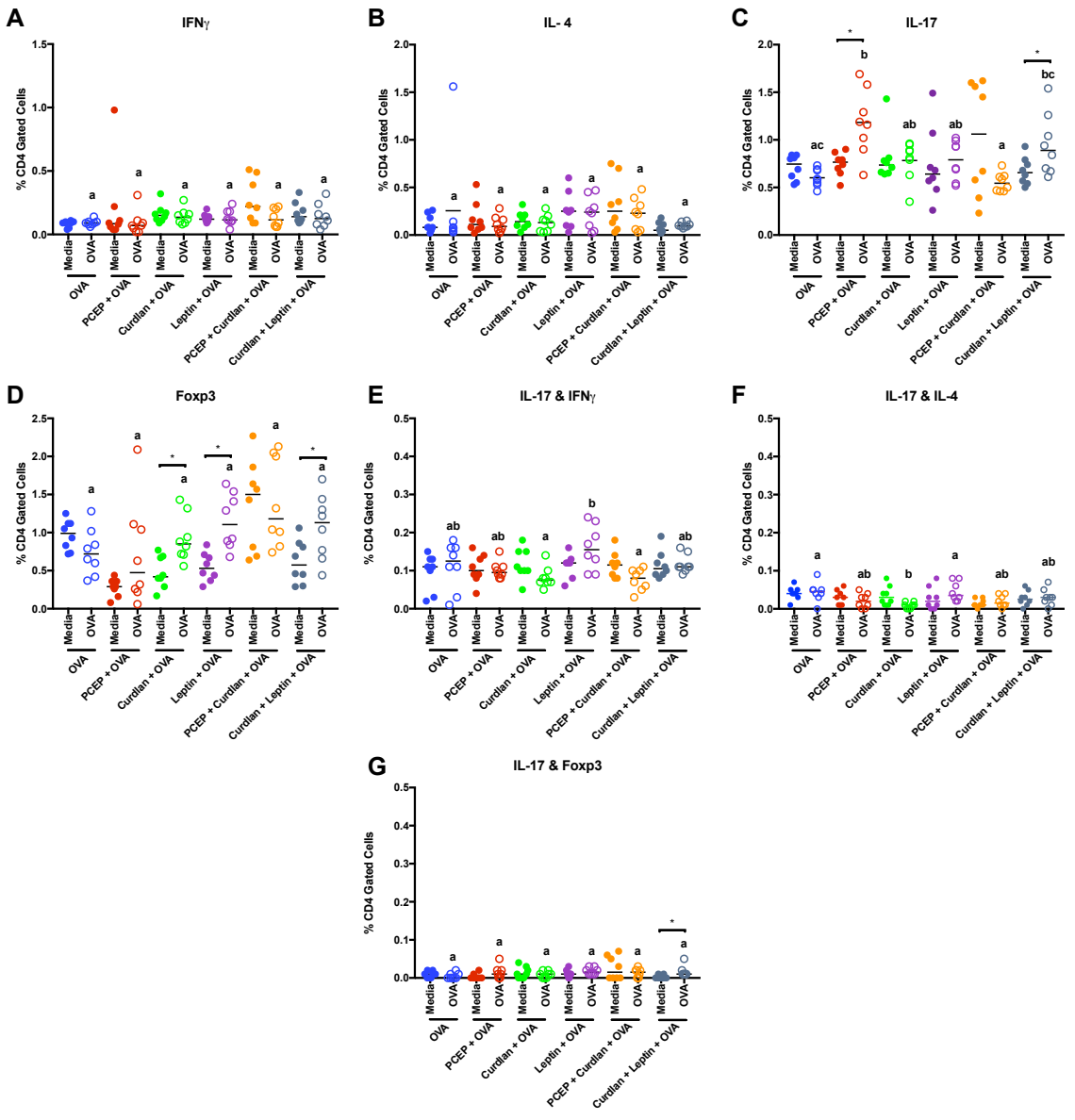

Figure 5-6. Expression of IFNγ, IL-4, IL-17, Foxp3, IL-17 & IFNγ IL-17 & IL-4 and IL-17 & Foxp3 on CD4$^+$ cells of immunized mice. BALB/c were immunized and boosted with 50 µL (25 µL per leg) of OVA or OVA + Adjuvant as: PBS + OVA (10 µg/mL), PCEP + OVA (50 + 10 µg/mL, respectively), Curdlan + OVA (10 + 10 µg/mL, respectively), Leptin + OVA (10 + 10 µg/mL, respectively), PCEP + Curdlan + OVA (50 + 10 + 10 µg/mL, respectively) and Curdlan + Leptin + OVA (10 + 10 + 10 µg/mL, respectively). Spleen cells were isolated and mock-stimulated with media or restimulated with OVA (1 µg/mL) and incubated for 48 hours. Splenocytes were then stained with antibodies for CD4$^+$ markers as well as A) IFNγ B) IL-4 C) IL-17 D) Foxp3 E) IL-17 & IFNγ and F) IL-17 & IL-4 G) IL-17 & Foxp3 markers to determine the percent of

cytokine producing CD4 cells, associated with Th1, Th2, Th17 and Tregs, respectively as well as analyzing for double positive cells, indicating a mixed response.

Cytokine ELISA analysis indicated that PCEP + OVA immunization group produced significant IL-17 than did the splenocytes from mice immunized with Curdlan + OVA. Through FCM we observed that the PCEP + OVA immunization group expressed significantly more IL-17 relative to the OVA formulation group. OVA-specific $CD4^+$ IL-17 T cells were more abundant in the mice immunized with PCEP + OVA and Curdlan + Leptin + OVA immunization groups which suggests that these vaccines induced antigen-specific Th17-type cell differentiation.

Figure 5-7 displays the percent of OVA-induced $CD4^+$ and $CD8^+$ splenocytes expressing IFN γ (Figure 5-7A and 5-7B, respectively) 35 days post immunization, which indicates OVA-specific Th1 cells or CTLs post-immunization. Neither $CD4^+$ nor $CD8^+$ cells showed a significant increase in the frequency of OVA-specific IFNγ producing cells.

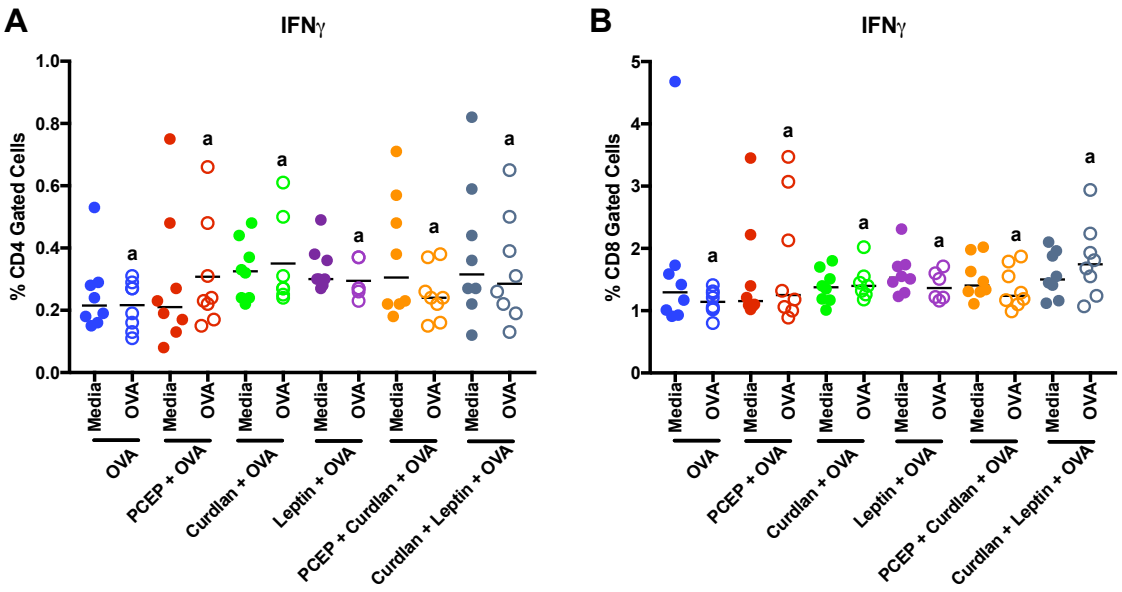

Figure 5-7. Expression IFNγ on $CD4^+$ and $CD8^+$ cells of immunized mice. BALB/c were immunized and boosted with 50 µL (25 µL per leg) of OVA or OVA + Adjuvant as: PBS + OVA (10 µg/mL), PCEP + OVA (50 + 10 µg/mL, respectively), Curdlan + OVA (10 + 10 µg/mL, respectively), Leptin + OVA (10 + 10 µg/mL, respectively), PCEP + Curdlan + OVA (50 + 10 + 10 µg/mL, respectively) and Curdlan + Leptin + OVA (10 + 10 + 10 µg/mL, respectively). Spleen cells were isolated and mock-stimulated with media or restimulated with OVA (1 µg/mL) and incubated for 48 hours. Splenocytes were then stained with antibodies for **A)** $CD4^+$ and **B)** $CD8^+$ markers as well as IFNγ markers to determine the quantity of cytokine producing CD4 and CD8 T cells.

