CELLULAR AND MOLECULAR MECHANISMS OF ACTION OF THE NOVEL ADJUVANT POLYPHOSPHAZENE

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ABSTRACT

Adjuvants are critical components of modern vaccines. They are added to improve the host's immune responses to the vaccine antigens. Understanding the mechanisms of action of adjuvants is critical in the rational design of vaccines. The novel adjuvant poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) has shown great potential as a vaccine adjuvant, but the mechanisms that mediate its adjuvant activity have not been investigated. Hence, the present investigations were undertaken to understand the molecular and cellular mechanisms of action of PCEP. First, we investigated *in vivo* the capacity of PCEP to induce innate immune responses at the site of injection. PCEP induced time-dependent changes in the gene expression of various "adjuvant core response genes" including cytokines, chemokines, innate immune receptors and interferon-induced genes. We also observed that PCEP enhanced production of various cytokines including pro-inflammatory cytokines and chemokines such as CCL2, CCL4, CCL12 and CXCL10 locally at the injection site but no systemic responses.

Due to the potent chemotactic potential of local cytokines and chemokines produced post-injection of PCEP, we observed increased recruitment of various myeloid and lymphoid cells at the injection site. Neutrophils and macrophages were recruited in significantly higher numbers followed by monocytes and dendritic cells (DCs). In addition, there was increased recruitment of T and B lymphocytes at the injection site. Further, confocal studies revealed intracytoplasmic lysosomal localization of PCEP in recruited immune cells at the site of injection. Whole body *in vivo* imaging of mice injected intramuscularly with PCEP revealed localized distribution of PCEP post-injection in the muscle tissue. Approximately 70% of PCEP was cleared from the injection site within 24 h post-injection, but there was evidence of PCEP retention up to 12 weeks post-injection. Although we could not detect PCEP in the draining lymph nodes, we observed significant increase in neutrophil, macrophage, monocyte and DC numbers, with the latter cell population being most abundant.

We observed that *in vivo* PCEP upregulates NLRP3 gene and pro-inflammatory cytokine expression at the injection site. Since caspase-1 is a critical component of NLRP3 inflammasome and known to plays an important role in the release of IL-1β and IL-18, we examined the role of caspase-1 in PCEP-mediated secretion of IL-1β and IL-18 by splenic DCs. Pre-treatment of splenic DCs with the caspase-inhibitor YVAD-fmk significantly inhibited IL-1β and IL-18 secretion in response to PCEP. Although PCEP was taken up by the DCs, it failed to induce DC maturation (expression of MHC class II and co-stimulatory molecules CD86 and CD40). In addition, PCEP did not induce direct activation of naïve T cells. However, when naïve B cells were directly activated, PCEP induced significant production of IgM and IL-6. Further, immunization of mice with OVA plus PCEP significantly increased the production of OVA-specific IFN-γ by $CD4^+$ T cells and $CD8^+$ T cells suggesting that PCEP can generate antigen-specific T cell responses.

Taken together, these results suggest that adjuvant activity of PCEP depends on creating a strong immunocompetent environment at the site of injection by activating innate immune responses, which involves modulation of adjuvant core response genes, production of cytokines and chemokines, recruitment of various immune cells and presumably activation of inflammasomes. Together, all these mechanisms might contribute to the adjuvant activity of PCEP.

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DEDICATION

To my Father and Mother

Who instilled in me the value of education and unconditionally supported me throughout my life

And

To my Husband

For his endless love and support

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIM2	Absent in melanoma 2
α-GalCer	Glycolipid α-galactosylceramide
ANOVA	Analysis of variance
APC	Antigen presenting cell
AS03	Adjuvant system 03
AS04	Adjuvant system 04
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BMDC	Bone marrow dendritic cells
CAF01	Cationic liposome 01
CARD	Caspase activation and recruitment domain
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDCs	Conventional DCs
CDPs	Common DC precursors
CFA	Complete Freunds Adjuvant
CIITA	Major histocompatibity complex II transactivator
CL	Cationic liposomes
CLIP	Class II-associated invariant chain peptide
CLR	C-type lectin receptor
CpG ODN	Cytosine-phosphate-guanosine oligodeoxynucleotides
CMI	Cell-mediated immunity
CTL	Cytotoxic T lymphocytes
CTLA4	Cytotoxic T lymphocyte antigen 4

CXCL	Chemokine (C-X-C motif) ligand
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DDA	Dimethyldioctadeclammonium
diC14-amidine	3-tetradecylamino-tert-butyl-N-tetradecylpropion-amidine
dLNs	Draining lymph nodes
DNA	Deoxyribonucleic acid
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTIM	Octadecenoyloxy(ethyl-2-heptadecenyl-3-hydroxyethyl imidazolinium
DTaP	Diphtheria, Tetanus, acellular Pertusis vaccine
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
Flt3L	Feline McDonough sarcoma-like tyrosine kinase ligand
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gD2	Glycoprotein D2
GMCSF	Granulocyte macrophage colony-stimulating factor
GPCRs	G protein coupled receptors
GSK	GlaxoSmithKline
HBs-Ag	Hepatitis B surface antigen
HBV	Hepatitis B virus
Hib B	Haemophilius influenzae type B
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSC	Hematopoietic stem cells
HSV	Herpes simplex virus
ICAM	Intercellular adhesion molecules
ICE	IL-1 beta-converting enzyme

iDCs	Immature DCs
IFN	Interferon
IFNGR	Interferon-gamma receptor
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IgM	Immunoglobulin M
IL-1β	Interleukin-1 beta
IL	Interleukin
i.m.	Intramuscular
iNKT	Invariant natural killer T cells
IPAF	Interleukin-1 beta converting enzyme (ICE) protease-activating factor
IPC	Type 1 interferon producing cells
IRAK	Interleukin-1 receptor-associated kinase
IRDye	Infra-red dye
ISCOMs	Immune stimulating complexes
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
KC	Keratinocyte-derived chemokine
kDa	Kilodalton
LDC	Langerhans dendritic cell
LFA-1	Lymphocyte function-associated antigen 1
LPs	Lymphoid precursors
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
mDCs	Mature DCs
MDP	Muramyl dipeptide
MDPs	Macrophages and DC precursors

MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MMR	Measles Mumps Rubella
MPs	Myeloid precursors
MPL	3-O-desacyl-4'-monophosphoryl lipid A
mRNA	Messenger Ribonucleic acid
MSU	Mono-sodium ureate
MyD88	Myeloid differentiation factor 88
NAIP	NLR family, apoptosis inhibitory protein
NFκB	Nuclear factor κB
NK	Natural killer (cells)
NKT	Natural killer T cells
NLR	NOD-like receptors
NLRC	NLR family CARD domain-containing protein 4
NLRP	NACHT, LRR and PYD domains-containing protein
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PBS	Phosphate buffered saline
PAMPs	Pathogen associated molecular patterns
PCEP	Poly[di(sodium carboxylatoethylphenoxy)phosphazene]
PCPP	Poly[di(sodium carboxylatophenoxy)phosphazene]
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PLGA	Poly-lactide-co-glycolide acid
PMN	Polymorphonuclear
PRR	Pattern recognition receptor
PSA	Porcine serum albumin

PYD	Pyrin domain
RIG	Retinoic acid-inducible gene 1
RLR	RIG-1 like receptors
ROR	Retinoic acid receptor related orphan receptor
ROS	Reactive oxygen species
RPL19	Ribosomal protein L19
rRNA	Ribosomal RNA
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
TAP	Transporter associated with antigen processing
TBX21	T-box transcription factor 21
TDB	Trehalose dibehenate
TDM	Trehalose-6-6-dimycolate
tgD	Truncated glycoprotein D
TGF-β	Transforming growth factor beta
Th1	T-helper lymphocyte type-1
Th2	T-helper lymphocyte type-2
TIMPs	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF-α	Tumor necrotic factor alpha
Tr1	Type 1 regulatory T cells
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAF	TNF receptor associated factor
VCAM1	Vascular cell adhesion molecule 1
WHO	World Health Organization

CHAPTER 1. LITERATURE REVIEW

1.1 Overview of vaccine adjuvants

1.1.1 Vaccine history

Vaccines are undoubtfully one of the most valuable public health tools, which have saved millions of lives from highly infectious and deadly diseases. The term vaccine is derived from the Latin word "*vacca*" meaning the cow, which is in reference to the work done by the English Physician, Edward Jenner. During the smallpox epidemic in Europe in 1796, Dr. Jenner extracted the infectious material from a cowpox-infected maid and inoculated it into the arms of an 8-year-old boy named James Philips, who developed symptoms of smallpox. After 48 h, the boy was inoculated with infectious smallpox material again but this time he did not develop the disease. The practice of using disease material for the prevention of the disease dates back to 200 B.C. in India and China. The ancient people in these countries used to preserve dried smallpox materials and injected them into skin or nose to help prevent smallpox. Almost 200 years after Dr. Edward Jenner's work, the World Health Organization (WHO) declared eradication of smallpox in 1980 after successful vaccination campaign. Another vaccination success story is the near-eradication of polio. Since the launch of the Global Polio Eradication Initiative in 1988, Polio has almost been successfully eradicated (99%). This year Polio has been reported only in three countries (Afghanistan, Nigeria and Pakistan). Every year millions of lives are saved due to vaccination against various infectious diseases including measles, mumps, diphtheria, haemophilius, meningitis, tetanus, hepatitis and pertussis in children and adults. However, many people continue to die from vaccine preventable diseases due to lack of vaccination.

Two important vaccine components are antigen and adjuvant. Adjuvants are natural or synthetic compounds that have been used in vaccines since the early 1920s to enhance or modulate the immunogenicity of co-administered antigen. The new vaccines, which include subunit, DNA, vectored, and genetically engineered vaccines, have higher safety profiles. However, the major drawback of these novel vaccines is that they are poorly immunogenic and therefore require addition of adjuvants to induce effective and sustainable immune responses.

1.1.2 Types of vaccines

1) *Live-attenuated vaccines*: To generate this type of vaccines, the pathogenic organisms are passaged in culture to reduce replicating ability and pathogenicity. Live-attenuated vaccines are replicating and highly immunogenic because they closely mimic the natural infection. However, the major drawback of live vaccines is that they have the potential of reverting back to their pathogenic nature and cause disease. Therefore, attenuated vaccines have a high safety risk especially in newborns, elderly, and immunocompromised individuals. Examples of successful live-attenuated vaccines include smallpox, polio, yellow fever, tuberculosis, typhoid, anthrax, MMR (measles, mumps, rubella), varicella (chickenpox), rotavirus and recently influenza vaccines. As live-attenuated vaccines are highly immunogenic, they do not require co-administration with adjuvants.
2) *Killed or inactivated vaccines*: These vaccines are made by killing or inactivating nucleic acids or cross-linking the proteins of pathogens by heat or chemical treatment to make them harmless. Since the pathogens are non-replicating, they cannot revert to their virulent forms. They often induce poor, short-lived immune responses and therefore require co-administration with adjuvants. Typhoid, cholera, hepatitis A, polio and rabies are some of the examples of this type of vaccines.
3) *Toxoid vaccines*: Toxoids are the weakened and chemically detoxified forms of toxins produced by some pathogens. Some toxoids are also poorly immunogenic and require adjuvants. The best example of a toxoid vaccine is DTaP (Diphtheria, Tetanus, and acellular Pertussis).
4) *Subunit/recombinant vaccines*: Contain purified protein antigens of pathogenic microorganisms. Since these vaccines are highly purified and contain only part of the pathogen instead of whole organism, they are poorly immunogenic and require addition of adjuvants. Higher doses of vaccine may be required to elicit protective responses. Hence, the major drawback of this vaccine is cost of production and requirement for booster doses to maintain immunity. Examples of subunit vaccines are hepatitis B and pertussis (whooping cough).

5) *Conjugated vaccines*: Pathogenic bacteria with polysaccharide coating easily escape immune recognition. The polysaccharide capsules are poorly immunogenic and hence require adjuvants. To induce immune responses against such pathogens, it has to be conjugated with immunogenic antigens. Some examples of conjugated vaccines are Haemophilus influenzae type B (Hib B) and pneumococcal vaccine.
6) *DNA vaccines*: In DNA vaccines, instead of injecting proteins or peptide antigens directly, genes encoding for pathogenic antigens are delivered. Expression vectors (e.g. plasmids) are used to clone the gene of interest. These vaccines are completely safe and cost-effective but poorly immunogenic. Hence, adjuvants or delivery methods are required to enhance the immunogenicity of DNA vaccines. Intramuscular inoculation of DNA vaccines results in poor transfection efficiency. However, novel delivery methods, such as electroporation, have shown higher transfection of antigen presenting cells (APCs), which then leads to enhanced humoral and cellular immune responses.
7) *Recombinant vector vaccines:* are similar to DNA vaccines, but they use an attenuated virus or bacteria to introduce microbial DNA into a host cell for production of antigenic proteins that can be tailored to stimulate immune responses. Viral vector vaccines, unlike DNA vaccines, also have the potential to actively invade host cells and replicate, much like a live attenuated vaccine, further activating the immune system. Various organisms have been utilized for vector vaccines including adenoviruses, attenuated poliovirus and vaccinia virus.

1.1.3 Adjuvants

Adjuvants (Latin word *adjuvare*, meaning “to help/aid”) were first described by Ramon as “substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone” (Ramon, 1924). Various natural and synthetic compounds have been investigated for their adjuvant properties. However, very few have been approved for human use due to safety concerns such as adverse local and systemic effects. Some examples of adjuvants are discussed below.

1.1.3.1 Alum

For almost a century, alum-based mineral salts were the only adjuvants approved for human vaccines. In 1926, Alexander T. Glenny and colleagues reported for the first time that precipitation of aluminium potassium sulfate (Potash Alum) to diphtheria toxoid greatly enhanced its antibody response (Glenny et al., 1926). Two decades later, Ericsson used aluminium phosphate as an adjuvant with diphtheria toxoid. Alum is chemically aluminium potassium sulfate. However, the two types of aluminium-containing adjuvants that are used in human licensed vaccines are aluminium hydroxide adjuvant and aluminium phosphate adjuvant (Hem and HogenEsch, 2007; Hem et al., 2007). Since then, alum has been used in numerous types of vaccines for humans and animals. However, aluminium salts mainly induce humoral (Th2) responses and are inefficient adjuvants with several vaccine formulations especially against those infections that require Th1 immune responses for protection (Hunter, 2002). Therefore, there is a clear need for specific adjuvants that promote both antibody and cell-mediated immunity. In this regard, several adjuvants have been developed or are under investigation. In the past decade, apart from alum, two other oil-in-water emulsions (MF59 and AS03) and one combination adjuvant (a Toll-like receptor (TLR) agonist, MPL plus alum [AS04]) have been approved for use in human vaccines.

1.1.3.2 MF59

MF59 is an oil-in-water emulsion of a squalene, polyoxyethylene sorbitan monooleate (Polysorbate 80) and sorbitan trioleate (Span 85). MF59 was licensed in Europe for a commercialized vaccine against influenza (Fluad®, Chiron Vaccines, Siena, Italy). MF59 is a potent adjuvant with an acceptable safety profile that has been approved in more than 20 countries for influenza vaccines in elderly people (De Donato et al., 1999; Menegon et al., 1999). Another emulsion adjuvant, AS03, was approved for a vaccine against prepandemic influenza strain H5N1 (Prepandrix™, GlaxoSmithKline Biologicals [GSK], Rixensart, Belgium). MF59 significantly enhanced the potency of influenza vaccine antigens as well as the breadth of immune responses (O'Hagan et al., 2011). Recently, MF59 has also been shown to be safe in infants and young children. MF59 enhanced the immune responses against influenza vaccines from 43% to 89% in young childrens (Vesikari et al., 2011). Various studies have shown that MF59 is a more potent

vaccine adjuvant compared to alum in induction of both humoral (antibody titers) and cell-mediated (CD4+ T cell) responses (O'Hagan, 2007; Wack et al., 2008). However, none of the MF59 components (squalene oil or citrate buffer) tested alone resulted in higher immune responses (Mosca et al., 2008). Currently MF59 has been tested with various other vaccine antigens. Hence, MF59 is a potent vaccine adjuvant with a broad safety profile that has the potential to be used with wide range of antigens across all age groups.

1.1.3.3 AS04

AS04 (Adjuvant System 04; GSK) is a combination adjuvant containing 3-O-desacyl-4'-monophosphoryl lipid A (MPL) and alum (aluminium hydroxide or aluminium phosphate). MPL is a "detoxified" derivative of lipopolysaccride (LPS) isolated from the gram-negative bacterium *Salmonella Minnesota* R595 strain. Due to the toxic properties of LPS and its ability to cause septic shock, it is not safe for human use. Detoxified MPL has reduced toxicity, but still retains the immuno-stimulatory properties of LPS. In 2009, AS04 was licensed in the USA for human use with HPV (Cervarix® [GSK]), hepatitis B virus (Fendrix® [GSK]) and herpes simplex virus (HSV)-2 vaccines. Clinical data with the AS04 formulation showed sustained antibody levels and 2.2 to 5.2-fold higher memory B cell responses compared to alum formulations alone (Giannini et al., 2006). Stronger B and T cell responses are attributed to the AS04 adjuvant in the Cervarix™ vaccine. The HBV vaccine (Fendrix® [GSK]) was the first AS04 formulated vaccine approved for human use in Europe. A previously used HBV vaccine formulated with alum has been used effectively in persons <40 yrs of age. Improved immuno-protection in elderly and immuno-compromised people was achieved by formulating HBV with AS04. Fendrix® induced higher and sustained antibody titers, increased seroprotection with fewer vaccine doses and stimulated better cell-mediated immune responses compared to HBV vaccine with alum alone (Ambrosch et al., 2000). Genital herpes caused by HSV-2 is a sexually transmitted disease affecting a large population. A vaccine containing glycoprotein D from HSV-2 and AS04 (gD2/AS04) provided better immune protection, significantly reduced the viral load and viral shedding than the gD2 vaccine with aluminum salts alone (Bourne et al., 2005). However, this vaccine was recently terminated due to low efficacy in clinical trails (Garçon and Van Mechelen, 2011; Stanberry et al.,

2002).

1.1.3.4 Liposomes

Liposomes have been used as vaccine delivery vehicles (e.g. for diphtheria toxoid) for several decades (Allison and Gregoriadis, 1974). Liposomes are poorly immunogenic and fail to efficiently activate APCs. Hence, they are incorporated with TLR or non-TLR ligands to potentiate the immune responses. Viral membrane protein based liposomal vaccines (virosomes) have been approved for use in humans as delivery platforms for hepatitis A and influenza vaccines (Ambrosch et al., 1997). Cationic liposomes are also effective liposomal delivery systems for vaccine antigens and have potent immuno-stimulatory properties (Vangasseri et al., 2006). Cationic liposomes prevent antigen degradation and enhance antigen presentation to APCs (Christensen et al., 2007). CAF01 is a cationic liposome that consists of 5:1 ratio of dimethyldioctadeclammonium (DDA) and trehalose dibehenate (TDB). TDB, a synthetic analogue of trehalose-6-6-dimycolate (TDM, also called mycobacterial cord factor) is less toxic and showed good adjuvant activity (Numata et al., 1985). CAF01 has been shown to induce potent humoral and cell-mediated immune responses against tuberculosis (TB), malaria, hepatitis B and Chlamydia (Agger et al., 2008; Vangala et al., 2006). Currently, CAF01 is in Phase I clinical trials for TB vaccine.

1.1.3.5 CpG DNA

Innate immunity is activated by recognition of pathogen-associated molecular patterns (PAMPs) including bacterial DNA. Unmethylated CpG DNA (contains central unmethylated CG dinucleotides plus flanking regions) released from bacteria, triggers mammalian immune responses via direct activation of APCs (Hemmi et al., 2000). Synthetic oligodeoxynucleotides (ODNs) expressing unmethylated CpG motifs mimic the immuno-stimulatory properties of bacterial DNA (Wagner, 1999). CpG ODNs have shown great potential as vaccine adjuvants in many animal models of infectious disease, allergy and cancer. Studies using TLR9 knock out mice confirmed that CpG ODNs signals through endosomal TLR9 (Hemmi et al., 2000). CpG ODNs have been tested in various Phase I-III clinical trials (Bode et al., 2011). The ability of CpG ODNs to promote

Th1 type immune responses and to produce $CD8^+$ T cell differentiation makes them ideal targets for cancer vaccines. Due to their very short half-life *in vivo* and transient activation of immune responses, CpG ODNs need to be formulated with other adjuvants (including other TLR ligands) to enhance their adjuvant effects in large animals and humans (Ioannou et al., 2002a; Ioannou et al., 2002b; Rankin et al., 2002). The reason behind the species differences in activity of CpG is partly due to differences in distribution of TLR9 on APCs. In humans, only B cells and pDCs express TLR9, whereas, in mice TLR9 is expressed by various myeloid cells including conventional DCs (Barchet et al., 2008).

1.1.3.6 Polyphosphazenes

Polyphosphazenes are high molecular weight, water-soluble, synthetic polymers consisting of a backbone with alternating phosphorus and nitrogen atoms, and organic side groups attached to each phosphorus (Fig 1.1) (Andrianov et al., 2006; Andrianov et al., 2004). Polyphosphazenes have been used in many applications and are under investigation as vaccine adjuvants and delivery systems (Lakshmi et al., 2003). They can be chemically modified by incorporating chemical moieties in their structure to make water-soluble salts. Polyphosphazenes have been shown to be potent immunological vaccine adjuvants. They dramatically enhance the magnitude, quality and duration of immune responses to a variety of bacterial and viral antigens in mouse models. A few studies have also shown the potential of polyphosphazenes as an effective and safe adjuvants in large animals (Mutwiri et al., 2009; Taghavi et al., 2009). Polyphosphazenes can be formulated as aqueous solutions or as microparticles by cross-linking with divalent cations. These microparticles have been shown to act as effective mucosal delivery systems (Payne et al., 1995; Shim et al., 2010). Hence, polyphosphazenes are versatile polymers that can act both as delivery systems as well as vaccine adjuvants.

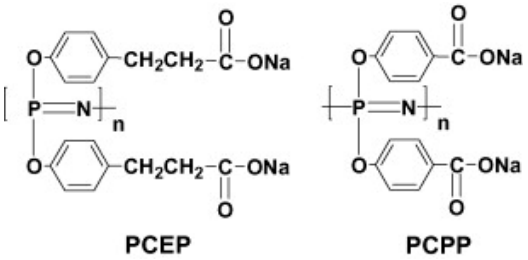


Fig 1.1 The structures of the polyphosphazene polyelectrolytes, PCEP and PCPP.

1.1.3.6.1 Polyphosphazenes as vaccine adjuvants

One of the most investigated polyphosphazenes polyelectrolytes, poly[di(sodium carboxylatophenoxy)phosphazene] (PCPP) has shown promise as a vaccine adjuvant with a variety of antigens including hepatitis B surface antigen (HBsAg) (Mutwiri et al., 2008), influenza (Mutwiri et al., 2007a; Payne et al., 1998a), rotavirus (McNeal et al., 1999), and cholera (Wu et al., 2001a) vaccines. Influenza antigens when injected with an aqueous formulation of PCPP, showed 10-fold enhanced antibody titers compared to influenza antigens without PCPP (Payne et al., 1998a). Antigen formulated with PCPP resulted in prolonged antibody titers for 6 months suggesting that PCPP promotes vaccine longevity (Andrianov and Payne, 1998).

A new generation polyphosphazene polyelectrolyte, poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) has significantly higher adjuvant activity compared to PCPP (Mutwiri et al., 2008; Mutwiri et al., 2007b) and 1000-fold higher immune responses compared to alum (Mutwiri et al., 2007a). A single immunization with PCEP induces sustained antibody responses for at least 6 months (Mutwiri et al., 2007a) suggesting that PCEP can be formulated with single-shot vaccines which avoids the need for booster immunization, and would reduce the cost of vaccination due to reduction in vaccine doses. PCEP may also increase cost-effectiveness of vaccines as PCEP reduced the dose of influenza X:31 antigen used in

vaccination by 25-fold, without significantly reducing the magnitude and quality of the IgG2a antibody responses (Mutwiri et al., 2007a). This will be an important property especially during pandemic outbreaks such as influenza, where antigen-sparing effects can help improve vaccine coverage.

Polyphosphazenes have also been shown to modulate the quality of immune responses. Both PCPP and PCEP induce Th2 type immune responses, but PCEP also induces Th1 responses. Subcutaneous injection of influenza antigen formulated with PCEP resulted in enhanced IgG1 and IgG2a antibody titers compared with PCPP and alum, which only induced IgG1 responses (Mutwiri et al., 2007a). This indicates that PCEP can promote a mixed Th1/Th2 type response giving broad-spectrum immunity (Dar et al., 2012; Mutwiri et al., 2008; Mutwiri et al., 2007b). Polyphosphazene adjuvants may be used to influence the quality of the immune responses as appropriate for the specific infections. For example, PCPP can be used to induce Th2 type responses that are required for protection against extracellular pathogens, whereas PCEP can be formulated to induce mixed Th1 and Th2 responses required for both intracellular and extracellular pathogens.

In addition to mice, polyphosphazenes have also been shown to be effective and safe adjuvants in large animals. In sheep, administration of porcine serum albumin (PSA) or truncated glycoprotein D (tgD) from bovine herpesvirus-1 formulated with PCPP, resulted in 100-fold and 10-fold higher antibody titers respectively, compared to antigen alone (Mutwiri and Babiuk, 2008). In recent studies, administration of *Actinobacillus pleuropneumoniae* outer membrane antigen with PCEP resulted in balanced Th1/Th2 immune responses in pigs (Dar et al., 2012). Overall, these studies indicate polyphosphazenes have potential as adjuvants in large animals.

Although the majority of the pathogens invade through mucosal routes such as the gastro-intestinal, respiratory and genital tract, there is a lack of an effective and safe mucosal adjuvant. Most of the licensed adjuvants fail to induce effective mucosal immune responses. Hence, there are many experimental adjuvants under investigation for mucosal vaccines. Cholera toxin is a potent

mucosal adjuvant; however it has been withdrawn from use in humans due to associated toxicity (Skene and Sutton, 2006). Intranasal immunization of mice with influenza X:31 antigen formulated with PCPP resulted in enhanced antibody responses. In addition to inducing immune responses as early as 2 weeks, PCEP also reduced the dose of influenza antigen by five-fold without compromising the antibody responses (Eng et al., 2009). Recently it has been shown that intranasal administration of influenza X:31 antigen with PCEP resulted in a significantly increased in antibody titers in nasal, lung and vaginal mucosal secretions (Eng et al., 2010a). Similar results were obtained following intranasal vaccination with several vaccine antigens such as pertussis toxoid, pneumococcal surface protein A and the influenza virus strain A/Puerto Rico/8 (PR8) formulated in PCPP (Shim et al., 2010). Influenza X:31 antigen + PCEP also induced antigen-specific IgA antibodies in nasal, saliva, vaginal and fecal secretions (Shim et al., 2010). Taken together, these results indicate that polyphosphazenes are effective as mucosal adjuvants (Eng et al., 2010a). Polyphosphazene properties such as hydrolytic degradability of their main chain, cost-effective production, dose-sparing effects, induction of prolonged and enhanced immune responses, easy formulation with more than one antigen/adjuvant and mucosal delivery systems makes them ideal adjuvant candidates for vaccine development.

1.1.3.6.2 Safety profile of polyphosphazenes

Many experimental adjuvants with great potential fail to cross the regulatory hurdles mainly due to safety concerns. However, PCPP has been tested in Phase I clinical trials in both young and elderly adults (Le Cam et al., 1998). Three influenza viral strains (A/H3N2, A/H1N1 and B strain) were tested with three doses of PCEP (100, 200 and 500 mcg) in 48 young and 41 elderly subjects. No serious adverse events were associated with any of the PCEP doses in the 89 subjects tested. Serum titers were increased by 15-fold when H3N2 was formulated with 500 mcg PCPP compared to 3-fold increase with non-adjuvanted vaccine. In addition, PCPP has also been tested in clinical trails against rgp 160 (MN/LA12) strain of HIV (Gilbert et al., 2003). In large animals, up to 1 mg of PCPP was injected in sheep (Mutwiri and Babiuk, 2008), cattle (Kovacs-Nolan et al., 2009b) and 500 μg in pigs (Dar et al., 2012) without any adverse reactions. Overall,

these results suggest that polyphosphazenes are well tolerated in humans and animals but more detailed safety studies are required.

1.1.3.7 Combinational adjuvants

Traditionally, only single adjuvants were used in vaccines. The new approach towards vaccine formulation to achieve improved immunoprotection is by using a combination of two or more adjuvants. Most adjuvant combinations include a delivery system and an immunostimulatory adjuvant. In general, delivery systems tend to induce Th2-type immune responses that are not protective against many intracellular pathogens. Alternatively, immuno-stimulatory adjuvants predominantly induce potent Th1-type immune responses by strongly activating the innate immune system. However, immuno-stimulatory adjuvants tend to have a very short half-life *in vivo* (Mutwiri et al., 2009). By using two adjuvants with complimentary actions, a vaccine may present with increased magnitude and also modulate the quality of immune responses. For example, alum can allow co-delivery of antigen and immunostimulatory adjuvants to the same APC, thereby potentiating the immune responses. When alum was combined with TLR agonists such as CpG oligonucleotide or LPS, it resulted in enhanced humoral and cellular responses compared to either adjuvant alone (Wack et al., 2008). Some examples of alum-based combinational adjuvants and their effects in immune responses are summarized in Table 1.1.

Polyphosphazenes have been extensively tested in combination adjuvant formulations. Mice immunized with a single subcutaneous injection of HBsAg plus the adjuvant combination of CpG ODN with PCPP or PCEP resulted in enhanced production of HBsAg-specific antibody responses compared with the mice immunized with HBsAg plus any of the three adjuvants alone (Mutwiri et al., 2008). In addition, mice immunized with PCPP microparticles encapsulating OVA and CpG ODN generated higher antigen-specific antibody responses compared to antigen alone (Garlapati et al., 2010; Wilson et al., 2010). Further, encapsulation of genetically detoxified pertusis toxoid (PTd) with triple adjuvant combination (synthetic cationic innate defense regulator peptide [IDR]-CpG ODN complexes into polyphosphazene-based microparticles) significantly lowered bacterial load and increased antigen-specific IL-17 secreting cells in comparison with each

adjuvant alone or any of the double adjuvant combinations when assessed in a *Bordetella pertusis* infection challenge model in mice (Garlapati et al., 2011). Co-formulation of PTd with IDR/CpG/polyphosphazene increased IgG1 responses in adult mice and induced superior serum IgG2a antibody titers in both adult and neonatal mice compared to immunization with each of the adjuvants and antigen alone (Gracia et al., 2011).

A recombinant truncated bovine respiratory syncytial virus (RSV) fusion protein (DeltaF) co-formulated with the triple combination of CpG, indolicidin and polyphosphazene (CpG/indol/pp) enhanced the secretion of antigen-specific serum IgG, IgG1 and IgG2a antibodies when compared with antigen alone in mice (Kovacs-Nolan et al., 2009c). *In vitro,* combination of CpG/indol/pp increased the secretion of tumor necrosis factor (TNF)-α, IL-12p40 and IL-6 by bone-marrow derived DCs *ex vivo* relative to the individual components (Kovacs-Nolan et al., 2009a). In cattle, CpG/indol/pp formulation with the antigen hen egg lysozyme (HEL) increased antigen-specific humoral responses and prolonged cell-mediated immune responses (Kovacs-Nolan et al., 2009b).

Table 1.1 Alum and its combination adjuvants

Combinations	**Species tested**	**Antigen**	**Effect on immune response/protection**	
Alum + MPL	Mice, humans, monkeys	HPV (licensed) HBsAg (licensed) HSV-2 (withdrawn)	↑ Antibody titers ↑ Memory ↑ Duration	(Didierlaurent et al., 2009; Giannini et al., 2006)
Alum + CpG ODN	Mice	HBsAg	↑ Antibody titers	(Davis et al., 1998; Ioannou et al., 2002a; Ioannou et al., 2002b)
Alum + MDP	Mice	*Helicobacter Pylori urease*	↑ Th1 responses ↑ IFN-γ/IL-2	(Moschos et al., 2006)

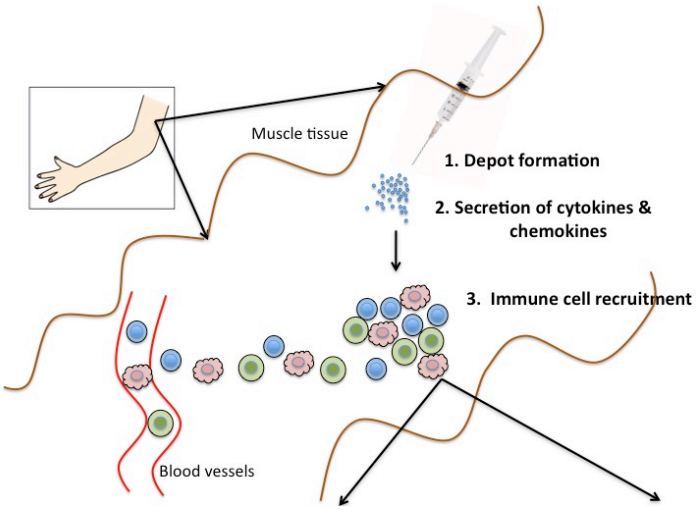
HBsAg: Hepatitis B surface antigen; HPV: Human papillomavirus; HSV: Herpes simplex virus; MDP: Muramyl dipeptide; MPL: Monophosphoryl lipid A; ODN: Oligodeoxynucleotide.

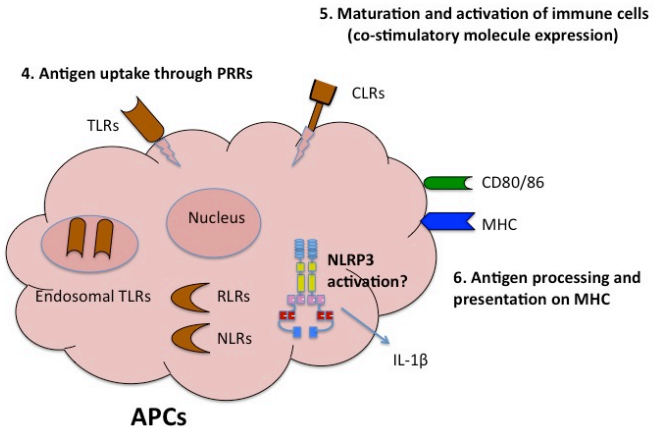
1.2 Mechanisms of action of vaccine adjuvants

1.2.1 Introduction

The goal of vaccination is induction of protective immunity and in some vaccines this can be enhanced by addition of adjuvants. Many diverse classes of compounds have been assessed as adjuvants including mineral salts, microbials products, emulsions, saponins, cytokines, polymers, microparticles and liposomes (Guy, 2007). Based on their proposed mechanisms of action, vaccine adjuvants have been broadly divided into delivery systems and immunostimulatory adjuvants (Singh and O'Hagan, 2003). In general, delivery systems were previously thought to act by providing a depot while immuno-stimulatory adjuvants activate cells of the innate immune system (Pashine et al., 2005). However, this classification is no longer appropriate since evidences have emerged that some delivery systems can activate innate immunity.

Surprisingly, despite the wide use of vaccine adjuvants in billions of doses of human and animal vaccines, the mechanisms of action by which they potentiate immune responses are not well characterized. This is well captured in a famous quote by Charles Janeway who observed that adjuvants are “the immunologists’ dirty little secret” (Janeway, 1989). However, recent advances in immunobiological research have unfolded several mechanisms by which adjuvants act. Available evidence suggests that adjuvants employ one or more of the following mechanisms to elicit immune responses: 1) sustained release of antigen at the site of injection (depot effect), 2) up-regulation of cytokines and chemokines, 3) cellular recruitment at the site of injection, 4) increase antigen uptake and presentation to APC, 5) activation and maturation of APC (increased MHC class II and co-stimulatory molecules expression) and migration to draining lymph nodes, 6) activation of inflammsomes, and 7) immunomodulation/priming of T cells or B cells (Fig 1.2) (Cox and Coulter, 1997; Fraser et al., 2007; Hoebe et al., 2004).


Muscle tissue
1. Depot formation
2. Secretion of cytokines & chemokines
3. Immune cell recruitment
Blood vessels


5. Maturation and activation of immune cells (co-stimulatory molecule expression)
4. Antigen uptake through PRRs
TLRs
CLRs
CD80/86
MHC
Nucleus
NLRP3 activation?
6. Antigen processing and presentation on MHC
Endosomal TLRs
RLRs
NLRs
IL-1β
APCs

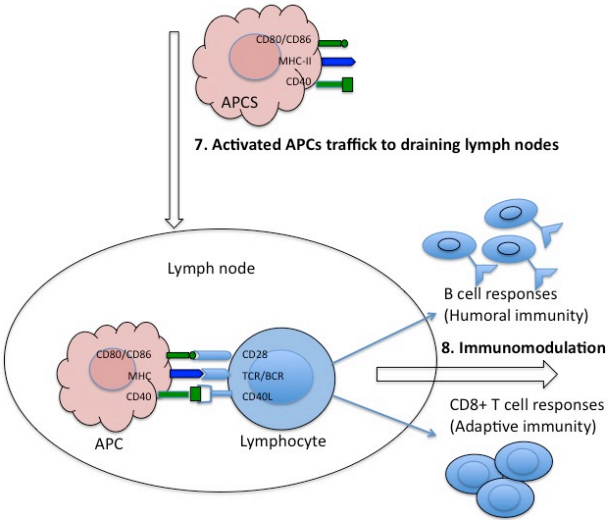

CD80/CD86
MHC-II
CD40
APCS
7. Activated APCs traffick to draining lymph nodes
Lymph node
B cell responses (Humoral immunity)
8. Immunomodulation
CD80/CD86
MHC
CD40
APC
CD28
TCR/BCR
CD40L
Lymphocyte
CD8+ T cell responses (Adaptive immunity)

Fig 1.2 Proposed mechanisms of action of adjuvants. 1) Some adjuvants presumably form a depot at the site of injection, which is associated with slow release of antigen. 2) Other adjuvants are associated with transient secretion of cytokines and chemokines. 3) Secreted cytokines and chemokines are involved in recruitment of various immune cells to the injection site. These recruited cells secrete cytokines and chemokines, in turn chemoattract other immune cells. All these events lead to formation of a local immunocompetent environment at the injection site. 4) The recruited APCs express various PRRs both on the surface (TLRs, CLRs) and intracellularly (NLRs and RLRs), which are recognized and/or are activated by the adjuvants. 5) This leads to maturation and activation of recruited APCs. Mature APCs upregulated the expression of MHC and co-stimulatory molecules. 6) They are also characterized by increased capacity for antigen processing and presentation. 7) Mature APCs then migrate to the draining lymph nodes to interact with antigen-specific B or T cell to (8) activate potent antibody secreting B cells and/or effector CD8+ T cell responses.

1.2.2 Formation of depot at the site of injection

The formation of a depot is perhaps the oldest and most widely recognized mechanism of action of adjuvants. Antigen trapping and slow release at the site of injection ensures constant stimulation of the immune system for production of high antibody titers (Siskind and Benacerraf, 1969). Until recently, depot effect was considered a classic mechanism of action of many adjuvants. Glenny et al. were the first to propose the importance of depot formation in the adjuvant activity of alum (Glenny et al., 1926). Antigen was detected for 2 to 3 weeks in alumina gel-induced granulomas (Osebold, 1982). Antigens are simply adsorbed onto the alum but the binding is proposed to be due to strong electrostatic interaction between antigen and alum (Burrell et al., 2000), which enhanced antigen uptake and presentation by APCs (Mannhalter et al., 1985). Various other adjuvants such as water-in-oil emulsions (Complete Freunds Adjuvant [CFA]) and biodegradable micro-and nano-particles were shown to act by depot effect to generate prolonged and sustained high antibody titers (Herbert, 1968; Kreuter, 1988). AS04, an adjuvant combination consisting of monophosphoryl lipid A (MPL) and alum was shown to induce optimal immune responses only when co-localized with antigen (Didierlaurent et al., 2009). The presence of alum in AS04 is important in stabilizing the MPL and antigen within the vaccine, along with providing a depot effect (Didierlaurent et al., 2009). The cationic adjuvant formulation (CAF) 01, a combination of dimethyldioctadeclammonium/trehalose-6,6-dibehenate (DDA/TDB), which is currently in phase I clinical trial, is also thought to induce long lasting depot effect (Henriksen-Lacey et al., 2010).

There is no definitive evidence that depot effect significantly contributes to adjuvant activity (Marrack et al., 2009). In various studies, it has been shown that surgical removal of the antigen-alum depot 14 days after immunization had no effect on the immune responses (Schijns, 2000). Apparently, the adsorption of antigen to alum was not required for alum adjuvant activity (De Gregorio et al., 2008; Iyer et al., 2003). Recently, removal of the injection site 2 h after antigen and alum administration had no effect on humoral or cell-mediated immunity (Hutchison et al., 2012). Similarly, MF59 was rapidly cleared and did not form a depot at the injection site (Ott et al., 1995). MF59 was distributed and cleared independent of antigen with a half-life of 42 h in the

muscle tissue (Dupuis et al., 1999). Likewise, ISCOMs tend to be rapidly transported to draining lymph nodes after administration (Morein and Bengtsson, 1999). Therefore, these studies clearly indicate that depot effect is not required for adjuvant activity of alum, and possibly MF59 or ISCOMs.

1.2.3 Upregulation of cytokines and chemokines leading to cellular recruitment at the injection site

Recent studies on the mechanisms of adjuvants have focused on recruitment of innate immune cells at the site of injection. Particulate adjuvants have been shown to create a local pro-inflammatory environment to recruit immune cells (Goto and Akama, 1982). Using genome wide micoarray analysis, Mosca *et al.* demonstrated that a cluster of genes encoding cytokines, chemokines, innate immune receptors, interferon-induced genes and gene encoding adhesion molecules defined as "adjuvant core response genes" were commonly modulated by alum, MF59 and CpG-ODN at the site of injection (Mosca et al., 2008). Compared with alum and CpG ODN (TLR9 agonist), MF59 was a strong modulator of adjuvant core response genes. Chemokines, which play a critical role in tissue specific migration of immune cells, were shown to be up-regulated by adjuvants at the injection site. MF59 significantly up-regulated the expression of CCR2, a receptor for CCL2, which is involved in $CCR2^{+}$ monocyte infiltration. This was in agreement with previous *in vitro* results showing that MF59 induced release of chemoattractants like CCL2, CCL3, CCL3 and CXCL8 (Seubert et al., 2008). Further, studies in CCR2-deficient mice showed that MF59-induced mononuclear cell recruitment is CCR2 dependent (Dupuis et al., 2001). Another oil-in-water emulsion AS03 co-localizes with antigen to trigger colony-stimulating factor 3 (CSF3) and IL-6, and leukocyte-recruiting chemokines CCL2, CCL3 and CCL5 at the site of injection (Morel et al., 2011). Similar cytokine and chemokine mRNA expression profiles were upregulated in the draining lymph nodes (Morel et al., 2011). Likewise, alum-induced infiltration of immune cells was accompanied by production of chemo-attractants like CCL-2, the neutrophil chemotaxin KC (CXCL1), and eosinophil chemotaxin eotaxin (CCL11) in the peritoneal cavity of mice (Kool et al., 2008c). Similarly, PCEP induced stronger expression of adjuvant core response

genes compared to CpG at the site of injection. Locally, PCEP triggered production of pro-inflammatory cytokines and chemokines including CCL2 (Awate et al., 2012).

Alum promotes Th2-type immune responses and differentiation of B cells resulting in robust antibody production (Grun and Maurer, 1989). However, the role of Th2 cytokines in the adjuvant activity of alum is not clearly defined. *In vitro* studies indicate that alum-induced activation of macrophages and up-regulation of co-stimulatory molecules did not depend on IL-4 (Rimaniol et al., 2004). However, in *in vivo* studies, alum induced priming of B cells through IL-4 producing Gr1+ cells in mouse spleen, which is required for proliferation of antigen-specific B cells and for optimal antibody production (Jordan et al., 2004). IL-4 producing Gr1+ cells were mainly eosinophils, which appeared within 24 h and induced expansion of B cells and enhanced IgM production (Wang and Weller, 2008). Further, studies with eosinophil-deficient mice showed that the priming of B cells was abolished after alum injection confirming the central role of eosinophils in alum-induced Th2 type immune responses (Jordan et al., 2004; Wang and Weller, 2008). In addition, a study by Serre et al revealed that the Th2 type immune responses generated by alum may signal through IL-25/IL-17RB and/or IL-6 pathways (Serre et al., 2008).

Alum has been shown to activate the complement cascade and recruit cells from blood to create an inflammatory environment at the site of injection (Goto et al., 1997; Ramanathan et al., 1979). Similar to alum, MF59 has been shown to recruit CD11b+ blood mononuclear cells in the mouse muscle (Mosca et al., 2008). Intra-peritoneal injection of alum induced rapid cell recruitment of inflammatory Ly6C+CD11b+ monocytes. The inflammatory monocytes take up antigen, differentiate into CD11c+ MHC class II+ DCs in a myeloid differentiation primary response gene 88 (MyD88)-dependent manner and migrate to draining lymph nodes, where they induced proliferation of antigen-specific T cells (Kool et al., 2008a). In similar studies by McKee et al, alum induced rapid recruitment of various polymorphonuclear (PMN) cells (eosinophils and neutrophils) and also monocytes,DCs, NK and NKT cells at the site of vaccination (McKee et al., 2009). Interestingly, in cell depletion studies in mice, alum-mediated humoral and cellular responses were independent of mast cells, macrophages and of eosinophils (McKee et al., 2009).

MF59-mediated immune cell recruitment to the injection site has been studied in detail (Calabro et al., 2011). MF59 induced recruitment of neutrophils, monocytes, eosinophils, macrophages followed by DCs after i.m. injection in mice. The recruited cells especially neutrophils, monocytes and B cells take up both antigen and adjuvant and traffick to draining lymph nodes. Neutrophils are the first cells to be recruited at the site of adjuvant injection and also one of the highest in numbers. However, depletion of neutrophils had no impact on the antigen-specific immune responses induced by MF59 (Calabro et al., 2011). Similar to MF59, administration of AS03 led to enhanced recruitment of neutrophils, eosinophils and monocytes at the site of injection, which take up antigen and traffick to the draining lymph nodes (Morel et al., 2011). At the injection site, neutrophils attract other immune cells by producing increased amounts of chemokines and transport antigen to draining lymph nodes (Calabro et al., 2011; Morel et al., 2011). However, the role of neutrophils in adjuvant activity is not completely clear.