CHAPTER 6 DISCUSSION

6.1 Discussion

Th17 responses are of much importance as they promote protection against fungal and bacterial infections as well as aid in cancer immunity. Therefore, it is important to study adjuvants or other tools in immunology to identify a means to promote a safe and effective Th17-type response. The mechanisms of action by which adjuvants direct an immune response towards one type of immune response or another type is not always known but induction of a specific cytokine environment may be one mechanism to induce Th17-type responses. As stated previously, Th17 cells express IL-17 as well as IL-21 and IL-22 cytokines [7, 132, 141]. Th17 cells differentiate from naïve T cells under a combined IL-6 and TGF-β cytokine environment [7, 132, 141]. IL-1β and IL-23 are important for the stability and upregulation of Th17 cell differentiation, and may be beneficial when seeking to make a vaccine to induce Th17 type immunity [7, 132, 141]. There is evidence that Th17 cells are highly plastic and the current research suggests that IL-12 in the environment can cause a switch in differentiation from a Th17 cell to a Th1 cell or a mixed cell Th1/Th17 and therefore IL-12 in the cytokine environment may also be not preferred when it is desirable to induce a Th17 type immune response to a vaccine [113, 135, 141]. My hypothesis was that we could identify PCEP or another adjuvant and their combinations would promote a Th17-type of response to a vaccine antigen. To test this hypothesis, we studied the effect that select adjuvants had on induction of IL-1β, IL-6, IL-12, IL-17, IL-23 and TGF-β in murine splenocytes *in vitro* and how they impacted the local cytokine environment when injected into muscle tissue. We also quantified IFNγ, IL-4, IL-10/Foxp3 and IL-17 production in response to immunization to assess

whether these adjuvants induced a Th1, Th2, Tregs, Th17 or mixed type immune response. We anticipated that a consistent cytokine profile would be induced by these adjuvants across tissues and in response to the vaccine.

Through *in vitro* studies (Figure 5-1, 5-2 and 5-3) we observed splenocytes cultured with adjuvants promoted different significant cytokine expression relative to splenocytes cultured in media alone. We were encouraged that PCEP alone did not promote IL-1β or IL-12 but promoted inhibition of IL-12 expression from splenocytes stimulated with PCEP or combinations with PCEP in these studies. IL-12 expression seemed to be present in these unstimulated splenocytes *in vitro*. However, it did not promote expression of IL-6, TGF-β or IL-17 either. This was inconsistent with findings from Awate et al (2012) where the IL-6 gene was observed to be expressed in response to PCEP but was consistent with the findings that IL-12 gene expression was produced from CpG but not PCEP [68]. In this same study, the IL-17 gene expression was increased at 96 hours; this may indicate that we needed to try a much longer time point to observe IL-17 expression by PCEP *in vitro*.

When we expanded our analysis to include the immunostimulants, Curdlan and Leptin, we observed that Curdlan +/- PCEP *in vitro* induced expression of splenocytes TGF-β and Curdlan + Leptin+/- PCEP induced expression of IL-17 which we speculated could mean that Th17 cells were being differentiated under these conditions. There has not been a lot of research behind IL-17 expression but there is evidence that innate αβ T cells, γδ T cells, NK cells and myeloid cells may produce IL-17A [149-154], while T helper cells are responsible for the production of IL-17F [7, 132]. So, because we did not directly investigate what cells were responsible for the expression of IL-17, we can only