ASO4 induces transient local NFκB activity and cytokine production (Didierlaurent et al., 2009). The TLR4 agonist MPL, one of the components of AS04, stimulated increased numbers of DCs and monocytes in the draining lymph nodes. Likewise CpG, a TLR9 agonist, signals through activation of MyD88, IRAK and TRAF-6, leading to recruitment of transcription factors, which in turn up-regulates the pro-inflammatory genes and protein expression (IL-1, IL-6, IL-12, IL-18 and TNF-α) within 3 h of injection (Klaschik et al., 2009; Klinman et al., 1996). Genes up-regulated by CpG included cytokines, cell signaling, cell movement and DNA damage response genes (Klaschik et al., 2010). One of the roles of cationic liposomes is to recruit immune cells and increase antigen presentation. Intra-peritoneal injection of cationic liposome (DDA/MPL) increases influx of neutrophils, monocytes, macrophages and activated natural killer (NK) cells in the peritoneal cavity (Korsholm et al., 2010). Another cationic liposome CAF01 induced recruitment of monocytes to the site of injection and increased trafficking of liposomes to the draining lymph nodes (Henriksen-Lacey et al., 2010).

Therefore, adjuvants induce recruitment of various immune cells to the site of injection, some of which then traffick the antigen to the draining lymph nodes to induce specific immune

responses. However, the relationship between these recruited cells and induction of immune responses is not very clear. Depletion studies suggest that the role of recruited innate immune cells at the injection site is redundant in the generation of adaptive immune responses (Calabro et al., 2011; McKee et al., 2009). Interestingly, these studies were performed by depleting single cell populations. Identifying the role of a specific cell population *in vivo* is even more challenging due to complex environment at the injection site. Injection of adjuvants often leads to recruitment of a variety of cell populations and due to high redundancy in the immune system, other recruited cells may compensate for the depleted single cell population. In this regard, mice whose specific cell populations have been depleted were shown to produce cytokines and chemokines to recruit innate immune cells and activate T cells (Calabro et al., 2011; Seubert et al., 2008). Further studies are required to investigate the detailed relationship between recruited immune cells and adjuvant activity.

1.2.4 Antigen presentation

Efficient antigen presentation by major histocompatibility complexes (MHC) on APCs is important for the induction of adaptive immune response. It was thought that many adjuvants including alum, oil-based emulsions and microparticles act by "targeting" antigens to APCs resulting in enhanced antigen presentation by MHC (Guéry et al., 1996; Schijns and Lavelle, 2011). Alum was shown to increase antigen uptake by DCs and alter the magnitude and duration of antigen presentation *in vitro* (Mannhalter et al., 1985; Morefield et al., 2005). Antigen adsorption on alum led to an increase in internalization of antigen (Morefield et al., 2005). Recent studies by Flach et al. have shown that alum does not enter DCs directly but rather delivers the antigen via abortive phagocytosis (Flach et al., 2011). In this regard, alum interacts with membrane lipids on DCs leading to lipid sorting, recruitment of ITAM containing molecules Syk and PI3 activation. These events eventually lead to uptake of antigen that is adsorbed on alum, DC activation and MHC class II expression (Flach et al., 2011).

The role of adjuvant-induced increased antigen presentation in development of adaptive immunity has not been clearly evaluated. Hence, our knowledge is limited regarding the role of this adjuvant mechanism. Recently, Ghimire et al investigated the impact of antigen presentation on alum adjuvanticity. In addition to confirming the ability of alum to increase the antigen internalization, the study also showed that alum plays an important role in reducing the rate of degradation of internalized antigen (Ghimire et al., 2012). Similarly, MF59 facilitated internalization of gD2 antigen from type 2 herpes simplex virus (HSV) by recruited APCs at the site of injection and increased phagocytosis in human PBMCs (Dupuis et al., 1999). Antigen size seems to play an important role in modulating the antigen presentation efficiency. Large lipid vesicles end up in early endosome/phagosomes and increases antigen presentation whereas smaller vesicles rapidly localize to late lysosomes leading to reduced antigen presentation (Brewer et al., 2004).

1.2.5 Activation and maturation of DCs

Activation of DCs is essential for induction of adaptive immune responses (Table 1.2). Increased expression of MHC class II, activation marker CD86 and maturation marker CD83 leads to enhanced ability of APCs to induce T lymphocyte activation and differentiation (Coyle and Gutierrez-Ramos, 2001). Freund's complete adjuvant, LPS, liposomes, CpG ODN, MF59, AS04 and α-galactosylceramide (α-GAL) have all been shown to induce DC maturation to enhance adaptive immunity (Copland et al., 2003; De Becker et al., 2000; De Smedt et al., 1996; Fujii et al., 2003; Shah et al., 2003). Intra-peritoneal injection of OVA and alum led to uptake of antigen and maturation of DCs (Kool et al., 2008a). However, *in vitro* studies on human cells have shown that alum and MF59 failed to directly activate DCs but enhanced the surface expression of MHC class II and co-stimulatory molecules (CD83 and CD86) on monocytes, macrophages and granulocytes that resulted in increased T cell proliferation (Seubert et al., 2008; Sun et al., 2003). Further, *in vitro* activation of DCs by alum has generated conflicting results. One study suggested that alum failed to induce maturation and antigen presentation (Sun et al., 2003) where as another study showed that the activation marker CD86 and antigen presentation was increased in DCs

(Sokolovska et al., 2007). The source of alum may have been a contributing factor in the conflicting results.

AS04 has been shown to induce maturation of DCs (via TLR4), which then trafficks to the draining lymph nodes to activate antigen-specific T cells (Didierlaurent et al., 2009). Similarly, CpG, induced up-regulation of CD40, CD54, CD80, CD86 and MHC class II molecules and antigen processing and presentation in plasmacytoid DCs (pDCs) (Kerkmann et al., 2003; Krieg, 2002). A novel class of TLR-independent adjuvants, mycobacterial cord factor trehalose-6-6-dimycolate (TDM) and TDB have been shown to directly activate DCs through the FcγR-Syk-Card9-Bcl10-Malt1 pathways, and up-regulates the expression of co-stimulatory molecules (Werninghaus et al., 2009). Microparticles such as Poly-lactic-co-glycolic acid (PLGA) did not induce co-stimulatory molecules expression on bone marrow derived DCs (BMDCs) but enhanced antigen presentation efficiency (Sun et al., 2003). DOTAP (1,2-dioleoyl-3-trimethylammonium-propane)-based cationic liposomes have been shown to induce maturation of DCs through activation of MAPK (extracellular signal-regulated kinase and p38), leading to up-regulation of co-stimulatory molecules (Yan et al., 2007). Likewise, diC14-amidine (3-tetradecylamino-tert-butyl-N-tetradecylpropion-amidine) based cationic liposomes up-regulates the expression of CD80 and CD86 on DCs through specific TLR4/MD2 ligation (Tanaka et al., 2008). Overall, adjuvants stimulate DC maturation and enhance the expression of MHC and co-stimulatory molecules, which is required for efficient T cell activation.

Table 1.2 Innate immune receptors activated by vaccine adjuvants.

PRRs		Adjuvants	Type of immune response induced	References
TLRs	TLR1/2	Triacyl lipopeptides Synthetic Pam_3Cys	Th1, Th2, CTL responses	(Deres et al., 1989; Schild et al., 1991)
	TLR2/6	Diacyl lipopeptides Pam_2Cys	Th1, Th2, CTL responses	(Moyle and Toth, 2008)
	TLR2	Pam_3Cys	Th1, Th2, CTL responses	(Deres et al., 1989; Schild et al., 1991)
	TLR3	Poly I:C	Both Th1 and Th2	(Choi et al., 2012; Tamura and Sasakawa, 1983)
	TLR4	LPS, AS04 (MPL)	Th1	(Casella and Mitchell, 2008; Sasaki et al., 1997)
	TLR5	Flagellin	Th1 and Th2	(Didierlaurent et al., 2004; McCarron and Reen, 2009)
	TLR 7	Imiquimod Resiquimod	Th1, $CD8^+$ T cell, CTL responses	(Stanley, 2002; Wagner et al., 1999)
	TLR8	Resiquimod	Th1, $CD8^+$ T cell, CTL responses	(Wagner et al., 1999; Wu et al., 2004)
	TLR9	CpG ODN	Th1, $CD8^+$ T cells, CTL responses	(Kobayashi et al., 1999)
NLRs	NOD1/ NLRC1	DAP	Th1, Th2, Th17	(Chamaillard et al., 2003; Fritz et al., 2007; Girardin et al., 2003a)
	NOD2/ NLRC2	MDP	Th1, Th17	(Girardin et al., 2003b; Shaw et al., 2009; van Beelen et al., 2007)
	NLRP1	Toxoids, MDP	Th1	(Hsu et al., 2008)
	NLRP3	Alum, MDP, ATP	Th2	(Eisenbarth et al., 2008; Li et al., 2007; Mariathasan et al., 2006)
	IPAF/ NLRC4	Flagellin	Th1 and Th2	(Lightfield et al., 2011; Zhao et al., 2011)
	NAIP5	Flagellin	Th1 and Th2	(Kofoed and Vance, 2011)
RLRs	RIG-1	DNA vectors	Th1, $CD8^+$ T cells	(Luke et al., 2011)
	MDA5	Poly I:C	Th1, $CD8^+$ T cells	(Wang et al., 2010)
CLRs	Dectin-1	Flagellin, β-glucan/zymosan	Th17	(LeibundGut-Landmann et al., 2007)
	Mincle	CAF01	Th1, Th17 $CD8^+$ T cells	(Gram et al., 2009; Rosenkrands et al., 2011)

Abbreviations: Pam3Cys: tri-palmitoyl-S-glyceryl cysteine, LPS: lipopolysaccharide, AS04: Adjuvant system 04, MPL: monophosphoryl lipid A, CpG ODN: cytidine-phosphate-guanosine oligodeoxynucleotides, Poly I:C: polyinosinic-polycytidylic acid, DAP: diaminopimelic acid, MDP: muramyl dipeptide, CAF01: Cationic adjuvant formulation-01, TLR: Toll-like receptor, NLR: NOD-like receptors, RLR: RIG-1 like receptors, CLR: C-type lectins, NOD: nucleotide-binding oligomerization domain, NLRP3: NOD-like receptor family, pyrin-domain-containing 3, IPAF: IL-1β-converting enzyme protease-activating factor, NAIP: neuronal apoptosis inhibitory protein, RIG-1: retinoic acid-inducible gene-1, MDA5: melanoma differentiation associated gene 5.

1.2.6 Activation of inflammsomes

Innate immune cells express various pathogen-recognition receptors (PRRs) to recognize infectious agents. In recent years, various new families of PRRs have been identified including TLRs, C-type lectin-like receptors (CLRs), nucleotide oligomerization domain (NOD) like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-1) like receptors (RLRs). Many immunological adjuvants signal via PRRs or act as ligands for innate immune receptors. In contrast to TLR agonists, particulate adjuvants are not recognized by specific PRRs but they still induce adaptive immune responses. The "danger" hypothesis was first discussed by Matzinger, who proposed that apart from self/non-self discrimination against infection, danger signals from damaged cells can trigger activation of the immune system (Matzinger, 1994). Molecules associated with tissue damage such as uric acid, nucleotides, adenosine triphosphate (ATP), reactive oxygen intermediates and cytokines are released at the injection site due to tissue damage (Shi et al., 2003). These non-infectious damage signals have now been named damage-associated molecular patterns (DAMPs) to distinguish them from PAMPs.

Particulate adjuvants cause local tissue damage and cell death at the injection site (Kool et al., 2008a). In addition, many adjuvants induce release of pro-inflammatory cytokines at the site of injection (Awate et al., 2012; Calabro et al., 2011; Didierlaurent et al., 2009). These damage signals trigger non-specific activation of the innate immune system, subsequently stimulating adaptive immunity. Recently inflammasomes have been one of the most widely investigated topics due to their potential role in adjuvant activity. The inflammasome receptors belongs to the NLR family, which also includes various other receptors, such as the nucleotide-binding oligomerization domain (NODs) (NOD1-5), NLRPs (NLRP1-14), NLRP1 (NAIP), NLRC4 (IPAF) and the major histocompatibity complex II transactivator (CIITA) (Martinon et al., 2009). Compared to others, NOD-like receptor family, pyrin-domain-containing 3 (NLRP3) is the most studied inflammasome receptor in regards to adjuvant mechanisms. NLRP3, also known as cryopyrin or NALP3 (NACHT, LRR and PYD domains-containing protein 3), is an intra-cytoplasmic multi-protein complex that consists of three components; a NLRP3 receptor, an apoptosis-associated speck-like protein containing a CARD (ASC) and a procaspase-1 (Schroder and Tschopp, 2010). Activation

of NLRP3 inflammasome induces caspase-1 activation, which in turn cleaves proforms of IL-1β and IL-18 to their bioactive forms (Martinon et al., 2009). The NLRP3 inflammasome can be activated by various stimuli including DAMPs, environmental irritants such as asbestos and silica, metabolic stress and UVB irradiation (Schroder and Tschopp, 2010). Apart from danger signals, inflammasomes can be activated by PAMPs such as bacterial flagellin through NLRC4 activation (Zhao et al., 2011).

Li and his colleagues reported for the first time that alum-induced secretion of IL-1β and IL-18 was caspase-1 dependent (Li et al., 2007). Subsequent *in vitro* studies by various groups showed that activation of NLRP3 is required for alum induced IL-1β and IL-18 secretion (Eisenbarth et al., 2008; Franchi and Nùñez, 2008; Hornung et al., 2008; Kool et al., 2008a). However, LPS priming to induce pro-IL-1β in APCs prior to alum stimulation was a pre-requisite for secretion of IL-1β. Contrary to *in vitro* studies, the role of inflammasomes in the adjuvant activity of alum *in vivo* has yielded conflicting results. Using NLRP3, ASC and caspase-1 knockout mice, Eisenbarth et al. showed that the NLRP3 inflammasome is a crucial component in the adjuvant activity of alum. NLRP3, ASC and caspase-1 knockout mice immunized with OVA adsorbed on alum, failed to induce antigen-specific antibody responses (Eisenbarth et al., 2008). Another study by Kool et al. showed that alum induced lower influx of inflammatory cells in the peritoneal cavity of NLRP3 deficient mice. They also showed that alum-mediated activation of adaptive immune responses was NLRP3-dependent (Kool et al., 2008a). Similar studies done by Li et al. showed that NLRP3 deficent mice injected with alum-adsorbed diphtheria toxoid or OVA vaccine elicited impaired levels of antigen-specific antibody responses (Li et al., 2008). All these studies indicate that NLRP3 inflammsome is critical in the adjuvant activity of alum *in vivo*. In contrast, Franchi and Núñez clearly showed that antigen-specific IgG production was not impaired in NLRP3 deficient mice following intraperitoneal injection of human serum albumin (HSA) in the presence of alum (Franchi and Nùñez, 2008). However, NLRP3 did affect alum-mediated cellular recruitment suggesting that inflammsomes might play an important role in activating innate immunity, but the contribution of inflammasomes in activation of adaptive immunity remains elusive. The conflicting results with regard to the role of inflammasomes in adjuvant activity of

alum have been attributed to the differences in the nature of alum used in different studies, immunization protocols and the mouse strains used (De Gregorio et al., 2008; Marrack et al., 2009).

To date, the ligand for NLRP3 has not been identified. Some theories proposed for alum-mediated activation of NLRP3 include phagosomal destabilization and release of cathepsin B, low intracellular potassium (K+) concentrations and generation of reactive oxygen species (ROS) (Hornung et al., 2008; Kool et al., 2008a; Petrilli et al., 2007). It was proposed that a catabolic product of nucleotides, uric acid and ATP released at the site of alum injection due to cell damage or necrosis act as danger signals for activation of NLRP3. Saturation of uric acid due to tissue damage forms mono-sodium ureate crystals (MSU). Phagocytosis of crystalline particles such as MSU or alum results in phagosomal destabilization and lysosomal rupture releasing the protease cathepsin B in the cytosol (Hornung et al., 2008). The released cathepsin B led to activation of NLRP3 and secretion of pro-inflammatory cytokines IL-1β and IL-18. Treatment of mice using uricase, a uric acid degrading enzyme, led to reduced cellular recruitment to draining lymph nodes in mice injected with alum (Kool et al., 2008a). Similarly, ATP released by the damaged cells at the injection site has been shown to indirectly activate NLRP3. Extracellular ATP triggered stimulation of purinergic $P2X_7$ receptor, resulting in activation of cation channel for K+ efflux and opening of pannexin-1 pore for entry of danger signals generated by alum, activate NLRP3 and subsequently caspase-1 (Petrilli et al., 2007; Solle et al., 2001). Further, blocking ROS using chemical scavengers abolished NLRP3 activation in response to MSU suggesting a link between NLRP3 activation and ROS generation (Dostert et al., 2008).

Recently, the role of the inflammasome in adjuvant activity of MF59 was evaluated (Ellebedy et al., 2011; Seubert et al., 2011). Two independent studies using NLRP3 deficient mice demonstrated that NLRP3 is not required for the adjuvant activity of MF59. However, an adaptor molecule required for the assembly of inflammasome, ASC was found to be crucial for MF59 adjuvant activity (Ellebedy et al., 2011). A recent study by Embry et al showed that MPL failed to

induce intra-cytoplasmic assembly of NLRP3 inflammasome leading to failure of caspase-1 activation and maturation of pro-inflammatory cytokines IL-1β and IL-18 (Embry et al., 2011).

1.2.7 Activation of adaptive immune responses

Selection of an adjuvant for a vaccine formulation depends on its ability to stimulate innate immune responses and subsequently induce potent and antigen-specific adaptive immune responses. Different adjuvants induce remarkably different types of adaptive immune responses. Most agonists to endosomal TLRs such as TLR3, TLR7, TLR8, and TLR9 (Poly I:C, imiquimods, CpG, MPL) promote development of Th1 type immune responses. The liposomal adjuvant CAF01 and CFA stimulate mixed Th1 and Th17 type immune responses while MF59, AS03, saponins (ISCOMs and ISCOMATRIX), and agonists for cell surface TLRs (TLR2/TLR6 and TLR5) promote the development of mixed Th1/Th2 type immune responses (Coffman et al., 2010).

Alum is known to enhance potent antibody responses against vaccine antigens but fails to stimulate CMI responses (Bomford, 1980). Alum preferentially stimulates Th2 type responses including B cell differentiation, production of Th2 type cytokines IL-4, IL-5 and IL-13, and B cell-mediated IgG1 and IgE antibody production (Brewer and Alexander, 1997). In studies using IL-4 and IL-13 deficient mice, alum-induced Th2 type immune responses were not affected but instead there was an increase in Th1 type antibody responses (IgG2a) and IFN-γ production (Brewer et al., 1999; Brewer et al., 1996; Kopf et al., 1993). Similar results were observed using IL-6 deficient mice (Brewer et al., 1998). These results suggest that alum-induced Th2 type cytokines play an important role in inhibiting the Th1 type immune responses. Particulate adjuvants ISCOMs and ISCOMATRIX are derived from saponins purified from the bark of *Quillaia saponaria* tree. ISCOMs induce strong $CD8^+$ T cell responses via efficient cross presentation of antigens on DCs in humans (Schnurr et al., 2009). ISCOMATRIX triggers activation of DCs and induces efficient MHC class II presentation of antigens to elicit strong Th1 cell responses (Duewell et al., 2011; Schnurr et al., 2009). Quil A and its derivative QS-21, another saponin based adjuvant, not only stimulate Th1 cytokines IL-2 and IFN-γ, but also induces production of cytotoxic T lymphocytes

(CTLs) (Kensil et al., 1995; Sun et al., 2009; Takahashi et al., 1990). However, high toxicity caused by Quil A makes it undesirable for use in human vaccines (Waite et al., 2001).

The oil-in-water emulsion MF59 activates balanced humoral and cellular immune responses. Although MF59 does not activate any TLRs, studies with MyD88-deficient mice have shown that MF59-induced Th1 and Th2 responses are dependent on MyD88 (Seubert et al., 2011). Recently it was shown that B cell activation and class switching can be triggered via MyD88 adaptor (He et al., 2010). Therefore, MF59-induced antibody responses could be activated through MyD88 signaling pathways in B cells (Seubert et al., 2011). The novel mucosal adjuvant glycolipid α-galactosylceramide (α-GalCer) has been shown to induce both Th1 and Th2 type cytokines thereby inducing B cell and T cell responses (Cerundolo et al., 2009). GalCer, presented through CD1d, induced strong iNKT activation leading to B cell responses and memory (Fujii et al., 2003; Galli et al., 2007). Later it was shown that expression of CD1d on B cells was not required for generation of antigen-specific antibody responses (Tonti et al., 2009).

Agonists for endosomal TLRs are strong type 1 IFN inducers and are able to elicit Th1 cell differentiation and CD4+ T cell activation (Blander and Medzhitov, 2006; Kadowaki et al., 2001; Loré et al., 2003). Efficient cross presentation of antigens by Poly I:C activates $CD8^+$ T cell responses (Schulz et al., 2005). In contrast, AS04, which contains the TLR4 agonist MPL, did not directly activate B or $CD4^+$ T lymphocytes (Didierlaurent et al., 2009). CpG-ODN induces strong Th1 cytokine production including IL-12 and IFN-γ, and $CD8^+$ T cell responses to vaccine antigens via activation of TLR9 signaling pathway in plasmacytoid DCs (Kobayashi et al., 1999; Overstreet et al., 2010; Salio et al., 2004; Stern et al., 2002). CTL responses induced by CpG-ODN against tumor antigens makes them ideal candidates for development of cancer immunotherapy (Miconnet et al., 2002).

A liposome-based adjuvant, CAF01, has shown potential as a vaccine adjuvant against various disease models in mice (Christensen et al., 2011; Lindenstrøm et al., 2009). CAF01 induced antigen-specific IFN-γ^+TNF-α^+IL2$^+$ multifunctional $CD4^+$T cells responses that were

detected for more than one year after injection (Lindenstrøm et al., 2009). CAF01 also induces functional $CD8^+$ T cell responses and CTL responses against HIV-1 antigens in mice (Gram et al., 2009). The adjuvant CAF01 is in clinical trails for HIV and TB vaccines (Christensen et al., 2009; Davidsen et al., 2005; Gram et al., 2009; Henriksen-Lacey et al., 2011). DOTIM (octadecenoyloxy(ethyl-2-heptadecenyl-3-hydroxyethyl imidazolinium)-based cationic liposome-DNA complexes (CLDCs), diC14-amidine based adjuvants and DDA/MPL induce strong Th1 responses and promote increased production of pro-inflammatory cytokines including IL-6, TNF-α, IL-12 and interferons (Ireland et al., 2010; Tanaka et al., 2008). DDA-based cationic liposomes are frequently combined with immuno-stimulator adjuvants (MPL/TDB/Poly I:C) to enhance the quality and quantity of immune responses (Christensen et al., 2011). Combinations of CAF01 and Poly I:C, termed CAF05, have also induced enhanced $CD8^+$ T cell responses, making them ideal adjuvants against viral antigens (Nordly et al., 2011). In addition, combinations of MPL and CAF01 resulted in increased numbers of IFN-γ^+CD8^+ T cells (Nordly et al., 2011). Overall, these studies indicate that there is a huge potential of exploiting various adjuvants alone or in combination to induce desired antibody and cell-mediated immunity.

1.2.8 Mechanisms of action of polyphosphazenes

The mechanisms by which polyphosphazenes induce higher immune responses at the molecular and cellular levels are poorly understood. The adjuvant activity of polyphosphazenes does not appear to depend on depot formation, since excision of the site of injection 24 h post-injection had no effects on antibody production (Payne et al., 1998a). However, polyphosphazenes form water-soluble, non-covalent complexes with protein antigens (Andrianov et al., 2005). This physical association with antigen might help to deliver antigens to APCs.

Available evidence suggests that the potent adjuvant activity of PCEP may be a consequence of its activation of innate immunity. *In vitro* studies by Mutwiri *et al.* 2008 have shown that polyphosphazenes stimulate the production of innate cytokines, which might contribute to its adjuvant activity. However, no *in vivo* mechanistic studies were done to understand the mechanisms of action of polyphosphazene.

In conclusion, a detailed knowledge of adjuvant mechanisms is very important in the rational design of vaccines. In recent years, considerable advances have been made in understanding the mechanisms of action of various adjuvants, particularly the activation of innate immunity via various mechanisms (Table 1.3). Safety is the biggest concern when it comes to adjuvant approval for human use and our knowledge of the same is very limited. Detailed understanding of the adjuvant mechanisms will provide some insight into its safety profile. In the coming years, we hope to get more intrinsic details of various adjuvant mechanisms that might help in rational formulations of vaccines and we finally hope to see more adjuvants approved for human use.

Table 1.3 Mechanisms of action of adjuvants licensed for human use.

Adjuvants	Proposed mechanisms of action	Immune response activated	Licensed vaccines	References
Alum	• No depot effect • NLRP3 activation *in vivo*? • Independent of TLR signaling • ↑ Local cytokines & chemokines • ↑ cell recruitment (eosinophils, monocytes, macrophages) • ↑ Ag presentation	↑ Ab responses ↑ Th2 responses Poor Th1 responses	Many human vaccines (e.g. DTap, Hib, Hepatitis A, Hepatitis B)	(Franchi and Nùñez, 2008; Gavin et al., 2006; Hutchison et al., 2012; Kool et al., 2008a; McKee et al., 2009)
MF59	• No depot effect • NLRP3 independent but ASC-dependent • Independent of TLR signaling but MyD88-dependent for Ab responses • ↑ Local cytokines & chemokines • ↑ Cell recruitment (neutrophils, macrophages & monocytes) • ↑ Ag uptake • Activate muscle cells • ↑ Ag-loaded neutrophils & monocytes in dLNs	Balanced Th1 and Th2 responses	Licensed for influenza vaccine (Fluad®), H5N1 pre-pandemic vaccine (Aflunov®), H1N1 pandemic vaccines (Focetria® and Celtura®),	(Calabro et al., 2011; Dupuis et al., 1999; Ellebedy et al., 2011; Mosca et al., 2008)
AS04	• MPL signals through TLR4 to activate APCs • ↑ Local cytokines & chemokines • ↑ Cell recruitment (DCs & monocytes) • ↑ Ag-loaded DCs & monocytes in dLNs	↑ Ab responses ↑ Th1 responses	Licensed for human papilloma virus (HPV) (Cervarix™), hepatitis B virus (Fendrix®)	(Didierlaurent et al., 2009)
AS03	• Spatio-temporal co-localization with Ag • Transient ↑ cytokines locally & in dLNs • ↑ Cell recruitment (granulocytes & monocytes) • ↑ Ag-loaded monocytes in dLNs	↑ Ab responses ↑ Immune memory	Licensed for pandemic flu vaccine (Pandemrix®)	(Morel et al., 2011)
Virosomes	• Ag delivery vehicle • Bind APCs & induce receptor-mediated endocytosis. • Escape endosomal degradation • Ag presentation via MHC class II and MHC class I to CD4+ T cells & CD8+ T cells respectively. • Immunopotentiator	↑ Ab responses ↑ CTL responses	Licensed for Inflexal® V and Invivac® influenza vaccine and hepatitis A vaccines (Epaxal®)	(Bungener et al., 2002a; Bungener et al., 2002b; Glück et al., 1992; Khoshnejad et al., 2007)

Abbreviations: Ab: antibody, Ag: antigen, CTL: cytotoxic T lymphocytes, dLNs: draining lymph nodes

1.3 Dendritic cells

1.3.1 Introduction

Innate immune cells recognize evolutionary conserved PAMPS (lipids, nucleic acids, cell wall polysaccharides) or DAMPS (heat shock proteins, purine metabolites such as ATP, adenosine and uric acid), through PRRs. APCs express many PRRs both extracellularly (TLRs and CLRs) and intracellularly (NLRs and RLRs). Recognition of PAMPs leads to activation of APCs and secretion of cytokines and chemokines, which further stimulate and recruit innate immune cells (Kensil et al., 2004; Pashine et al., 2005). Activated APCs possess enhanced antigen uptake, processing and presentation capacities and migrate to the draining lymph nodes. Before reaching lymph nodes, APCs mature and up-regulate the expression of MHC class II and co-stimulatory molecules (CD86, CD83 and CD40) that are required for activation and differentiation of adaptive immune responses (Hoebe et al., 2004; Medzhitov and Janeway Jr, 1997; Singh and O'Hagan, 2002).

In 1868, a German Scientist Paul Langerhans first identified DCs while staining human skin cells. Due to their dendritic morphology, he believed them to be nerve cells but they were actually skin DCs (called Langerhans DCs). Almost 100 years later, a Canadian Scientist Ralph Steinman discovered DCs as a part of the innate immune system and described their role in adaptive immunity (Steinman and Cohn, 1973). This discovery won him the Nobel Prize in Physiology and Medicine in 2011. Due to their critical role in activation of adaptive immune responses or induction of tolerance, DCs are becoming promising targets for treatment of various diseases including cancer, allergies and autoimmune disorders.

Compared to other immune cells, DCs are the only APCs specialized in antigen uptake, processing and presentation to adaptive immune cells. Due to this property DCs are also called professional APCs. Immature DCs (iDCs) act as sentinels in peripheral tissues, where they continuously sample antigenic environment. Upon encounter with pathogenic organisms, byproducts of tissue damage or danger signals, DCs process and present antigens on the MHC class II and up-regulate co-stimulatory molecules expression that are required for effective

interaction with B, T, NK or NKT cells. DCs then migrate and when they reach lymph nodes, the now mature DCs (mDCs) guide the development of immune responses or immune regulation resulting in immunity or tolerance.

1.3.2 Generation and development of DCs

1.3.2.1 *In vivo* generation of DCs

Hematopoietic stem cells (HSC) in bone marrow differentiate into myeloid (MPs) and lymphoid precursors (LPs). MPs give rise to monocyte, macrophages and DC precursors (MDPs), which in turn differentiate into common DC precursors (CDPs) that later differentiate into plasmacytoid DCs (pDCs) and pre-conventional DCs (pre-cDCs). Pre-cDCs circulate in blood and enter lymphoid organs and differentiate into $CD8\alpha^+$ and $CD8\alpha^-$ DCs and in peripheral tissues they give rise to $CD103^+$ lamina propria DCs (Geissmann et al., 2010). During inflammatory conditions, $Ly6C^+$ monocytes might differentiate into DCs that produce copious amounts of TNF and inducible nitric oxide synthase (iNOS), hence called TipDCs (Serbina et al., 2003). DCs have short life span and are non-replicating cells, hence need to be continuously replenished *in vivo* (Kamath et al., 2000).

1.3.2.2 *In vitro* generation of DCs

The use of DC in cancer immunotherapy has gained a lot of interest in recent years. Effective transfection of tumor antigens or pathogens to DCs leads to generation of high quality and quantity of immune responses. However, the pre-requisite in DC therapy is *in vitro* generation of DCs and subsequently maturation of DCs (mDCs), as immature DCs (iDCs) are poor inducers of immune responses. Human DCs can be generated *in vitro* from many sources. The most common source are circulating peripheral $CD14^+$ blood monocytes, $CD34^+$ stem cells from the cord blood, circulating DCs from blood, and bone marrow $CD34^+$ cells. *In vitro* generated bone marrow DCs differentiate into both cDCs and pDCs. Various cytokines are used for *in vitro* generation of DCs such as granulocyte macrophage colony stimulating factor (GM-CSF), IL-4, IL-3, IL-15 and transforming growth factor beta-1 (TGF-β1). GM-CSF and IL-4 are used in combination for generation of DCs from human monocytes (Rossi et al., 1992). Both GM-CSF and IL-4 promote the development and differentiation of bone-marrow stem cells into

DCs. IL-4 plays an role in inhibiting macrophage differentiation and provides a bias towards generation of DCs (Sallusto and Lanzavecchia, 1994). Haemopoietic growth factor, feline McDonough sarcoma (a family of DNA sequences; fms)-like tyrosine kinase ligand (Flt3L) was used *in vivo* to expand early hematopoietic progenitors and DCs. Recently, Flt3L was used *in vitro* to culture human bone marrow stem cells and for expansion of DC precursors (Daro et al., 2000). Flt3L-stimulated BMDCs express CD11c, MHC class II and co-stimulatory molecules and differentiate into both myeloid and lymphoid DC subsets. The expression of co-stimulatory molecules was further up-regulated upon treatment with LPS or IFN-α (Brasel et al., 2000).

1.3.3 Dendritic cell subsets

DC subtypes are classified based on their location within the body, the type of pathogen to which they are exposed, surface markers and distinct immune responses modulated by them (Shortman and Naik, 2007). The developmental relationship between these subtypes, generation of immunity or tolerance in response to pathgens and factors influencing the adaptive immunity are areas of intense research. There are considerable differences between the murine and human DC subsets. However, generally in steady state, we can categorize DCs into tissue resident cDCs and migratory type 1 IFN producing pDCs. cDCs are immature DCs with high phagocytic abilities that are present in the peripheral tissues and continuously sample pathogens. Their main function is to guide the adaptive immune system to mount specific immune responses against harmful invading pathogens or maintain self-tolerance against non-harmful microorganisms (Banchereau and Steinman, 1998). cDCs express myeloid lineage markers (CD11c and CD11b) and are capable of inducing Th1 and Th2 type immune responses. cDCs and pDCs differ drastically in their shape, surface markers, function and location. cDCs show characteristic dendritic morphology whereas pDCs are round cells without dendrites and they resemble antibody producing plasma cells (Shortman and Liu, 2002). Compared to cDCs, pDCs have negligible phagocytic ability and are normally not found in the peripheral tissues. Their main function is to secrete type 1 IFN in response to viral infection, hence were originally known as IFN-producing cells (Siegal et al., 1999; Svensson et al., 1996). pDCs express high levels of CD123 and low levels of CD11c. CD123 is an IL-3 cytokine receptor, hence IL-3 is very important for *in vitro* differentiation of pDCs.

The oldest known subset of DC family is LDCs, which are present in the epidermis of the skin. They typically express intracytoplasmic rod-shaped Birbeck granules (Wolff, 1967). They can be easily identified based on the high expression of CD11c, DEC205 and langerin (CD207) (Pulendran et al., 2008; Valladeau et al., 2000).

Secondary lymphoid organs harbor a DC-like cell population known as follicular DCs (FDCs) in their germinal center and B cell follicles. However, they are distinct from cDCs and produce IL-6 and CXCL13 in large amounts when activated by antigen (Allen and Cyster, 2008; Suzuki et al., 2010).

Tolerogenic DCs (tolDCs) have the capacity to induce antigen-specific unresponsiveness or tolerance in the body. They gain the capacity to induce tolerance in central lymphoid organs and in the periphery via T cell silencing, T cell deletion, immune deviation (polarization of the T cell cytokines) or by induction of regulatory T cells (Tregs) (Steinman et al., 2003). Due to their role in maintaining immune tolerance, immunomodulation of tolDCs have become potential targets for treatment of auto-immune diseases and increasing transplantation efficiency in humans (Thomson, 2010).

The new and most informative way of classifying DCs is based on their phenotypic characteristic, function and location. Based on the location, DCs are classified as splenic and lymph node DCs. Mouse spleen contains three subsets of DCs: 1) $CD8\alpha^{+}$ $CD4^{-}$ $CD11c^{high}$ $CD11b^{-}$ $DEC205^{+}$ lymphoid DCs, 2) $CD8\alpha^{-}$ $CD4^{+}$ $CD11c^{high}$ $CD11b^{+}$ $DEC205^{-}$ myeloid DCs, 3) $CD8\alpha^{-}$ $CD4^{-}$ $CD11b^{-}$ $B220^{+}$ $Gr1^{+}$ plasmacytoid DCs. In addition to these three subsets, lymph nodes contains two more subsets: $CD11c^{high}$ $CD8\alpha^{low}$ $DEC205^{high}$ $langerin^{+}$ langerhans DCs and $CD11c^{high}$, $CD8\alpha^{-}$ $DEC205^{+}$ langerin- dermal DCs (Pulendran, 2004; Shortman and Naik, 2007).

Although CD8 molecules are usually present on T cells, CD8 molecules present on the DC surface are $\alpha\alpha$-homodimers whereas CD8 on classical T cells are $\alpha\beta$-heterodimers. CD4 and CD8 are present on mouse DCs but human DCs express only the CD4 marker. $CD8^{+}$ DCs localize mainly in the T cell area of the spleen and lymph nodes whereas $CD4^{+}$ DC are concentrated in the marginal zones of the spleen and subcapsular sinus of the lymph nodes.

However, $CD4^+$ DCs have been shown to migrate into T cell zones upon stimulation (Iwasaki and Kelsall, 2000; Pulendran et al., 1997; Sousa et al., 1997). Functionally both DC subsets have cross-presentation capacity *in vivo* (den Haan and Bevan, 2002), however $CD8^+$ DCs secrete copious amounts of IL-12 and induce Th1 type immune responses while $CD4^+$ DCs secrete more IL-10 than IL-12 and induce Th2 type responses (Maldonado-López et al., 1999; Pulendran et al., 1999). Further, the functional significance of CD4 and CD8 on DCs has not been reported yet. They are used only for characterization of mouse DC subsets (Shortman and Liu, 2002).

In conclusion, DCs play an important role in determining the type of immune response, which in turn depends on the pathogen encountered, type and level of DC maturation and the cytokine microenvironment in the lymph nodes. Balancing the relationship between various subtypes is important in regulating immunity, maintaining homeostasis and limiting harmful reactions or damage to the host such as allergic conditions and autoimmune diseases.

1.3.4 Phagocytosis

DCs are the only cells known as professional APCs because of their unique ability to phagocytose, process and present various antigens. DCs express diverse receptors (TLRs, CLRs, etc.) on their surface that aid in detecting and phagocytosing a variety of pathogens via clathrin-dependent and clathrin-independent mechanisms (Barral et al., 2008; Savina and Amigorena, 2007; Steinman et al., 1999). Liquid phase particles are taken up via macropinocytosis (Norbury, 2006). However, DCs can also recognize danger signals and DAMP molecules/alarmins released from necrotic cells and leukocytes via various intracellular receptors. Except DCs, all other APCs are involved in destroying and clearing pathogens from the body. However, DCs do not clear the pathogens directly but instead are involved in processing and presentation of specific antigenic peptides via MHC to T helper (Th) cells and subsequently initiating adaptive immune responses.

1.3.5 Maturation

Once DCs take up antigen, they undergo a maturation process to become specialized APCs. Mature DCs attain the following features: reduction of endocytic abilities (which is increased in the first few hours), reduced expression of CCR5 and CCR6 and increased expression of CCR7, which helps trafficking to lymph nodes and increased ability to present

antigen (Ohl et al., 2004; Willimann et al., 1998). Mature DCs also have increased expression of MHC and co-stimulatory molecules (CD80, CD86 and CD40), maturation marker (CD83) and chemokine receptors (CCR7 and CXCR4) (Lechmann et al., 2001; Ohl et al., 2004). They also gain the ability to secrete copious amounts of cytokines and chemokines. On the contrary, iDCs express low levels of MHC and co-stimulatory molecules (Table 1.4). Sometimes DCs do not undergo the maturation process even after pathogen recognition and phagocytosis. Such iDCs are shown to be involved in induction of T cell tolerance (Hawiger et al., 2001; Tan and O'Neill, 2005).

Table 1.4 Characteristics of iDCs and mDCs.

S.no.	Immature DCs (iDCs)	Mature DCs (mDCs)
1.	High intracellular expression of MHC	High surface expression of MHC I and II molecules
2.	High surface expression of PRRs	Low surface expression of PRRs
3.	High phagocytic abilities	Less phagocytic abilites
4.	Low/absent costimulatory molecules (CD40/80/86)	High costimulatory molecules (CD40/80/86)
5.	Low cytokine expression	High cytokine expression
6.	Express CCR5 and CCR6	Express CCR7

1.3.6 Antigen processing and presentation

Antigen presentation by DCs plays a crucial role in linking innate and adaptive immune responses. The type of immune responses activated by DCs depends on the pathogen encountered, tissue environment and maturation signals. Exogenous pathogens are endocytosed and presented mainly on MHC class II molecules on DCs. In the endoplasmic reticulum (ER) of DCs, newly synthesized MHC class II are associated with preformed invariant chain (Ii), which occupies the peptide-binding site (Lamb and Cresswell, 1992; Roche et al., 1991). Later they are transported to the acidic endosmal compartments, where Ii is degraded by lysosomal proteases into contiguous internal segment of Ii (CLIP). Internalized proteins are processed into peptides in endosomes and later loaded on to newly synthesized MHC class II molecules by replacing CLIP with the aid of the non-conventional MHC class II molecule HLA-DM (Bryant and Ploegh, 2004). Degradation of proteins and loading on MHC class II process is further enhanced in mature DCs by enhancement of acidification of endosomal compartments via activation of a proton pump (Trombetta et al., 2003). The antigen-loaded MHC class II complexes are transported to plasma membranes via tubular organelles (Kleijmeer et al., 2001; Savina and Amigorena, 2007).

Endogenous antigens (viral proteins, defective ribosomal products) and exogenous antigens that are processed internally are exclusively presented on MHC class I molecules (Savina and Amigorena, 2007). Intracellular proteins often have ubiquitin attached to them. These ubiquitin-conjugated proteins are degraded into small peptides of eight to ten amino acids by multifunctional protease complexes called proteasomes and later transported into the ER via transporter associated with antigen processing (TAP) (Yewdell and Nicchitta, 2006). An MHC class I molecule consist of α chain and β2-microglobulin and this complex leaves the ER after peptide binding. The assembly of this complex requires various chaperones such as calnexin, calreticulin, ERp57 and tapasin that assist in folding and loading of peptides on the MHC 1 peptide-binding groove (Cresswell et al., 2005; Rock et al., 1990). Peptide-MHC class I complexes exit from the ER and are transported to the cell surface via the Golgi apparatus.

Apart from the traditional pathway, sometimes APCs can switch these pathways resulting in cross-presentation of antigens such as presentation of exogenous antigens via the MHC class I pathway. DCs are the only APCs that have the capacity of cross-presentation (Jung et al., 2002). Cross-presentation of antigens could occur due to destruction and release of acidic compartments carrying exogenous antigens into cytoplasm or fusion of MHC class I molecules with endosomes carrying exogenous antigens (Grommé et al., 1999). This pathway is especially important for cross-presentation of tumor-associated antigens (TAAs) to produce strong cell-mediated immunity against tumor cells. DC-mediated cross-presentation could be further exploited clinically to generate desired immune responses against diseases by vaccination.

1.3.7 Migration of DCs

DCs are strategically positioned throughout the body as immune sentinels to protect against invading pathogens. Upon antigen exposure, DCs traffic to secondary lymphoid organs, where they induce adaptive immune responses. DCs have been shown to continuously migrate towards lymph nodes even in the absence of pathogens or tissue damage probably to induce peripheral tolerance (Kamath et al., 2000). The DCs migration process can be divided into six steps: 1) recognition of mobilizing signals: DCs are mobilized in response to various inflammatory signals including PAMPs, DAMPs and pro-inflammatory cytokines TNF-α and IL-1β (Antonopoulos et al., 2001; Cumberbatch et al., 1999; Enk and Katz, 1992). Exposure to such stimuli induces maturation of DCs, rearranges their chemokine receptor expression, alters expression of adhesion molecules and eventually induces mobility (Granucci et al., 1999). Pro-inflammatory cytokine exposure induces expression of CCR7, which is generally not present on iDCs (Yanagihara et al., 1998). 2) Detachment from the surrounding tissues: adhesion molecules such as E-cadherin are responsible for retention of DCs in the peripheral tissues. TGF-β plays an indirect role in DC retention as it upregulates the expression of E-cadherin on DCs and inhibits its maturation and CCR7 expression (Geissmann et al., 1999). Pro-inflammatory cytokines TNF-α and IL-1β induces DC detachment by reducing E-cadherin expression (Jakob and Udey, 1998). 3) Interstitial migration: Once DCs are detached from tissue they must migrate through extracellular matrix proteins (collagens, fibronectins and laminins) and basement membranes before entering the lymphatic system. To overcome these barriers, mDCs up-regulate matrix-metalloproteinases (MMP)-2 and MMP-9 (Ratzinger et al., 2002), and down-regulate tissue

inhibitors of metalloproteinases (TIMPs) (Darmanin et al., 2007). DC entry into lymphatics is guided by chemokine CCR7 and its ligands, CCL19 and CCL21 (Ohl et al., 2004) and CXCR4 and its ligand CXCL12 (Kabashima et al., 2007). Secretion of TNF-α further increases CCL21 expression by lymphatic endothelial cells, which further chemoattracts DCs towards lymph vessels (Martin-Fontecha et al., 2003). 4) Transit through afferent lymphatic endothelium: steady state migration of DCs through lymphatic endothelium is guided by a chemokine scavenging receptor, D6 and CCX-CKR1 expressed on the lymphatic endothelium (Heinzel et al., 2007; Mantovani et al., 2006). 5) Transit through the afferent lymph: sheer physical force can transport DCs into lymph nodes which is supported by the intercellular adhesion molecules (ICAM)-1 and vascular cell adhesion protein (VCAM)-1 expressed on the lymphatic endothelium (Johnson et al., 2006). However, this mechanism is not clear. 6) DC migration in lymph node: CCR7-CCL19/CCL21 and CCR8/CCL1 axes control DC migration in the lymph node parenchyma (Nakano and Gunn, 2001; Qu et al., 2004).