speculate what cells may have been responsible during this *in vitro* study. Others have shown that neutrophils from cultured murine lungs produced IL-17A in response to Dectin-1 signalling so it is possible that Curdlan signalling through Dectin-1 receptor induced expression of IL-17 from spleen neutrophils [155]. However, because Dectin-1 receptor is also present on macrophages/monocytes, and DCs, Curdlan could have activated these innate immune cells, which in turn induced activation of memory Th17 cells [86, 88, 156-158]. Human DCs stimulated by Dectin-1 agonists have been shown to induce secretion of IL-1β, IL-6 and IL-23, with low levels of IL-12 which prime naïve $CD4^+$ cells to differentiate into Th17 and Th1 cells [158]. Like Dectin-1 receptor, Leptin receptors are also present on neutrophils, monocytes, and lymphocytes but Leptin signalling may be critically required for Th1 or Th17 differentiation [101, 159]. Both IL-6 and Leptin are able to induce RORγt, Th17's master regulator, which could make it possible that Leptin is acting as /similar to IL-6 in facilitating Th17 cell development [93, 160, 161]. Leptin induces functional and morphological changes in human dendritic cells (DCs), directing them towards Th1 priming and promoting DC survival [162]. Further, mouse $CD4^+$ and $CD8^+$ T lymphocytes express the Leptin receptor and respond to Leptin with promotion of T cell number and activation [163]. The Leptin receptor is highly expressed on the cell surface of human Tregs and acts as a negative signal for their proliferation [164]. Thus, Leptin may act on the murine splenic Th17 cells directly or indirectly through induced innate immune cell activation. Finally, PCEP was shown to induce robust secretion of IL-1β and IL-18 in murine splenic DCs as well as promote increased production of IFNγ from $CD4^+$ and $CD8^+$ T Cells [165]. Therefore we speculate that it is unlikely that PCEP triggered induction of IL-17 production from

$CD4^+$ T cells in the spleen from naïve mice. Together Curdlan, Leptin and PCEP may have induced IL-17 production from effector Th17 type immune cells, from innate immune cells or they promoted Th17 type immune cell differentiation.

Our ultimate goal was to use adjuvants in an immunization study to induce an antigen-specific Th17-type of immune response. Thus, we wanted to understand how these adjuvants impacted the local cytokine environment in the muscle in the period after injection. Again we predicted that the effect of the adjuvants on the muscle cells would show a similar pattern of induction of the local cytokine environment as was observed in splenocytes. Through a muscle tissue injection study, where adjuvants were injected without antigen (Figure 5-4), we observed which adjuvants were able to promote significant expression of cytokines in cells recruited to or already present at the site of injection. PCEP induced IL-6 expression at the site of injection and Curdlan + Leptin induced IL-12, IL-17 and TGF-β expression. Similar to the *in vitro* splenocyte stimulation, no injections were able to induce both IL-6 and TGF-β expression at the site of injection which others have shown is necessary for Th17 cell development [7, 132]. As we saw in the splenocyte culture *in vitro* studies, injection of Curdlan+/-Leptin+/- PCEP induced significant IL-17 expression after injection into muscle and therefore must be impacting induction of innate immune cell-mediated expression of IL-17 or they are activating local effector T cell production of IL-17.

It is known that IL-6 and IL-12 can be produced by DCs through the activation of TLR9, MyD88 and TRAF6 by CpG [40], therefore it is likely that any combination of adjuvants that include CpG will produce both IL-6 and IL-12. IL-6 promotes the cell differentiation switch from Treg to Th17, while IL-12 promotes the cell differentiation

switch from Treg to Th1 or Th17 to Th1 [113, 166]. Thus, we predict that it would be most beneficial to identify an adjuvant able to induce expression of IL-6 and TGF-β without expression of IL-12. All conditions including CpG in this *in vitro* study showed some production of IL-12. So, we speculated that they would not be desirable as Th17 cell -inducing adjuvants. Therefore, we removed the adjuvants promoting IL-12 expression from the immunization studies as well as the adjuvants not able to induce IL-17 characteristics. This elimination left us primarily with PCEP, Curdlan and Leptin to continue on to our immunization study. Because the quantification of cytokines through an ELISA does not allow us to determine which cells in the spleen are expressing cytokines, we preformed both ELISA and FCM to determine if the cytokines being quantified in the ELISAs were being produced from $CD4^+$ T helper cells.