1.3.8 Development of adaptive immune responses

Once DCs reach lymph nodes, naïve T cells recognize the antigen presented on MHC molecules via specific T cell receptor (TCR). The activation state of DCs, subset of DC presenting antigen as well as presentation of antigen on MHC class I or class II are critical factors in determining the outcome of T cell responses (Mellman and Steinman, 2001). Presentation of antigen by DCs to naïve T cells can lead to induction of effector function or development of tolerance. Three signals are important for generation of effective T cell activation (Fig 1.3). The first signal is recognition of antigen displayed on MHC molecules through TCR on T cells. Antigens presented by MHC class I are recognized by TCR on $CD8^+$ T cells, whereas antigens on MHC class II interact with TCR on $CD4^+$ helper T cells. Formation of prolonged and dynamic interaction between DCs and T cells, called immunological synapse, is required for effective T cell activation. Initially, ICAM-1 on DCs forms a limited interaction with leukocyte function associated antigen 1 (LFA-1) on the T cells (Dustin et al., 2006). This interaction is further stabilized by ligation of co-stimulatory molecules CD80/CD86 with CD28 on T cells, which provides the second signal (Lotze and Thomson, 2001). Additionally, interaction between CD40-CD40L activates DCs to further up-regulates co-stimulatory molecules that in turn helps in stabilizing the immunological synapse (Lanzavecchia and Sallusto, 2001). Cytokines released

from DCs provide the third signal, which determines the differentiation status of the T cells **(**Fig 1.3). A T cell that proliferates in the absence of the third signal fails to induce cytotoxic T cell (CTL) responses (Curtsinger et al., 2003).

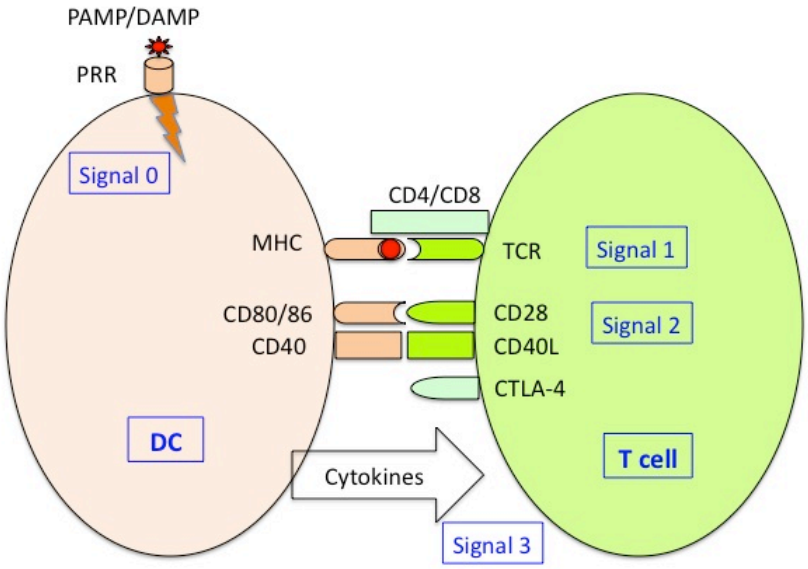


Fig 1.3 Three signals are required for the activation of naïve T cells. In the peripheral tissues DCs recognize pathogens via PRRs, which initiates the activation and maturation of DCs (signal 0). Binding of the antigen-MHC complex on DCs to the antigen-specific TCR on T cells provides signal 1. Signal 2 comprises of binding of co-stimulatory molecules present on the surface of DCs and T cells. Interaction of CD80/86 and CD40 on DCs with CD28 and CD40L on T cells generate activation signals, whereas interaction with CTLA4 leads to inhibitory signals. Secretion of cytokines by DCs gives signal 3 to T cells, which polarizes them, to differentiate towards various phenotypes.

Efficient presentation of antigens from intracellular viruses and bacteria results in Th1-biased responses while Th2-biased responses are generated against extracellular bacteria and parasites (Mosmann et al., 1986). DC-mediated naïve T cell differentiation into various populations depends on the cytokine microenvironment (Heufler et al., 1996). Various T cell types are distinguished based on their cytokine production profiles and immune functions. IL-12 secreted by DCs activates transcriptional factor TBX21 (T-box transcription factor 21), which is responsible for development of Th1 type cells. Th1 cells secrete high amounts of IL-2 and IFN-γ resulting in strong cellular responses (Magram et al., 1996; O'Garra and Robinson, 2004). In addition, Th1 cells modulate B cell isotype switching to immunoglobulin G2a (IgG2a) and can provide help to CTLs, NK and NKT cells. Interleukin-4 is the main cytokine that drives the development of Th2 cells via activation of transcription factor GATA3 (GATA-binding factor 3). Th2 cells secrete IL-4, IL-5 and IL-13, and modulate isotype switching to IgG1 and IgE, thus facilitating induction of potent humoral responses (Kopf et al., 1993; Murphy and Reiner, 2002). Since IL-4 secretion by DCs has not been reported, it was believed that induction of Th2 biased immune responses was due to absence of IL-12 production by DCs. This concept was later disapproved when Ohshima et al showed that ligation of OX40 receptor, a member of TNF receptor family, induces the production of IL-4 by naïve T cells and promotes the differentiation of Th2 type cells (Ohshima et al., 1998).

Fourteen years after Th1 and Th2 responses were identified, another independent type of response, Th17 was described (Infante-Duarte et al., 2000). Differentiation of Th17 cells requires both IL-6 and TGF-β, which induces transcription factors retinoid-related orphan receptor (ROR) RORα and RORγt. Th17 cells produce various cytokines including IL-17, IL-17F, IL-21 and IL-22 (Bettelli et al., 2006). In the absence of IL-6, TGF-β induces the differentiation of regulatory T cells (Tregs) while addition of IL-6 to Tregs results in differentiation into IL-17 producing cells (Xu et al., 2007). Similarly, TGF-β in the presence of IL-4 has been shown to induce differentiation of naïve $CD4^+$ T cells into a new T cell subtype called Th9 cells, which are characterized by production of high levels of IL-9 and IL-10 and no IFN-γ, IL-4 and IL-17 cytokines. Despite production of high levels of IL-10, Th9 cells do not have any regulatory properties (Dardalhon et al., 2008). Follicular helper T (Tfh) cells are yet another $CD4^+$ T cell

population that provides help to B cells, aid in development of germinal centers and promote immunoglobulin class switch recombination and affinity maturation (O'Shea and Paul, 2010).

Although immature DCs do not have the ability to activate adaptive immune responses, they play an important role in maintaining immune tolerance by programming $CD4^+$ $CD25^+$ regulatory T cells (Tregs) (Mahnke et al., 2007). Even mature DCs have been shown to induce *de novo* proliferation of Treg cells (Reis e Sousa, 2006). Treg cells function by producing immunosuppressive cytokines IL-10 and TGF-β, up-regulating forkhead box protein3 (foxp3) expression and inhibiting effector T cell functions (Levings et al., 2002). Broadly, tolerance can be divided into two types: central tolerance, which occurs through clonal deletion in thymus during early development and peripheral tolerance, which is required to control stimulation of the immune system against extra-thymic self-antigens and non-pathogenic antigens (Bluestone and Abbas, 2003). Apart from Tregs, another T helper cell type, Th3 cells have been shown to play a role in peripheral tolerance (Groux et al., 1997). Chronic activation in the presence of IL-10 induces differentiation of $CD4^+$ T cells into Th3 type cells which are characterized by low proliferative ability, high levels of IL-10 and TGF-β production and absence of IL-4 production (Carrier et al., 2007). Th3 type cells are MHC class II restricted T cells that suppress the antigen-specific $CD4^+$ T cell proliferation by production of high levels of IL-10 and TGF-β-mediated activation of the Foxp3 gene in T cells. A high level of IL-10 production is also associated with another regulatory cell known as T regulatory subset 1 (Tr1), however Tr1 cells produce low amounts of TGF-β (Groux et al., 1997).

1.3.9 Cytokine and chemokine production

1.3.9.1 Cytokines

Cytokines are involved in almost every aspect of immunity from growth, activation and differentiation of innate and adaptive immune cells to inducing cell recruitment and determining the nature of the immune response and regulation. Various cytokines are produced by DCs but the predominant ones are TNF-α, pro-inflammatory cytokines IL-1β, IL-18, IL-6, IL-12, IL-10 and IFNs. Tumor necrosis factor including TNF-α derived from mononuclear phagocytes and TNF-β from lymphocytes (therefore formerly known as Lymphotoxin-α) (Beutler and Cerami,

1989). Both TNF-α and TNF-β bind to the same TNF receptor on the cell surface but the outcome of the response (apoptosis or inflammation) depends on the adaptor proteins such as TNF receptor type 1-associated death domain (TRADD) and TNF receptor associated factors (TRAF) (Banner et al., 1993). TNF is involved in various immune functions including activation of neutrophils, induction of intracellular adhesion molecules (ICAM-1, VCAM-1 and E-selectin) thereby mediating chemotaxis and degranulation. It is also one of the cytokines used for *in vitro* DC maturation (Rieser et al., 1997). The adverse effects of TNF are induction of cachexia and fever and it is the primary mediator of sepsis and shock (Tracey et al., 1987).

The interleukin-1 family consists of four important molecules namely, IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra) and IL-18 (Dinarello and Wolff, 1993). Totally there are now 11 members of the IL-1 family (IL-1F1-IL-1F11). IL-1α and IL-1β are pro-inflammatory cytokines with similar biological activities and both of them (along with IL-1ra) interact with the same IL-1 receptor (IL-1R) (Sims et al., 1993). Apart from APCs, IL-1 is produced by endothelial cells, fibroblasts, neutrophils, iNKT cells and γδ T cells. IL-1 and IL-18 are involved in generating inflammatory responses against infection. Similar to TNF, they up-regulate expression of adhesion molecules, recruit immune cells to sites of inflammation/infection and induce fever (Dinarello, 2009). The difference between biological activities of TNF and IL-1 is that TNF is not directly involved in lymphocyte function whereas IL-1 promotes the production of IL-2 and IL-2 receptor thereby activating T cells and IL-1 acts on B cells to induce proliferation and Ig production (Ben-Sasson et al., 2011).

IL-1 and IL-18 are produced as inactive precursors, which are later cleaved by IL-1 converting enzyme (ICE) or caspase-1 to release the active forms (Nett et al., 1992). Neutrophils and macrophages can also process pro-IL-1β and pro-IL-18 via serine proteases such as proteinase-3, elastase and cathepsin-G to secrete active forms (Coeshott et al., 1999; Netea et al., 2010; Sugawara et al., 2001). Both IL-1 and IL-18 play an important role in regulation of immune responses and are considered Th1 cytokines. The inflammatory process induced by IL-1β and IL-18 is controlled by another member of the IL-1 family, IL-1ra, which binds to IL-1R and modulates the immune responses. Due to its important function in controlling inflammation, IL-1ra has been used in the treatment of autoimmune diseases and rheumatoid arthritis.

IL-6 is secreted by DCs, macrophages, osteoblasts, hepatocytes, endothelial cells, T and B lymphocytes; however IL-6 is also produced by smooth muscle cells (hence sometimes called "myokine"). IL-6 acts as pro-inflammatory cytokine and shares various functions with IL-1. In addition, IL-6 has an anti-inflammatory role that includes production of anti-inflammatory cytokines (IL-10 and IL-1ra) thereby antagonizing the effects of TNF-α and IL-1 (Dodge et al., 2003; Kubo et al., 2004).

The IL-12 family consists of three closely related cytokines, IL-12, IL-23 and IL-27. IL-12 consist of two subunits IL-12A (p35) and IL-12B (p40) and the biological active form is a heterodimer. Stimulated APCs produce copious amounts of IL-12, which promotes induction of Th1 type immune responses (Berberich et al., 2003; Koch et al., 1996). IL-12 activates and induces proliferation of T helper, CTL and NK cells. IL-23 specifically acts on memory T cells whereas IL-27 on naïve T cells (Oppmann et al., 2000; Pflanz et al., 2002). In addition, IL-23 is a potent inducer of IFN-γ and like IL-12 it is a Th1 inducing cytokine. Biological activities of IL-12 closely resemble those of IL-18 including IFN-γ release. IL-12 up-regulates the expression of IL-18 receptor (IL-18R), thereby synergizes the effect of IL-18 to stimulate IFN-γ production (Novick et al., 2001).

The IFN family consists of 3 members (IFN-α, IFN-β and IFN-γ). Plasmacytoid DCs are also known as interferon producing cells (IPCs) due to their natural ability to produce high levels of type I IFNs. IFN-α and IFN-β are primarily secreted by APCs whereas T cells, NK cells and NKT cells are the major sources of IFN-γ. IFNs play significant roles in antiviral and antitumor activity (Müller et al., 1994). IFN-γ stimulates APCs to produce cytokines, present antigens via MHC class I and modulates the other effector functions including phagocytosis and killing of intracellular pathogen. IFNs signals through binding to IFN gamma receptors (IFNGR1 and IFNGR2) that leads to downstream signaling of JAK-STAT pathways and induction of immune-related genes (Platanias, 2005). Similar to IL-1, uncontrolled IFN-γ expression is associated with a number of inflammatory and autoimmune diseases.

TGF-β and IL-10 are two important anti-inflammatory cytokines. They are produced by APCs and some T cell subsets. TGF-β producing T helper cells are Tr1, Th3 and Tregs. In addition to inhibiting B and T cell effector responses, TGF-β induces stimulation of fibrosis and wound healing (Sporn and Roberts, 1992). Unlike TGF-β, IL-10 is produced by all subsets of T helper cells including Th1, Th2, CTL and B cells. IL-10 inhibits secretion of cytokines by all T helper cells and APCs (Del Prete et al., 1993). In addition, it inhibits expression of MHC and co-stimulatory molecules leading to inefficient activation of T cells (Ding et al., 1993).

1.3.9.2 Chemokines

Chemokines belong to a large superfamily of mostly small (8-12 KDa) cytokines or proteins that are involved in leukocyte trafficking and recruitment in normal and patho-physiological conditions. Various chemokines are constitutively produced in the peripheral tissues to retain APCs for immune surveillance and in the lymph nodes for regular trafficking of lymphocytes. However, during inflammatory or disease conditions, damaged endothelium and necrotic tissues release inflammatory chemokines to recruit immune cells. Chemokines are classified into four sub-families: C, CC, CXC, and CX3C based on positioning of cysteine residues, where X is a variable amino acid. The CC and CXC families are the largest containing 28 and 16 members respectively. The CC family is exclusively involved in the chemotaxis of monocytes and lymphocytes whereas CXC largely attracts neutrophils.

Chemokines regulate their biological activity by interacting with G protein-coupled transmembrane receptors (GPCRs) called chemokine receptors. Chemokine receptors are expressed on various cells and tissues throughout the body to influence bidirectional cellular movement. Chemokines play a major role in the movement of immune cells across the tight endothelial junction. The magnitude of cellular movement depends on the level of receptor expression on the endothelium (Boldajipour et al., 2008). Chemokine binding to GPCRs leads to intracellular-signalling cascade such as activation of phospholipase C (PLC) and small GTPase, which in turn regulates integrin avidity by increasing integrin affinity and valency (Constantin et al., 2000; Laudanna et al., 2002). Chemokine-mediated increase in integrin avidity arrests the rolling leukocytes on the endothelium. Leukocyte arrest gives them a chance to further prepare to

migrate the endothelial layers. Positive chemokine gradient is required for further movement of leukocytes to the site of infection or damage.

DCs continously patrol the peripheral tissues and carry the information back to the lymph nodes to generate adaptive immune responses. Various chemokines play a critical role in executing DCs functions. DCs utilize various chemokine receptor-ligand pathways such as CCR2-CCL2 (Geissmann et al., 2003; Merad et al., 2002), CCR5-CCL5 (Yamagami et al., 2005) and CCR6-CCL20 (Merad et al., 2004) to retain and migrate in the peripheral tissues. Once DCs encounter pathogens, they undergo maturation and down-regulate these chemokine receptor-ligand pathways. Mature DCs up-regulate CCR7 and CXCR4 receptor to migrate to draining lymph nodes. CCL19 and CCL21 are ligands of CCR7, which are expressed by lymphatic endothelial cells as well as lymph nodes to guide DC migration (Britschgi et al., 2010; Ricart et al., 2011; Saeki et al., 1999; Schumann et al., 2010). CCR7 is also believed to guide DC migration within the lymph nodes, which express high concentrations of CCL19 and CCL21. After entering the T cell zone, DCs themselves start expressing CCL19 to attract naïve and memory T cells as well as other mDCs to transfer antigenic information (Alvarez et al., 2008; Cyster, 2000).

pDCs patrol the blood and enter lymph nodes even in the steady state condition. They engage CXCR3, CXCR4 and CCR5 in steady state whereas upon activation they express high amounts of CCR7, which guide pDCs migration to lymph nodes (Penna et al., 2001; Seth et al., 2011).

Neutrophils provide the first line of defense during infections and hence, are the first cells to reach infection site. Neutrophil chemoattractants such as CXCL1 (also called neutrophil activating protein (NAP)-3 or KC), CXCL2 (also called macrophage-inflammatory protein (MIP-2a), CXCL3 (MIP-2b), CXCL5 and CXCL-8 are mainly produced by the tissue resident macrophages (De Filippo et al., 2008). The receptor CXCR2 binds to most of the neutrophil chemoattractants and it plays a critical role in the earliest recruitment of neutrophils during microbial infections (Reichel et al., 2006; Souto et al., 2011). CCR2-dependent pathways mainly mediate monocyte trafficking during acute inflammatory conditions. CCL2 (also called monocyte

chemoattractant protein (MCP)-1) and CCR7 (MCP-3) are ligands for CCR2 and are involved in monocyte infiltration during inflammatory conditions (Ingersoll et al., 2011; Jia et al., 2008).

Overall, chemokines play a critical role in tissue specific migration of immune cells in normal and diseased conditions. Chemokine-dependent DC migration is crucial to pass antigenic information to lymphocytes and induce adaptive immune responses.

CHAPTER 2. HYPOTHESIS AND OBJECTIVES

2.1 HYPOTHESIS

PCEP is a strong modulator of innate immune responses.

2.2 OBJECTIVES

Our main objective was to investigate the cellular and molecular mechanisms of action of PCEP.

Objective 1. To investigate whether PCEP is a strong modulator of 'adjuvant core response genes' at the site of injection. The following were specific aims to achieve this objective:

1.1) To identify the genes regulated by PCEP at the site of injection by qRT-PCR.
1.2) To investigate the systemic effects of PCEP by assaying serum cytokine concentrations.
1.3) To investigate the local production of cytokines and chemokines post-injection of PCEP by ELISA.

Objective 2. To investigate whether PCEP recruits immune cells at the site of injection. The following were specific aims to achieve this objective:

2.1) To identify the immune cell recruitment at the site of injection of PCEP by flow cytometry.
2.2) To determine changes in cell composition in the draining lymph nodes post-injection of PCEP by flow cytometry.
2.3) To identify the specific cell targets of PCEP by examining the PCEP uptake by recruited immune cells by flow cytometry.
2.4) To determine localization of PCEP in the recruited immune cells by confocal microscopy.
2.5) To evaluate the distribution of PCEP at the injection site by *in vivo* imaging.

Objective 3. To investigate whether PCEP directly activates dendritic cells (DCs) and lymphocytes *in vitro*. The following are specific aims to achieve this objective:

3.1) To assess induction of pro-inflammatory cytokines by PCEP in splenic DCs.

3.2) To determine whether the induction of pro-inflammatory cytokines by PCEP is caspase-1 dependent.

3.3) To determine whether PCEP induces activation and maturation of DCs.

3.4) To determine whether PCEP induces activation and proliferation of T cells and B cells.

3.5) To assess the potential of PCEP to induce antigen-specific $CD8^+$ T cell responses and IFN-γ production.

CHAPTER 3. ACTIVATION OF ADJUVANT CORE REPSONSE GENES BY THE NOVEL ADJUVANT PCEP

(Molecular Immunology (2012); 51: 292-303)

Relationship of this study to the dissertation

Polyphosphazene PCEP has shown great potential as a vaccine adjuvant with various viral and bacterial antigens but their mechanisms of action are poorly understood. Hence, in this first study we investigated the capacity of a novel adjuvant PCEP to induce adjuvant core response genes (cytokines, chemokines, innate immune receptors, interferon-induced genes and adhesion molecules) at the site of injection and local production of cytokines and chemokines after intramuscular injection in mice. These studies suggest that PCEP adjuvant activity depends on strongly activating early innate immune responses and promoting a strong immuno-stimulatory environment at the site of injection.

3.1 Introduction

Adjuvants are natural or synthetic substances that can enhance or modulate immune responses to a co-administered antigen. Ideally, an effective adjuvant will promote strong cell-mediated as well as humoral immunity, and contribute to antigen sparing and/or eliminate the need for booster immunizations. Many diverse classes of compounds have been assessed as adjuvants including microbial products, mineral salts, emulsions, microparticles and liposomes (Fraser et al., 2007). For over 80 years, insoluble aluminium salts have been the most widely used vaccine adjuvant in humans and animals primarily due to their high safety profile. However, their safety might be questionable since alum has been associated with causing vaccine-associated feline sarcomas in cats (Hendrick et al., 1992; Morrison and Starr, 2001). Secondly, alum mainly induces Th-2 type immune responses (humoral response) and is a poor Th-1 adjuvant (Grun and Maurer, 1989). Therefore alum is not an appropriate adjuvant with many vaccines such as HIV or TB that require Th-1 type immune responses for protection (Hunter, 2002). In addition to alum, two oil-in-water emulsions and one combinational adjuvant have recently been approved for use

in humans (De Gregorio et al., 2008). These licensed adjuvants do not induce optimal immune responses with all the vaccine antigens, promote primarily antibody production and have limited ability to induce cell-mediated immune responses. Therefore, novel vaccine adjuvants with a good safety profile and an ability to induce cell-mediated immunity are under investigation.

Particulate adjuvants such as liposomes, virosomes, ISCOMs (immune stimulatory complexes), or virus like particles are immunostimulatory in nature and act by encapsulating and enhancing the delivery of antigen to antigen presenting cells (APCs) (Wilson-Welder et al., 2009). CpG oligodeoxynucleotides (ODN), a strong immunostimulatory adjuvant, has been shown to induce Th1 type of immune response and enhance production of pro-inflammatory cytokines through activation of TLR9 (Klinman, 2004).

Despite the wide use of adjuvants in billions of vaccine doses, the mechanisms that mediate adjuvant activity remain poorly understood. Understanding the mechanisms of action of adjuvants will provide critical information on how innate immunity influences the development of adaptive immunity. Recent studies are beginning to unveil the mystery behind adjuvant action. It was proposed that at the injection site, alum adsorbs antigen and increases uptake by antigen presenting cells (APCs) (Morefield et al., 2005). It is now known that *in vitro,* alum activates NLRP3 inflammasome complex leading to caspase-dependent production of IL-1β, IL-18 and IL-33 in dendritic cells (DCs) and macrophages (Kool et al., 2008a; Li et al., 2007). Despite the compelling *in vitro* evidence on alum-induced inflammasome activation, various *in vivo* studies yielded conflicting results on the role of inflammasomes in mediating the adjuvant activity of alum (Eisenbarth et al., 2008; Franchi and Nùñez, 2008; Li et al., 2008; McKee et al., 2009). An emulsion based adjuvant, MF59, promotes antigen uptake by APCs, induces secretion of cytokines and chemokines at the site of injection, increases recruitment of immune cells and promotes the differentiation of monocytes towards dendritic cells (Calabro et al., 2011; Mosca et al., 2008; Seubert et al., 2008). Taken together, these studies suggest adjuvants activate complex molecular and cellular pathways within the innate immune system to enhance antigen specific immune responses and this primarily involves activation of DCs.

One promising class of new vaccine adjuvants are polyphosphazenes. They are high molecular weight water-soluble synthetic polymers with a backbone comprised of alternating phosphorus and nitrogen atoms and organic side groups attached to each phosphorus (Andrianov et al., 2006; Andrianov et al., 2004). The two most investigated polyphosphazene polyelectrolytes are poly[di(carboxylatophenoxy)phosphazene] (PCPP) and poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) (Mutwiri et al., 2009). They have been shown to drastically enhance the immune responses when co-administered with a variety of bacterial and viral antigens (McNeal et al., 1999; Payne et al., 1998a; Payne et al., 1998b; Wu et al., 2001b). PCEP has been shown to possess significantly stronger adjuvant activity that exceeds that of PCPP and alum in mice immunized with influenza virus X:31 or HBsAg (Eng et al., 2010a; Mutwiri et al., 2008; Mutwiri et al., 2007b). PCEP efficiently promotes a mixed Th1/Th2 type response, both mucosally and systemically, giving broad-spectrum immunity (Eng et al., 2010b; Mutwiri et al., 2007b).

The mechanisms that mediate the adjuvant activity of polyphosphazenes are poorly understood. A study by Payne et al suggested that polyphosphazenes do not mediate their adjuvant activity through formation of a depot at the site of injection (Payne et al., 1998a). Rather, their adjuvant activity has been attributed to their physical association with antigen (Andrianov et al., 2005). It has been proposed that polyphosphazenes form a water-soluble, non-covalent protein-polymer complex that delivers or transports antigen to immune cells but this remains to be proven (Andrianov et al., 2005). It has recently been proposed that the powerful adjuvant activity of polyphosphazenes is a consequence of strong activation of innate immunity (Mutwiri et al., 2008). In this regard, *in vitro*, PCEP stimulates significant production of the innate cytokines IL-12 and IFN-γ suggesting that activation of innate immunity may be important in mediating its adjuvant activity (Mutwiri et al., 2008). However, whether polyphosphazenes induce any innate immune responses *in vivo* has not been investigated. Hence, the present studies were undertaken to determine whether PCEP has any effect on local innate immune responses *in vivo*.

3.2 Materials and methods

3. 2.1 Animal experiments

Four to six week old female BALB/c mice (Charles River Laboratories, North Franklin, CT, USA) were injected intramuscularly (i.m.) in the quadriceps muscle with 25 μl of either phosphate-buffered saline (PBS) as control, 50 μg PCEP, or 10 μg of CpG-1826 (a potent adjuvant in mice) per animal. These doses were selected based on previous experiments (Mutwiri et al., 2008; Mutwiri et al., 2007a). Muscle tissue from the site of injection and sera were taken from all six mice per group at 3, 6, 12, 24, 48 and 96 hours after treatment. The animal experiments were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane use of animals.

3.2.2 Adjuvants

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). PCEP was dissolved in Dulbecco's PBS (Sigma, St. Louis, MO, USA) before using for animal experiments. The CpG ODN 1826 (5'-TCCATGACGTTCCTGACGTT-3') was provided by Merial (Lyon, France).

3.2.3 Quantitative Real-time PCR *(qRT-PCR)*

Immediately after mice were euthanized, whole muscle tissues from the thigh were collected in TRIzol (Invitrogen) and aseptically homogenized with 2.3 mm Zirconia microbeads (Biospec Products Inc., Bartlesville, OK) in a Mini-Beadbeater™ (Biospec Products Inc.). The homogenates were centrifuged for 1 min at 10,000 x g, and the supernatents were collected for total RNA extraction as per the manufacturer's instruction. The extracted RNA was quantified and treated with DNase (Invitrogen). The cDNA was synthesized using random hexamers (Applied Biosystems) and SuperScript® II Reverse Transcriptase (Invitrogen) as per manufacturer's instruction. All PCR reactions were carried out in duplicate in 96-well plates with optical quality tape (Bio-Rad) using an iCycler iQ® Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each PCR reaction contained 1 μl target cDNA, 0.2 μM each of forward and

reverse primers, 7.5 µl of iQ SYBR® Green Supermix (Invitrogen) and distilled water to make 15 µl of final volume according to manufacturer's instruction. The negative control contained all the reagents except cDNA. All the primers used in qRT-PCR are shown in Table 3.1. Reference genes GAPDH, RPL19 and18s rRNA were analyzed and the best (GAPDH) was selected for further analysis. Amplification was performed by initial denaturation at 95 °C for 3 min in cycle 1, followed by cycle 2 (95 °C, 15 s; 55 °C, 30 s; 72 °C, 30 s) ×45 and then cycle 3, the Melt curve analysis, was preset at 55 °C ramping to 95 °C with 1 °C increase each 10 s and final hold at 20 °C. A Melt Curve analysis was performed to ensure that any product detected was specific to the desired amplicon.

Table 3.1 List of primers used for qRT-PCR

Gene symbol	*Forward sequence (5'→3')*	*Reverse sequence (5'→3')*
IL-1β	5'-GTGTGGATCCCAAGCAATAC-3'	5'-GTCCTGACCACTGTTGTTTC-3'
IL-2	5'-CCTGGAGCAGCTGTTGATGG-3'	5'-CAGAACATGCCGCAGAGGTC-3'
IL-4	5'-ATGGGTCTCAACCCCCAGC-3'	5'-GCTCTTTAGGCTTTCCAGG-3'
IL-6	5'-TGTCTATACCACTTCACAAGTC-3'	5'-GCACAACTCTTTTCTCATTTCCA-3'
IL-10	5'-TAGTTCCCAGAAGCCATGTG-3'	5'-AGAGGGAGCAGTTTGTAAGC-3'
IL-12	5'-TGCCAGCCTGCCTTATATTG-3'	5'-TCCACCAGGACCACTAAATG-3'
IL-13	5'-CAGCAGCTTGAGCACATTTC-3'	5'-CATAGGCAGCAAACCATGTC-3'
IL-17	5'-ACCTCAACCGTTCCACGTCA-3'	5'-CAGGGTCTTCATTGCGGTG-3'
IL-18	5'-ATGGCTGCCATGTCAGAAGAC-3'	5'-CTAACTTTGATGTAAGTTAGT-3'
IFN-γ	5'-TGAACGCTACACACTGCAT-3'	5'-CGACTCCTTTTCCGCTTCCT-3'
TNF-α	5'-GACCCTCACACTCAGATCATCT-3'	5'-CCACTTGGTGGTTTGCTACGA-3'
NLRP3	5'-TCTACTCTATCAAGGACAGG-3'	5'-CCCAATGTGCTCGTCAAAGG-3'
NFκB	5'-AGAAGACACGAGGCTACAAC-3'	5'-TCACAGACGCTGTCACTATC-3'
BCL-2	5'-CAGAAGATCATGCCGTCCTT-3'	5'-GTCTACTTCCTCCGCAATGC-3'
CCL-2	5'-TCACCTGCTGCTACTCATTC-3'	5'-TCTGGACCCATTCCTTCTTG-3'
CCL-4	5'-CCAGCTGTGGTATTCCTGAC-3'	5'-GAGCTGCTCAGTTCAACTCC-3'
CCL-5	5'-CTCCCTGCTGCTTTGCCTAC-3'	5'-CACACTTGGCGGTTCCTTCG-3'
CCL-12	5'-TGCCTCCTGCTCATAGCTAC-3'	5'-GGCTGCTTGTGATTCTCCTG-3'
CXCL-10	5'-GTCACATCAGCTGCTACTCC-3'	5'-CGCACCTCCACATAGCTTAC-3'
TLR-4	5'-TCCCAGTGATGGCTGATTAG-3'	5'-GCACCCAACATTGTGTTACC-3'
TLR-9	5'-GAAGGGACAGCAATGGAAAG-3'	5'-GCCAAGTGCTACCATTAACC-3'
IL-1RA	5'-GGCAACTGGTAACCGTTGAG-3'	5'-AGAGGCAGGAGATGACAAGG-3'
Fcgr1	5'-TGAGGTGTCACGGATGGAAG-3'	5'-TGCCTGAGCAGTGGTAGATG-3'
Fcgr4	5'-ACAATGACAGTGGCTCCTAC-3'	5'-TCCTATCAGCAGGCAGAATG-3'
Fcer1g-M	5'-GCCGCAGCTCTGCTATATCC-3'	5'-GTGTTCAGGCCCGTGTAGAC-3'
Fcer1g	5'-CAGCCGTGATCTTGTTCTTG-3'	5'-TTTCGGACCTGGATCTTGAG-3'
Lilrb3	5'-GGTAACTTCAGGAGGGTATG-3'	5'-CGTGGTACTTCCTTGTAGAG-3'
Lilrb4	5'-TCCCAGCCTGTCAGTCTATC-3'	5'-GAGAGGCCATGCTTTCCTTC-3'
Ltb4r1	5'-CATGAGTCTGGACCGATCAC-3'	5'-GGTACACAAGGACCGGTATG-3'
Klra18	5'-AACAGAGCTGCCAGAATTCC-3'	5'-AGATGGGCGATTGTCAATCC-3'
Msr1	5'-AGGGCTTACTGGACAAACTG-3'	5'-TGATCTTGATCCGCCTACAC-3'
Tnfrsf1b	5'-CCTGTGGATGCTGAGGAAAC-3'	5'-GGCTTCCGAGATGACAGAAC-3'
Csf2rb1	5'-CAGCACTGTCAGGCTCCTTG-3'	5'-CTGGACCCACACTGCACATC-3'
Hrh2	5'-AGGCCAAGAAGTGAGTGTAG-3'	5'-TGCCAGCAACAGTGATGAAG-3'
Ifit2	5'-GCACTGCAGAGGTCTAAATG-3'	5'-CAGATAAGCCTGAGCCTTTG-3'
Ifit3	5'-GTGCCGTTACAGGGAAATAC-3'	5'-TCTCTACTTCCGGGAAATCG-3'
Ifi-47	5'-ATGAATCCGCTGATGTTGGG-3'	5'-AAGCGTCTGCGTGGAAATTG-3'
Mx1	5'-CTCTGCTGTACTGCTAAGTC-3'	5'-GCCTTGGTCTTCTCTTTCTC-3'
Mx2	5'-ATTACCAGGGTGGCTGTAGG-3'	5'-ACCACCAGGTTGATGGTCTC-3'
Oasl1	5'-TGGACCTTGGGCTCAGTAAC-3'	5'-GCACAACGGTGACAGTGATG-3'
Oasl2	5'-CTAAGACACCTGCACAGATG-3'	5'-GGGTTAGGCTAGGTTATTCC-3'
Oas1f	5'-ACTGCACTCAAGAGCAAGTC-3'	5'-AGCTCTGCACCTCAAACTTC-3'
Ifi202b	5'-CATCTGTCCCAGGCAATGTC-3'	5'-GAGAGGCTTGAGGTTGATCC-3'
Ifi204	5'-CAGGTGCCAGTCACCAATAC-3'	5'-CAGTGAGCACCATCACTGTC-3'
Igtp	5'-GACTCTGGCAATGGCATGTC-3'	5'-AGGAGTAGCAGGCTGGTTTC-3'

Iigp1	5’-GCAGATGGCAAACCTCAAAC-3’	5’-AGATTGGTGGCTCAGCAATG-3’
Irf7	5’-GTTTGGAGACTGGCTATTGG-3’	5’-AGATCCCTACGACCGAAATG-3’
Garg49	5’-GAGGATGGCAGAACTGAGAC-3’	5’-GGGCTCTCCTTACTGATGAC-3’
Stat2	5’-CTTCCTACTGCGCTTCAGTG-3’	5’-GCGGATGATCTCTGTCAGTG-3’
Psmb8	5’-AGTTCCAGCATGGCGTCATC-3’	5’-ATGGTGCCAAGCAGGTAAGG-3’
Gvin1	5’-AGATGTGTCGTGTCTCTACC-3’	5’-GACAGAACCAGCAGATTTCC-3’
Ifnar2	5’-ACTACATCGTGCCTGCAAAC-3’	5’-GGCTCGTGCTTCTTCCTAAC-3’
Clec4a1	5’-CAAAGTCTGGAGCTGTTGTC-3’	5’-CTCTGGATCACCAGCAGATG-3’
Clec4a2	5’-TACCGTTGGAAGACTGGATG-3’	5’-TCCCAGGTGTCTGTGTAATG-3’
Clec4a3	5’-GGAAGCCGTTTGGTTCCTAC-3’	5’-CTGTGGATCACCACCAGATG-3’
Lgals3	5’-TCCCGAAGAATCGAGGTCAG-3’	5’-AAAGCCGTCCACAGTAGTCC-3’
Gapdh	5’-TTGATGGCAACAATCTCCAC-3’	5’-CGTCCCGTAGACAAAATGGT-3’
18s rRNA	5’-CGGCTACCACATCCAAGGAA-3’	5’-GCTGGAATTACCGCGGCT-3’
RPL19	5’-CTGAAGGTCAAAGGGAATGTG-3’	5’-GGACAGAGTCTTGATGATCTC-3’

3.2.4 Cytokine detection in mice

Cytokine concentrations were assayed in serum and in muscle tissue obtained from the site of injection from each mouse. To detect the cytokine concentration at the site of injection, the muscle tissues were homogenized in 1 ml PBS with protease-inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were centrifuged at 20,000 g for 10 min. Cytokine and chemokine concentrations were determined for IL-1β, IL-18, TNF-α, IL-12, IL-6, CXCL-10, and CCL-2. Saline-injected mice were used as controls. With the exception of IL-18, all the cytokine and chemokine concentrations were determined using DuoSet ELISA development system (R&D Systems, Minneapolis, MN, USA) following manufacturer's protocol. For IL-18, Immunol II microtitre plates (Dynex Technology Inc., VA, USA) were coated overnight with anti-mouse IL-18 antibody (MBL, Japan) at 2 μg/ml concentration at room temperature (RT). The microtitre plates were washed 3 times with Tris-buffered saline (pH 7.3) containing 0.05% Tween 20 (TBST) and 100 μl of diluted sera or tissue samples were added to the wells and incubated for 2 hrs. The wells were washed again 3 times with TBST and biotinylated rat anti-mouse IL-18 antibody (MBL, Japan, 1/1000) was added to the wells in a 100 μl volume and incubated for 2 h at RT. Wells were washed and horseradish-peroxidase conjugated streptavidin was added to each well followed by incubation for 20 min at RT. Wells were washed 3 times with TBST before addition of 3,3',5,5'- Tetramethylbenzidine (TMB) (Sigma-Aldrich Canada Ltd.). The microtitre plates were incubated for 20 min at RT before stopping the reaction with 2 N sulphuric acid. For all the cytokines and chemokines, the absorbance was read as optical density (OD) at 570 nm in a Microplate Reader (Bio-Rad Laboratories). The samples were assayed in triplicate, and the cytokine concentration was determined by extrapolation from a standard curve generated by serial dilution of the respective appropriate recombinant murine cytokines.

3.2.5 Statistical analysis

The increase in target gene expression levels in PCEP and CpG-1826 stimulated muscle tissues were calculated as fold change increase ($2^{-\Delta\Delta CT}$). Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). The differences in fold change of gene expression were calculated by using non-parametric student t-test and Mann-Whitney test. The results were assumed statistically significant at *P<0.05 and **P<0.005.

3.3 Results

3.3.1 *Temporal transcriptional responses elicited by CpG 1826, PCEP or saline at the site of injection.*

Mosca et al., reported that a set of common genes were stimulated by the adjuvants MF59, alum and CpG and referred these genes as "adjuvant core response genes" (Mosca et al., 2008). These genes included cytokines, chemokines, innate immune receptors, interferon-induced genes and adhesion molecules (listed in table 3.2). In the present study, we investigated whether PCEP-induced differential expression of the "adjuvant core response genes" at the site of injection. We expanded this list of genes to include IL-1β, IL-4, IL-10, IL-12, IL-17, IL-18, IFN-γ, IL-6, TNF-α, Bcl-2, NFκb, inflammasome receptor (NLRP3), TLR-4, and TLR-9 genes (listed in table 3.2).

The temporal expression profiles of the selected genes of interest were analyzed by qRT-PCR at 3 h, 6 h, 12 h, 24 h, 48 h and 96 h following administration of PBS, PCEP or CpG 1826. Global changes in gene expression were analyzed relative to the time matched saline controls. At the site of injection, PCEP injection lead to up-regulation of cytokines and chemokines (Fig 3.1), innate-immune receptors (Fig 3.2) and interferon-induced genes (Fig 3.3). PCEP up-regulated Th1 type IL-2 and IFN-γ (Fig 3.1a), Th17 type IL-17 and IL-6 (Fig 3.1a) and Th2 type IL-4, IL-10, and IL-13 (Fig 3.1b) cytokines with expression generally being increased over time. In a similar pattern, CpG up-regulated the gene expression of

cytokines at later time points. PCEP induced 14 to 21-fold gene expression of TNF-α at 12 to 48 h. However, CpG induced significant TNF-α expression as early as 3 h with 39-fold increase but the levels dropped sharply by 6 h (Fig 3.1a). None of the adjuvants had any effect on IL-12 gene expression pattern (Fig 3.1b). It was interesting that the major transcription factor NF-κB was up-regulated 10-fold by PCEP at a later time point (96 h), whereas its expression remained unchanged in CpG-injected muscle tissues (Fig 3.1c). Although CpG is a TLR9 agonist, PCEP more potently increased the expression of TLR9 as well as TLR4 genes at the site of injection (Fig 3.1c). Further, we observed that at the injection site, PCEP and CpG were potent inducers of chemokine genes CCL-2, CCL-4, CCL-5, CCL-12 and CXCL-10 (Fig 3.1d). CCL-2 and CCL-12 genes, also known as monocyte chemotactic protein-1 (MCP-1) and monocyte chemotactic protein-5 (MCP-5) respectively, were highly up-regulated in PCEP-injected mice by more than 300-fold at 96h but in contrast, were up-regulated to a substantially lesser extent of between 20-30-fold in CpG-injected mice. CXCL-10 gene was highly induced by CpG as early as 3 h by 480-fold, to a 1000-fold by 6 h and up to 100-fold by 96 hour (Fig 3.1d).

Table 3.2 List of adjuvant core response genes.

Gene symbol	*Accession no.*	*Gene name*
Cytokines		
IL-1β	NM_008361	Mus musculus interleukin 1 beta (Il1b), mRNA [NM_008361]
IL-2	NM_008366	Mus musculus interleukin 2 (Il2), mRNA [NM_008366]
IL-4	AF352783	Mus musculus IMAGE:578022 interleukin 4 mRNA, complete cds
IL-6	NM_031168	Mus musculus interleukin 6 (Il6), mRNA
IL-10	M84340	Mouse interleukin 10 (IL10) gene, complete cds
IL-12	AL669944	Mouse DNA sequence from clone RP23-388G23 on chromosome 11 Contains the 3' end of the Il12b gene for interleukin 12b (Il12b)
IL-13	NM_008355	Mus musculus interleukin 13 (Il13), mRNA [NM_008355]
IL-17	NM_010552	Mus musculus interleukin 17A (Il17a), mRNA
IL-18	NM_008360	Mus musculus interleukin 18 (Il18), mRNA
IFN-γ	AK089574	Mus musculus activated spleen cDNA, RIKEN full-length enriched library, clone:F830002I10 product:interferon gamma, full insert sequence
TNF-α	NM_013693	Mus musculus tumor necrosis factor (Tnf), mRNA
NLRP3	NM_145827	Mus musculus NLR family, pyrin domain containing 3 (Nlrp3), mRNA
NFκB	AY521463	Mus musculus nuclear factor kappa B (Nfkb1) mRNA, complete cds
BCL-2	AK042257	Mus musculus 3 days neonate thymus cDNA, RIKEN full-length enriched library, clone:A630075L21 product:inferred: B-cell leukemia/lymphoma 2 / bcl2-alpha, full insert sequence
TLR-4	AF177767	Mus musculus toll-like receptor 4 (Tlr4) gene, Tlr4A allele, complete cds
TLR-9	AY649790	Mus musculus strain BALB/c toll-like receptor 9 (Tlr9) gene, promoter region and partial cds
Chemokines		
CCL-2	NM_011333	Mus musculus chemokine (C-C motif) ligand 2 (Ccl2), mRNA
CCL-4	NM_013652	Mus musculus chemokine (C-C motif) ligand 4 (Ccl4), mRNA []
CCL-5	NM_013653	Mus musculus chemokine (C-C motif) ligand 5 (Ccl5), mRNA
CCL-12	NM_011331	Mus musculus chemokine (C-C motif) ligand 12 (Ccl12), mRNA
CXCL-10	NM_021274	Mus musculus chemokine (C-X-C motif) ligand 10 (Cxcl10), mRNA
Immune cell receptors		
IL-1ra	DQ383807	Mus musculus strain A/J IL-1 receptor antagonist (Il1rn) gene, complete cds
Fcgr1	AF143181	Mus musculus strain AB/H (Biozzi) high affinity immunoglobulin gamma Fc receptor I (Fcgr1) mRNA, Fcgr1-d allele, complete cds.
Fcgr4	NM_144559	Mus musculus Fc fragment of IgG, low affinity IIIa, receptor (Fcgr3a), mRNA
Fcer1g-M	AI326608	mm74d09.y1 Stratagene mouse macrophage (#937306) Mus musculus cDNA clone IMAGE:534161 5' similar to gb:J05020 Mouse mast cell high affinity IgE receptor (MOUSE)
Fcer1g	NM_010185	Mus musculus Fc receptor, IgE, high affinity I, gamma polypeptide (Fcer1g), mRNA

Lilrb3	NM_011095	Mus musculus leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3 (Lilrb3), mRNA
Lilrb4	NM_013532	Mus musculus leukocyte immunoglobulin-like receptor, subfamily B, member 4 (Lilrb4), mRNA
Ltb4r1	NM_008519	Mus musculus leukotriene B4 receptor 1 (Ltb4r1), mRNA
Klra18	NM_053153	Mus musculus killer cell lectin-like receptor, subfamily A, member 18 (Klra18), mRNA
Msr1	NM_031195	Mus musculus macrophage scavenger receptor 1 (Msr1), mRNA
Tnfrsf1b	NM_011610	Mus musculus tumor necrosis factor receptor superfamily, member 1b (Tnfrsf1b), mRNA
Csf2rb1	TC1516250	CYRB_MOUSE (P26955) Cytokine receptor common beta chain precursor (CDw131 antigen) (GM-CSF/IL-3/IL-5 receptor common beta-chain), partial (21%)
Hrh2	NM_008286	Mus musculus histamine receptor H 2 (Hrh2), transcript variant 2, mRNA
Interferon induced genes		
Ifit2	NM_008332	Mus musculus interferon-induced protein with tetratricopeptide repeats 2 (Ifit2), mRNA
Ifit3	NM_010501	Mus musculus interferon-induced protein with tetratricopeptide repeats 3 (Ifit3), mRNA
Ifi-47	NM_008330	Mus musculus interferon gamma inducible protein 47 (Ifi47), mRNA
Mx1	NM_010846	Mus musculus myxovirus (influenza virus) resistance 1 (Mx1), mRNA
Mx2	NM_013606	Mus musculus myxovirus (influenza virus) resistance 2 (Mx2), mRNA
Oasl1	NM_145209	Mus musculus 2'-5' oligoadenylate synthetase-like 1 (Oasl1), mRNA
Oasl2	NM_011854	Mus musculus 2'-5' oligoadenylate synthetase-like 2 (Oasl2), mRNA
Oas1f	NM_145153	Mus musculus 2'-5' oligoadenylate synthetase 1F (Oas1f), mRNA
Ifi202b	NM_011940	Mus musculus interferon activated gene 202B (Ifi202b), mRNA
Ifi204	NM_008329	Mus musculus interferon activated gene 204 (Ifi204), mRNA
Igtp	NM_018738	Mus musculus interferon gamma induced GTPase (Igtp), mRNA
Iigp1	NM_021792	Mus musculus interferon inducible GTPase 1 (Iigp1), mRNA
Irf7	NM_016850	Mus musculus interferon regulatory factor 7 (Irf7), mRNA
Garg49	AK077243	Mus musculus 11 days pregnant adult female ovary and uterus cDNA, RIKEN full-length enriched library, clone:5031412D17 product:interferon-induced protein with tetratricopeptide repeats 3, full insert sequence
Stat2	NM_019963	Mus musculus signal transducer and activator of transcription 2 (Stat2), mRNA
Psmb8	NM_010724	Mus musculus proteosome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7) (Psmb8), mRNA
Gvin1	NM_029000	Mus musculus GTPase, very large interferon inducible 1 (Gvin1), transcript variant A, mRNA
Ifnar2	NM_010509	Mus musculus interferon (alpha and beta) receptor 2 (Ifnar2), mRNA
Clec4a1	NM_199311	Mus musculus C-type lectin domain family 4, member a1 (Clec4a1), mRNA
Clec4a2	NM_011999	Mus musculus C-type lectin domain family 4, member a2 (Clec4a2), mRNA
Clec4a3	NM_153197	Mus musculus C-type lectin domain family 4, member a3 (Clec4a3), mRNA
Lgals3	NM_011150	Mus musculus lectin, galactoside-binding, soluble, 3 binding protein (Lgals3bp), mRNA