When we vaccinated mice i.m. with OVA + PCEP, Curdlan, Leptin and combinations thereof, the resultant antigen-specific immune response was distinct from that which we observed *in vitro* and *in vivo* in the acute period after injection. We specifically quantified the frequency of $CD4^+$ T cells expressing IL-4, Foxp3, IL-17 or IFNγ as a measure of Th2-type, Treg-type, Th17-type or Th1-type cell OVA-specific immunity, respectively. $CD4^+$ T cells expressing IL-17 were produced as a result of an antigen-specific immune response from the PCEP + OVA and Curdlan + Leptin + OVA immunization groups. The Curdlan + Leptin + OVA immunization group also produced Treg cells ($CD4^+$Foxp3 expressing cells) and a small but significant mixed response, Th17/Treg ($CD4^+$IL-17^+Foxp3^+ expressing cells).

These data indicate that we may be observing a mixed type of cell under these conditions but because of the low $CD4^+$ cell percentages it is unlikely that we are seeing a

mixed-type of immune response but that rather both Th17 cells and Tregs are both being produced in response to the antigen, OVA. Others have shown that it is possible to have a Th17 or Treg cell development if TGF-β is induced either alone or in combination with induction of IL-6 [113, 132]. It is also theorized that it is necessary to have a balanced ratio of Th17 to Treg cells to regulate a Th17-type of immune response to avoid promotion of an autoimmune disease in a largely increased Th17 cell environment [136, 137, 167].

Curdlan + Lepton were able to consistently promote significant IL-17 expression *in vitro*, in an adjuvant injection study and in an immunization study through an OVA-specific response. Both of these immunostimulants were added to this project as adjuvants because of their ability to promote the differentiation of Th17 cells. However, throughout this project we were not able to induce cytokine expression of IL-6 and TGF-β from any similar conditions *in vitro* or *ex vivo*. This portrays that we may not be able to predict immunization adjuvants through *in vitro* and *ex vivo* immunostimulants. Curdlan induces DCs to produce Th17 developmental characteristics: IL-6 and IL-23 and Leptin are able to signal Th17 regulators: RORγt and STAT3 [85, 88, 93]. Because we observed $CD4^{+}Foxp3^{+}$ cells production in response to Curdlan + OVA, Leptin + OVA and Curdlan + Leptin + OVA immunizations, we can speculate that Tregs are being differentiated. Tregs produce TGF-β, needed for Th17 cell development and one study suggested that adding Curdlan to a $CD4^{+}Foxp3^{+}$ environment produced an accumulation of $CD4^{+}IL\text{-}17^{+}Foxp3^{+}$ (double positive) cells [85]. This same study found that adding Curdlan to a naïve mouse cell culture also induced $CD4^{+}$ cells to express IL-17 [85]. Therefore, under the Curdlan + Leptin + OVA immunization conditions, we can speculate that because of

the presence of Tregs and other stromal cells, which produce TGF-β, and the addition of Curdlan and Leptin, both of which induce IL-6 production, we have attained suitable conditions for the development of Th17 cells.

Although we have attained optimal conditions for promoting a Th17-type of immune response, more research must be done. The murine model is not an ideal model for veterinary or human vaccine development, but they are rather a stepping-stone to finding future adjuvant and vaccine candidates. It would be ideal to test these findings on a different animal model, while also conducting dose titrations, safety and protection studies of the adjuvants. It would also be beneficial to consider antigens of diseases that may be mediated by a Th17-type of response, as OVA may not indicate proper antigen-specific responses. Therefore, there are many variables that will need to be reviewed for future studies.

6.2 General Conclusions

We conclude that significant IL-17 expression can be induced in murine splenocytes stimulated with Curdlan + Leptin and PCEP + Curdlan + Leptin, and in muscle tissue injected with Curdlan + Leptin +/-PCEP. Immunization with PCEP + OVA and Curdlan + Leptin + OVA showed induction of Th17-type immunity. Mice immunized with Curdlan and Leptin alone or combined showed induction of $Foxp3^{+}IL\text{-}17^{+}CD4^{+}$ T cells. Further studies are needed to assess whether $Foxp3^{+}IL\text{-}17^{+}CD4^{+}$T cells lead to the development of protective Th17-type of immunity against pathogens or autoimmune diseases.

Overall, the Curdlan + Leptin combination is able to induce a Th17-type of immune response *in vitro* and *ex vivo.* Curdlan + Leptin + OVA and PCEP + OVA are able to induce an antigen-specific Th17-type of immune response. These results portray potential safe and effective vaccine adjuvant abilities. However, it would be beneficial to conduct more studies with these adjuvants to determine their protective abilities in immunizations for both human and veterinary vaccines.

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