Fig 3.1a Th1 and Th17 cytokines

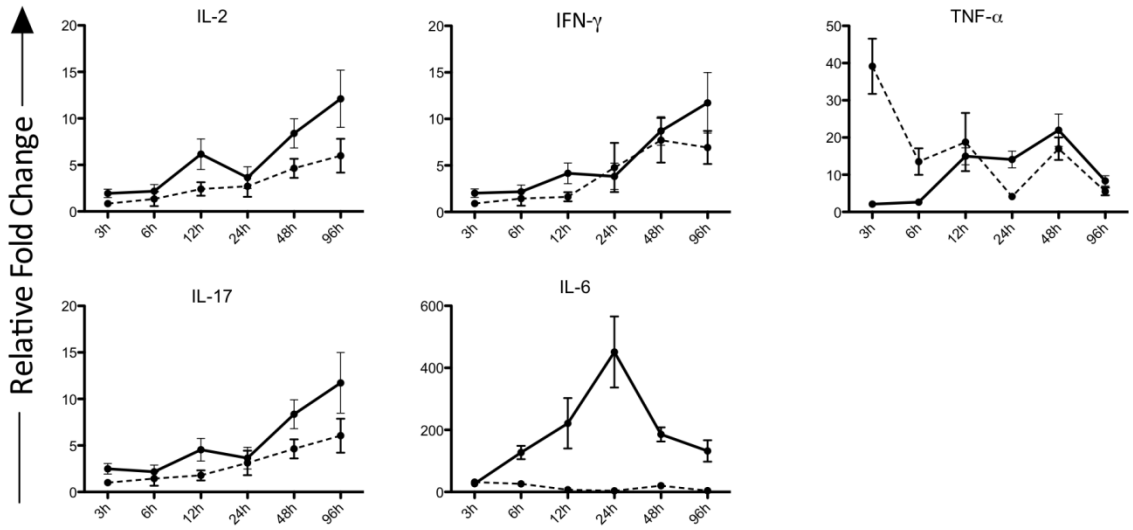


Fig 3.1b Th2 cytokines and IL-12

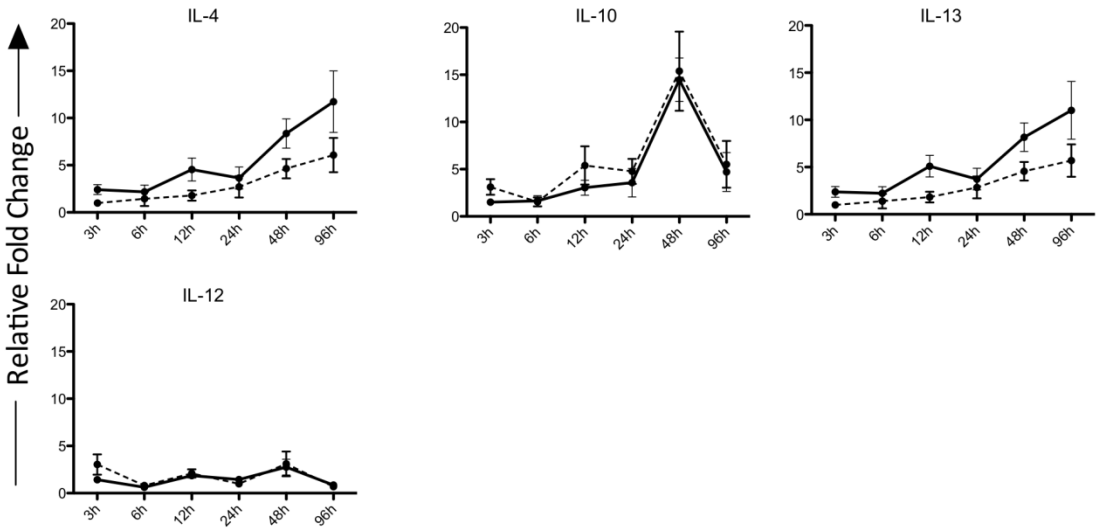


Fig 3.1c NFκB and TLRs

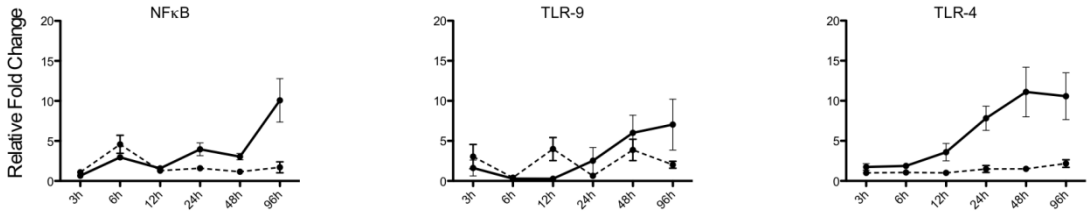


Fig 3.1d Chemokine

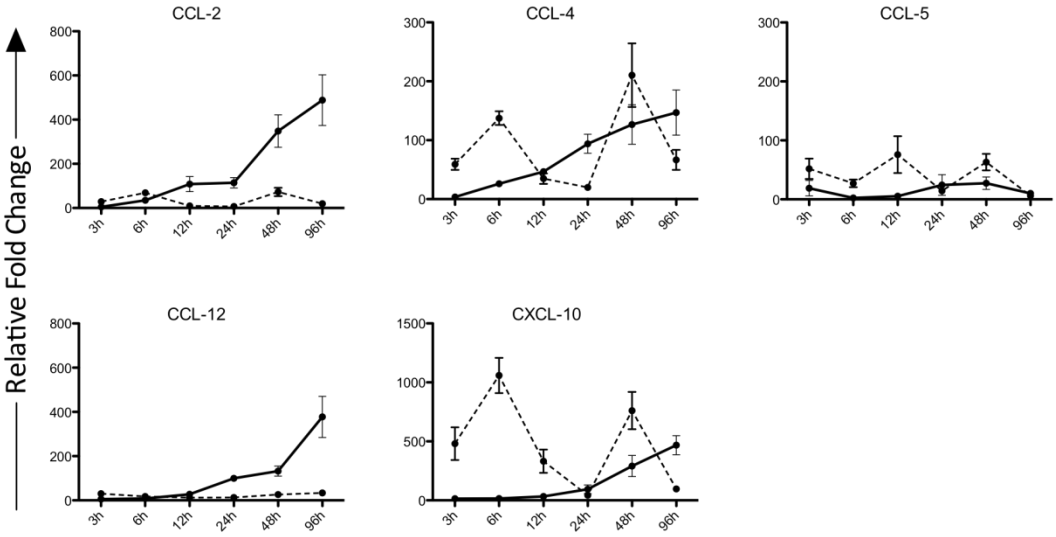


Fig 3.1 Cytokine and chemokine gene expression profiles elicited by PCEP and CpG at the site of injection after intramuscular injection in mice. Mice were injected with PBS, PCEP or CpG intramuscularly. Muscle tissue were collected at 3, 6, 12, 24, 48 and 96 h and analyzed for cytokine and chemokine genes by quantitative real-time PCR. Gene expression profiles in PCEP-injected muscle tissue are shown in bold line whereas CpG-injected muscle tissues are in dotted line. Results shown are the mean ± SE of six replicates at each time point. Relative fold changes (y-axis) for each gene were normalized to mouse GAPDH. Fold changes are calculated by the Ct method and are relative to the gene expression in PBS-injected muscle tissue.

Similarly, PCEP induced significant expression of immune cell receptor genes in a time dependent manner at the site of injection. In particular, the members of leukocyte immunoglobulin-like receptor family (Lilrb3 and Lilrb4), Leukotriene (Ltb4r1) (Fig 3.2a) and macrophage scavenger receptor 1 (Msr1) (Fig 3.2c) were up-regulated as high as 100-400 fold by 96 hours. Furthermore, PCEP and CpG administration induced high expression of FC receptors such as Fcgr1, Fcgr4 and Fcer1g at the injection site with CpG being strongest inducer of Fcgr4 with 1000-fold upregulation by 96 hour (Fig 3.2b).

Fig 3.2a Immunoglobulin-like and Leukotrienem receptors

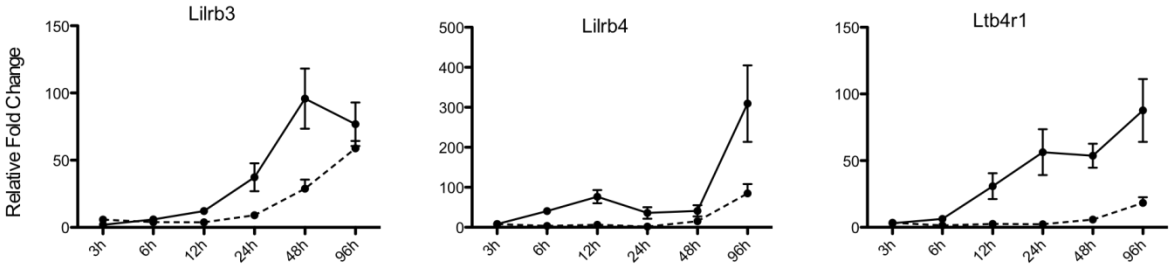


Fig 3.2b Fc receptors

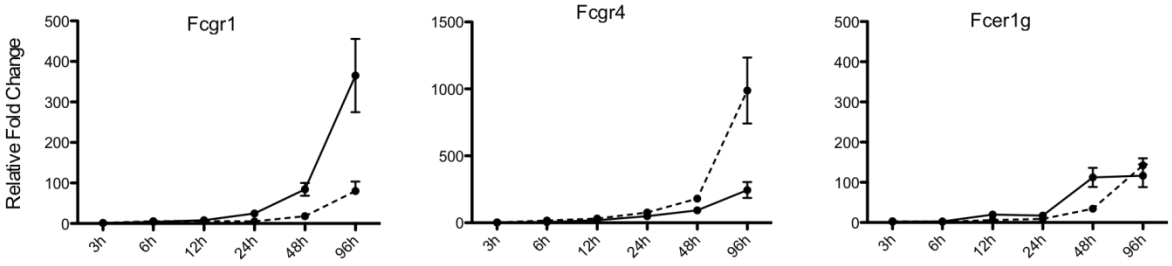


Fig 3.2c Other innate immune receptors

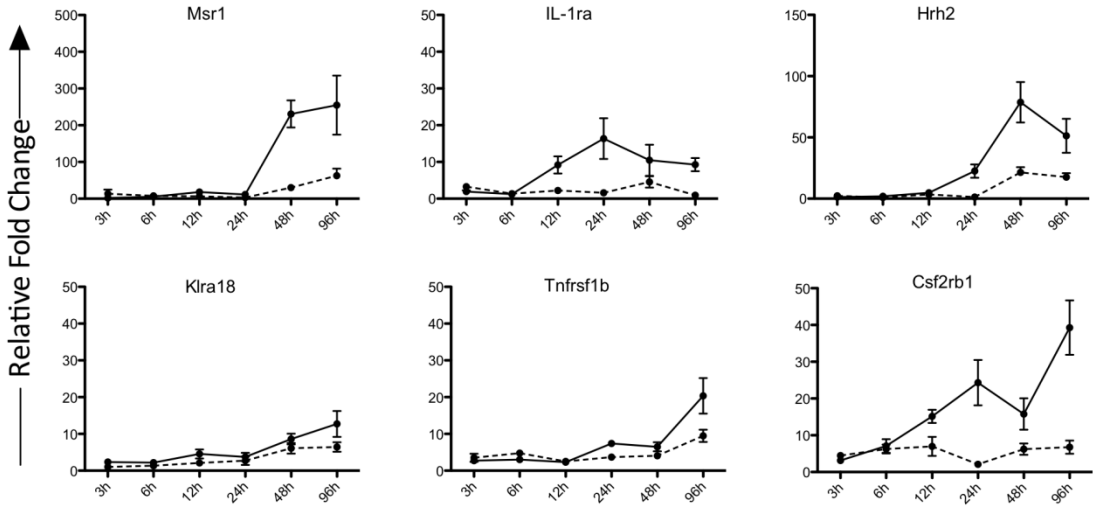


Fig 3.2 Innate immune receptor genes induced by PCEP and CpG at the site of injection after intramuscular injection in mice. Mice were administered with PBS, PCEP or CpG intramuscularly. Muscle tissue were collected at 3, 6, 12, 24, 48 and 96 h and analyzed for innate immune receptor genes by quantitative real-time PCR. Gene expression profiles in PCEP-injected muscle tissue are shown in bold lines whereas CpG-injected muscle tissues are in dotted lines. Results shown are the mean ± SE of six replicates at each time point. Relative fold changes (y-axis) for each gene were normalized to mouse GAPDH. Fold changes are calculated by the Ct method and are relative to the gene expression in PBS-injected muscle tissue.

The interferon pathway genes elicited by PCEP and CpG at the site of injection were clearly different. Compared to PCEP, CpG induced significantly higher expression of interferon-induced GTPase (Igtp, Iigp-1 and Ifi-47) (Fig 3.3a), interferon-induced genes with tetratricopeptide repeats (Ifit-2, Ifit3 and Garg49) (Fig 3.3b) and other interferon-induced genes (Oasl-1, Mx1, Ifi202b, Stat2, Psmb8 and Gvin1) (Fig 3.3c) at the site of injection. Iigp and Iigp-1 were up-regulated by 200 to 400-fold within 48 h of CpG administration (Fig 3.3a). Interestingly, PCEP followed a pattern of late expression at 96 h with interferon pathway genes that is similar to its expression pattern of innate immune receptors.

Fig 3.3a Interferon-induced GTPase.

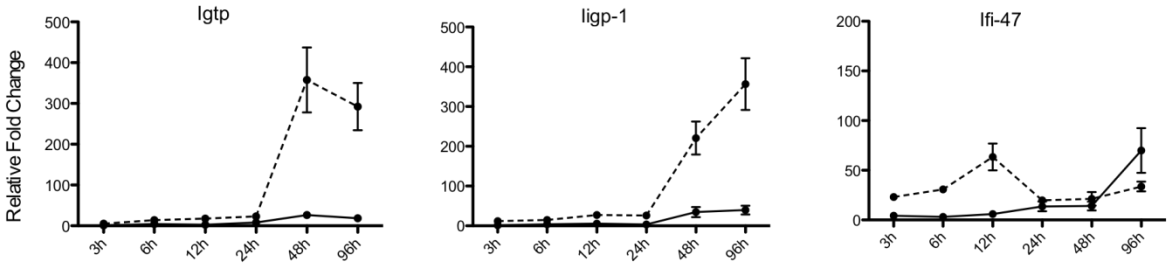


Fig 3.3b Interferon-induced genes with tetratricopeptide repeats

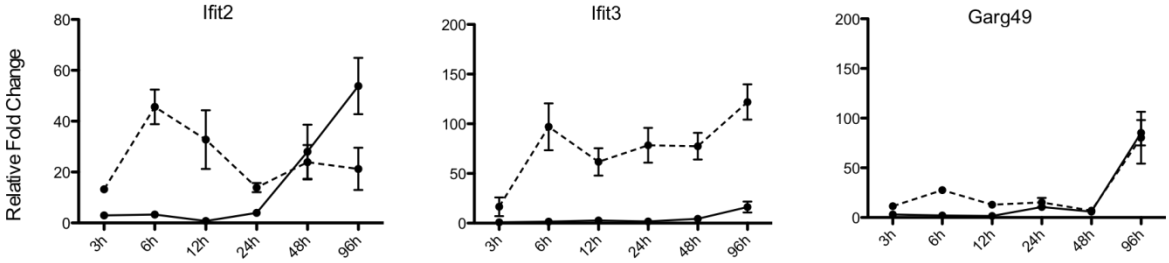


Fig 3.3c Other interferon-induced genes.

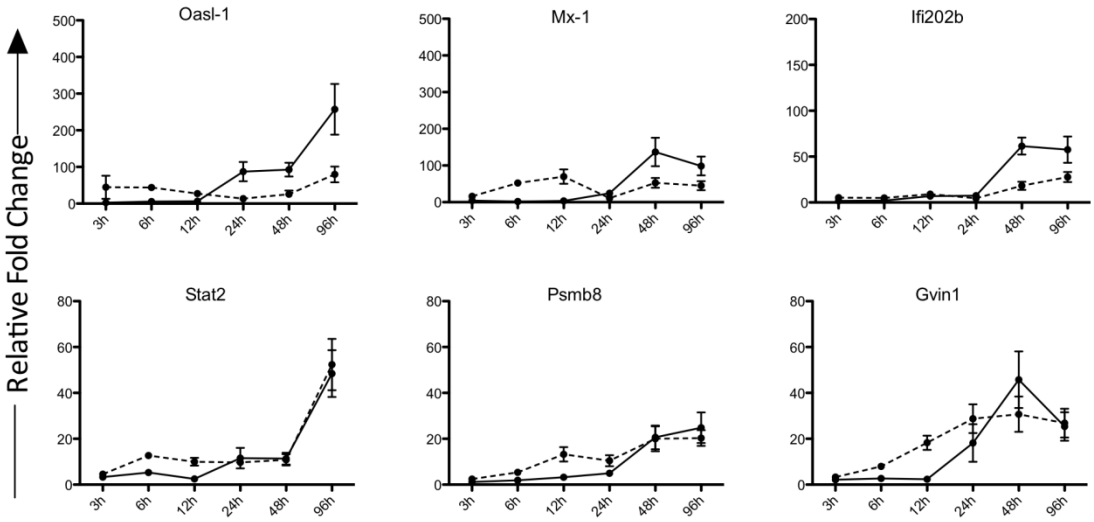


Fig 3.3 Interferon-induced gene profile elicited by PCEP and CpG at the site of injection after intramuscular injection in mice. Mice were administered with PBS, PCEP or CpG intramuscularly. Muscle tissues were collected at 3, 6, 12, 24, 48 and 96 h and analyzed for interferon-induced genes by quantitative real-time PCR. Gene expression profiles in PCEP-injected muscle tissue are shown in bold line whereas CpG-injected muscle tissues are in dotted line. Results shown are the mean ± SE of six replicates at each time point. Relative fold changes (y-axis) for each gene were normalized to mouse GAPDH. Fold changes are calculated by the Ct method and are relative to the gene expression in PBS-injected muscle tissue.

For the overall pictorial depiction, we also presented our data as relative mRNA expression (i.e. as $2^{-\Delta Ct}$ relative to the time matched expression of the housekeeping gene) so one can appreciate the effect that injection of PBS itself has on the gene expression (Fig 3.4). Thus, within the muscle tissues injected with PBS, PCEP or CpG, the highest relative mRNA values per gene (regardless of the time point) were ascribed the darkest red color (given the number 1) represented by the heat map (Fig 3.4). Each relative mRNA values for the remaining tissues injected with saline, PCEP or CpG were represented relative to this highest relative mRNA value and ascribed the color blue (0-0.25) indicating very low expression, turquoise (0.25-0.5) indicating low expression, yellow (0.5-0.7), indicating moderate expression, red (0.7-0.9) indicating high expression or very dark red (0.9-1.0) indicating very high expression.

By analyzing differences in mRNA expression across all the treatments, the majority of the adjuvant core response genes showed higher relative mRNA expression when injected with PCEP or CpG relative to the tissues injected with PBS. Generally, PCEP induced stronger mRNA expression at later time points (48-96 hrs) (as indicated by the larger pool of genes ascribed yellow or dark red color) than did tissues injected with CpG. As expected, the act of injecting the tissues with saline did trigger changes in mRNA expression with the most noticeable changes being expression of mRNA for IL-10, IL-12, Garg49, Clec4a3 and Lgals3bp. However, the majority of the adjuvant core response genes were up-regulated by PCEP and CpG at the site of injection but the expression levels and kinetics of the transcriptional profile elicited by PCEP and CpG are distinct. Generally, it is evident that PCEP induced higher overall expression of adjuvant core response genes relative to CpG.

Fig 3.4

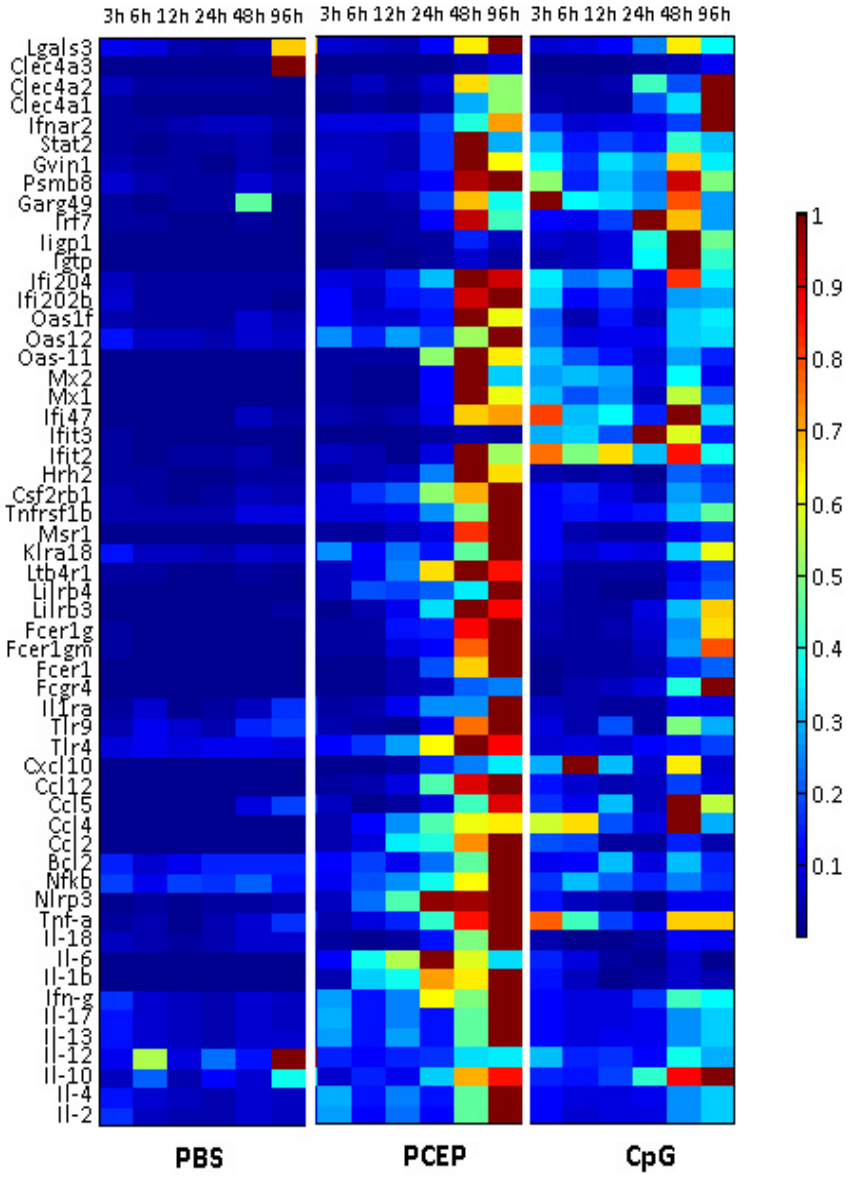


Fig 3.4 Comparison of relative mRNA expression in mouse muscle induced by administration of saline, PCEP and CpG at the site of injection. Mice were grouped into three treatment groups and injected with either PBS, PCEP or CpG intramuscularly. Muscle tissues were collected at 3, 6, 12, 24, 48 and 96 h and analyzed for gene expression by quantitative real-time PCR. For each gene, expression shown is the ratio of relative mRNA

expression at each time point divided by the maximum expression of that gene across the treatment at any time point. Each column represents one time point and each row represents the average kinetic of expression of one gene. Color ranges from blue to dark red indicating lowest to highest expression, respectively. Gene names are listed on the left of panel; time points on top of panel and treatments on the bottom of the panel.

3.3.2 *PCEP up-regulates NLRP3 and proinflammatory cytokine gene expression*

In addition to assessing the transcriptional profile of adjuvant core response genes, expression of inflammasome receptor (NLRP3) and proinflammatory cytokines was also assessed (Fig 3.5). CpG induced higher NLRP3 expression at 3 hr with an 11-fold increase compared to the 5 fold observed with PCEP but at 12 h, 24h, 48 hr and 96 hr, PCEP induced significant expression of NLRP3 compared to CpG at the site of injection (Fig 3.5). The highest NLRP3 expression by PCEP was observed at 12 h with 86-fold change. When comparing IL1β expression, CpG induced significantly higher expression compared to PCEP at the earliest time point (3h) but all subsequent time points showed significantly higher expression in the presence of PCEP (Fig 3.5). Similar to IL-1β gene expression pattern, PCEP induced significant expression of IL-18 at 48 h to 96 h (Fig 3.5).

Fig 3.5

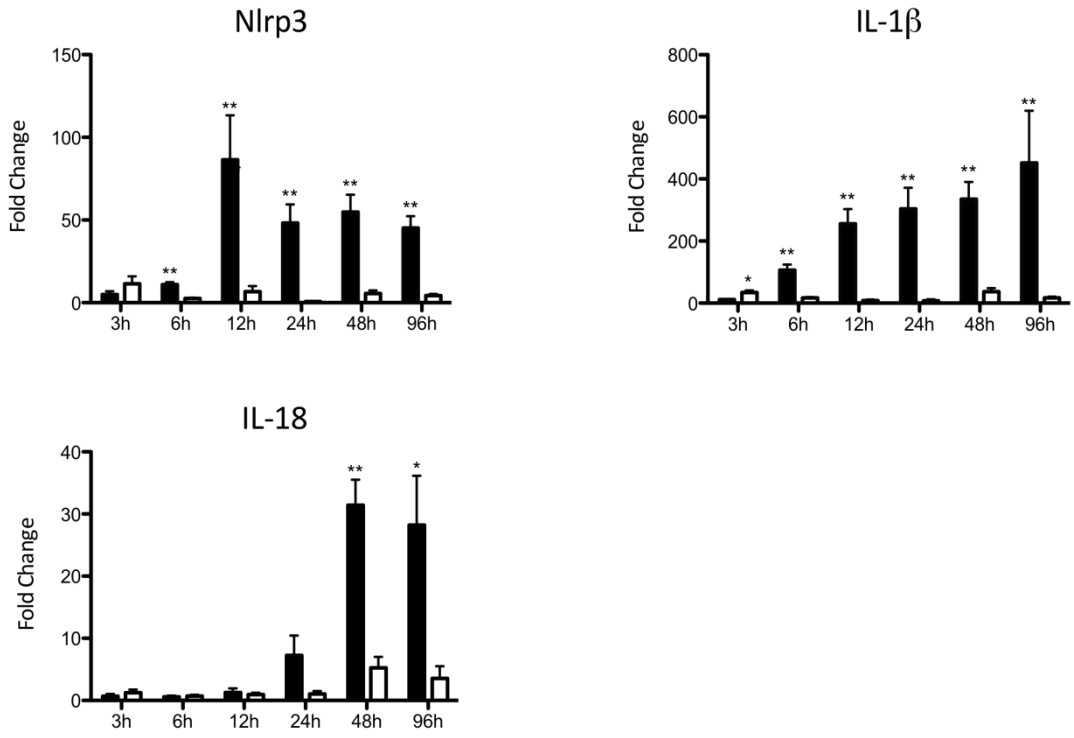


Fig 3.5 PCEP up-regulates the gene expression of inflammasome receptor NLRP3 and proinflammatory cytokines IL-1β and IL-18 at the site of injection. BALB/c mice were injected i.m. with either PBS, PCEP or CpG and tissues at the site of injection were analyzed by quantitative real-time PCR. Expression levels are shown in fold change compared to PBS-treated muscles. Black and white column indicate PCEP and CpG respectively. Groups with asterisks indicate significant differences from each other [$P<0.005$ (**) and $P<0.05$ (*)].

3.3.3 *Systemic responses to PCEP*

To determine the systemic effect, sera were collected from same mice at the time of muscle collection, to measure cytokine concentration. CpG was a powerful inducer of IL-12(p40) (Fig. 3.6). However, unlike gene expression at site of injection, PCEP and CpG did not induce any systemic increase in any of the other tested cytokines (IL-1β, IL-5, IL-6, IL-18, IFN-γ and TNF-α) and chemokines (CCL-2 and CXCL-10).

Fig 3.6

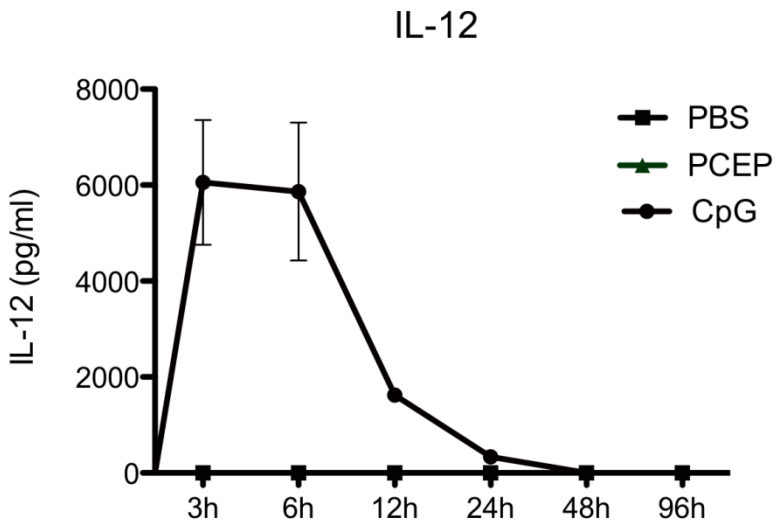


Fig 3.6 Systemic expression of IL-12 induced by PBS, PCEP and CpG in mice. BALB/c were injected i.m. with either PBS, PCEP or CpG and sera were collected at 3, 6, 12, 24, 48 and 96 h. Sera were assayed for IL-1β, IL-4, IL-6, IL-18, IFNγ, TNFα, CCL-2 and CXCL-10 expression by ELISA. Systemic expression of IL-12 is shown as the mean ± S.E.M of titers of six replicates at each time point.

3.3.4 *Local cytokine and chemokine production in response to PCEP*

The qRT-PCR results showed that PCEP was a strong inducer of cytokine and chemokine genes at the site of injection. PCEP also induced significant gene expression of inflammasome receptor (NLRP3) and proinflammatory cytokines. Because changes in gene expression do not always correlates with changes in protein expression, we examined the local production of cytokines and chemokines using site of injection thigh muscle by ELISA. High levels of cytokines and chemokines were detected at the site of PCEP injection (Fig 3.7). All cytokines and chemokines tested were detected as early as 3 h and sustained even at 96 h after injection. PCEP induced production of Th1 and proinflammatory cytokines IL-1β, Il-6, IL-12, IL-18 IFN-γ and TNF-α (Fig. 3.7a) as well as Th2 cytokine IL-4 (Fig 3.7b) at the site of injection. CCL-2, a strong chemoattractant of monocytes was significantly induced after PCEP injection (Fig. 3.7c). Locally, PCEP also induced early production of CXCL-10 also known as interferon gamma-induced protein 10, which sustained for 96 h (Fig 3.7c).

Fig 3.7a Th1 and pro-inflammatory cytokines

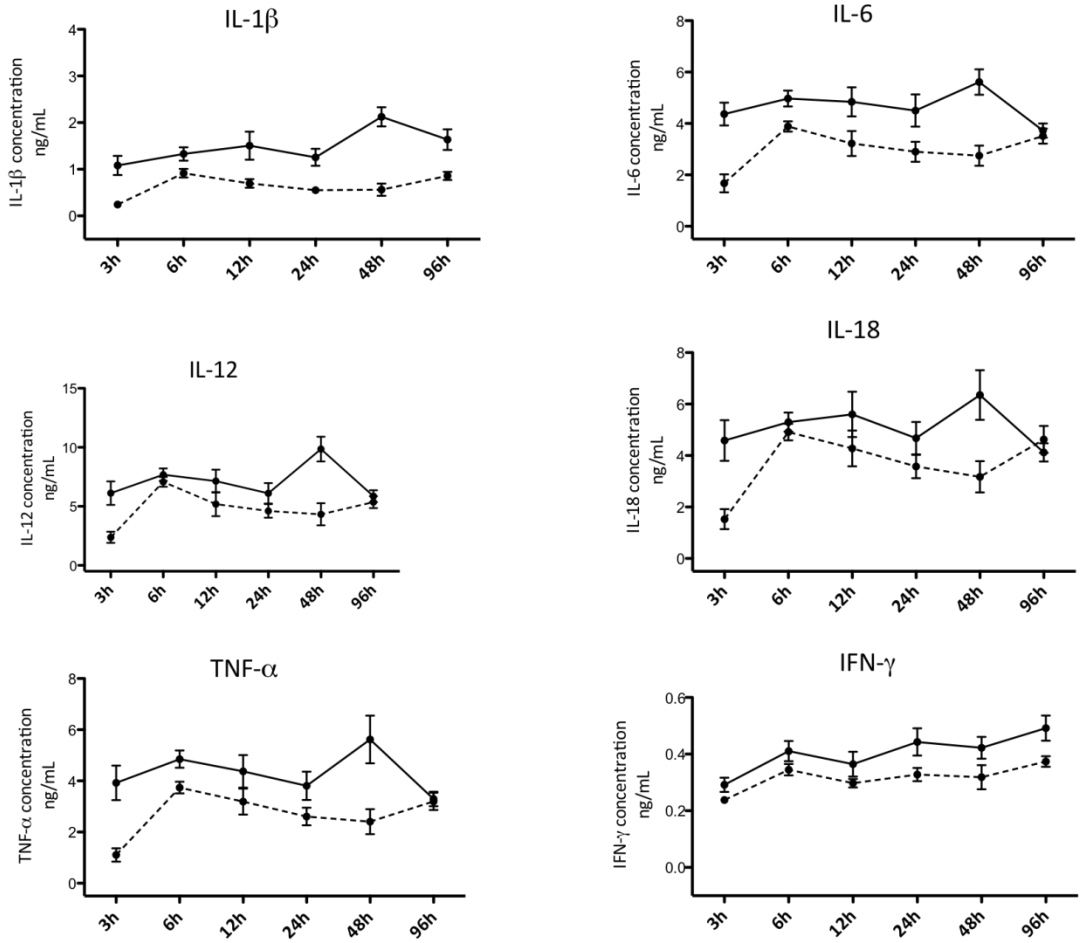


Fig 3.7b Th2 cytokine

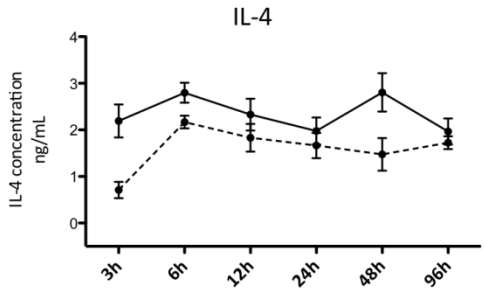


Fig 3.7c Chemokines

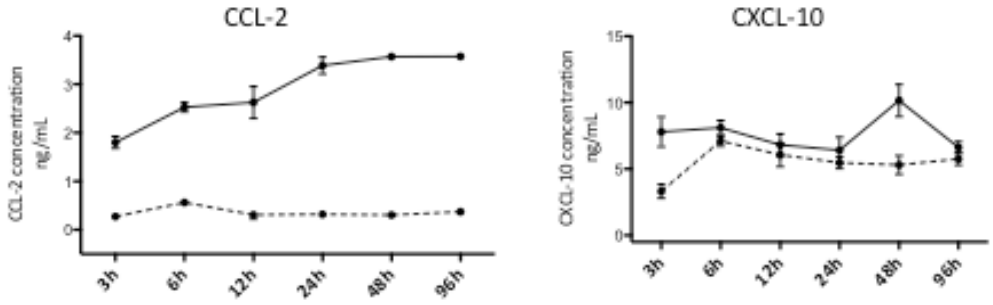


Fig 3.7 Local cytokine and chemokine profile induced by PCEP in mice. Mice were assigned to three treatment groups and injected intramusculary with PBS (dotted line) or PCEP (solid line) in the quadriceps muscle. Muscle tissues were collected at 3, 6, 12, 24, 48 and 96 hours after injection to measure cytokine and chemokines by ELISA. Local CCL-2 and CXCL-10 concentrations in PCEP-injected muscle tissues are shown in bold line whereas PBS-injected muscle tissues are in dotted line.

3.4 Discussion

In the present investigation we demonstrate that PCEP is a strong modulator of early innate immune responses at the site of injection. All these studies were performed in *vivo* to address the complex cellular interactions that occur when an adjuvant is administered. To our knowledge, this is the first report describing the local and molecular *in vivo* effects of PCEP.

It has been demonstrated that PCEP promotes superior antigen-specific Th1 and Th2 immune responses compared to the response observed when conventional adjuvant alum was used (Mutwiri et al., 2007a). The adjuvant activity of polyphosphazenes has been attributed to their ability to form water-soluble non-covalent protein-polymer complexes with antigen, which facilitates uptake by APCs (Andrianov et al., 2005). However, to date, no definitive proof has been provided. The result from the present investigations strongly suggests that activation of innate immune responses at the site of injection may be a potential mechanism of action for the adjuvant activity of PCEP.

Despite being used in billions of vaccine doses worldwide, the mechanisms that mediate adjuvant activity are not fully understood. Recent studies are beginning to unveil the mystery behind adjuvant action. Genome wide microarray analysis was applied to compare the local effects of the common adjuvants, MF59, alum and CpG after intramuscular injection in mice (Mosca et al., 2008). All three adjuvants modulated a cluster of common genes named “adjuvant core response genes” comprised of cytokines, chemokines, innate receptors, interferon-induced genes and adhesion molecules. Compared to CpG and alum, MF59 was the most potent activator of adjuvant core response genes at the injection site (Mosca et al., 2008). In this report, we demonstrate that PCEP is also a strong modulator of adjuvant core response genes at the site of injection, and was even more immuno-stimulatory than CpG. Apparently, some genes were also modulated in control groups injected with PBS alone including IL-10, IL-12, Garg49, Clec4a3 and Lgals3bp. This indicates that the trauma caused by injecting a liquid into the tissue is sufficient to alter the expression of a few genes locally, and emphasizes the importance of this control group.

It has been reported that MF59 activated many inflammatory genes including TNF-α, IL-1β and Ltbr4, forming an immuno-stimulatory environment at the site of injection, whereas CpG inhibited these genes (Mosca et al., 2008). Similarly, PCEP was a more potent activator of pro-inflammatory cytokine genes IL-1β, IL-6, IL-18 and Ltbr4 at the site of injection compared to CpG. Locally, MF59 induces secretion of IL-5 and CCL-3 whereas alum induces secretion of IL-5, CCL-2, eotaxin and KC (Calabro et al., 2011; Kool et al., 2008b; McKee et al., 2009). Thus, MF59 induces Th2 type immune responses that lead to prominent recruitment of immune cells principally neutrophils and monocytes (Calabro et al., 2011). In contrast, our studies show that PCEP induced significant production of cytokines (IL-1β, IL-18, IFN-γ and TNF-α) and chemokines (CCL-2) that tend to promote Th1-type immune responses. Up-regulation of the IFN-γ gene in response to PCEP is in agreement with previous *in vitro* studies where PCEP induced IFN-γ production in mouse splenocytes (Mutwiri et al., 2008).

In vitro studies suggest that MF59 and alum do not directly activate dendritic cells (DCs) (Li et al., 2007; Seubert et al., 2008). *In vivo,* these adjuvants activate TLR-independent pathways to indirectly activate DCs by creating an immuno-competent environment through up-regulation of various cytokines and chemokines (Calabro et al., 2011; Kool et al., 2008b; McKee et al., 2009). Conversely, PCEP significantly up-regulated TLR-4 and TLR-9 gene expression at the site of injection. Since, CpG is a TLR-9 agonist, this might explain the synergistic effect reported when CpG and polyphosphazene were used in combination (Mapletoft et al., 2008; Mutwiri et al., 2008; Taghavi et al., 2009).

CpG was a stronger inducer of interferon-induced genes at 3, 6, 12 and 24 h time points than PCEP. However, PCEP up-regulated the same genes on the second or fourth day after injection. This finding is consistent with previous data obtained from stimulating human peripheral blood mononuclear cells (PBMC) with CpG, which showed that CpG up-regulated interferon-inducible proteins, including IFIT1, OAS1 and MX1 predominantly at 6 to 24 hours after stimulation (Kato et al., 2003).

Previous studies have shown that CpG is a strong inducer of systemic immunity and may directly activate circulating blood cells (Mosca et al., 2008). This is in agreement with our results showing systemic IL-12 production as early as 3 h after CpG injection. On the other hand, PCEP failed to promote significant systemic cytokine production but it did strongly induce the production of cytokines and chemokines at the site of injection. PCEP significantly up-regulated chemokines especially CCL-2 and CCL-12 both of which are known as monocyte-chemotactic protein that are involved in the recruitment of monocytes at the site of tissue injury or inflammation (Sarafi et al., 1997). Likewise, monocytes and granulocytes recruitment induced by MF59 both *in vitro* and *in vivo* was shown to be dependent on chemokine receptor 2 (CCR-2), which is a receptor for CCL-2 and CCL-12 (Dupuis et al., 2001; Seubert et al., 2008). PCEP induced potent expression of "adjuvant core response genes" suggesting establishment of local immuno-competent environment at the injection site. This leads to effective recruitment of innate immune cells for better antigen presenting and processing resulting in stronger antigen-specific immune responses. Studies are in progress for assessing the PCEP-induced immune cell recruitment at the site of injection.

The inflammasome is a multi-protein complex that comprises of NACHT, LRR and PYD domains-containing protein 3 (Nlrp3), an NLR family member that interacts with CARD-domain-containing adaptor protein ASC and the protease caspase-1. Activation of inflammasome leads to processing of pro-inflammatory cytokines (IL-1β and IL-18) into their mature forms (Schroder and Tschopp, 2010). Alum has been shown to induce local inflammatory reaction at the site of injection (Goto et al., 1997), which resulted in recruitment of neutrophils, eosinophils and inflammatory $Ly6C^+$ $CD11b^+$ monocytes (Didierlaurent et al., 2009). Alum-induced proinflammatory cytokine production *in vitro* was dependent on activation of caspase-1, which in turn was mediated by NLRP3 (Eisenbarth et al., 2008; Li et al., 2007). However, the role of inflammasomes in mediating the adjuvant activity of alum *in vivo* remains controversial (Eisenbarth et al., 2008; Franchi and Nùñez, 2008; Li et al., 2007). Apart from alum, NLRP3 is activated by two other vaccine adjuvants namely, Chitosan and Quil A (Li et al., 2008). In the present investigations, PCEP up-regulated the expression of NLRP3 gene, a member of the multi-protein complex inflammasomes, at the site of injection.

In conclusion, the present study demonstrates that PCEP is a potent modulator of adjuvant core response genes at the site of injection. However, PCEP did induce genes that are distinct from CpG, suggesting that some differences exist in the mechanisms mediating the adjuvant activities of PCEP and CpG. In addition, PCEP induces strong local production of cytokines and chemokines at the site of injection. Our studies strongly suggest that one of the mechanisms that mediate the adjuvant activity of PCEP is the induction of a strong immuno-stimulatory environment at the site of injection. Understanding the mechanisms of action of adjuvants will provide critical information on how innate immunity influences the development of adaptive immunity. In addition, such knowledge will facilitate the rational development of new vaccine adjuvants.

CHAPTER 4. PCEP INDUCES RECRUITMENT OF MYELOID AND LYMPHOID CELLS IN MUSCLE AND THE DRAINING LYMPH NODE

Relationship of this study to the dissertation

In chapter 3, we have shown that PCEP is a strong modulator of adjuvant core response genes and induce potent cytokines and chemokines at the injection site. Due to potent chemotactic potential of cytokines and chemokines, we hypothesized that PCEP induces recruitment of immune cells at the site of injection. In this study, we examined PCEP-induced immune cell recruitment at the injection site and changes in cell composition in the draining lymph nodes. We also determined the cellular uptake and distribution of PCEP at the site of injection. Taken together, these results suggest recruitment of distinct immune cells to the site of injection site may be an important mechanism by which PCEP potentiates immune responses to antigens.

4.1 Introduction

Vaccination continues to be an important public health tool to decrease the mortality and morbidity caused by infectious diseases. Vaccines based on live microorganisms typically induce potent immune responses but have been associated with a number of safety concerns. Modern vaccines containing highly purified antigens are poorly immunogenic and require addition of adjuvants to induce effective immune responses. Despite their critical role in vaccines, the mechanisms of action of many adjuvants remain poorly understood. Understanding the mechanisms of action of adjuvants is important for development of safe and effective vaccines.

Polyphosphazenes are high-molecular weight, water-soluble polymers and promote enhanced and long lasting immune responses with a variety of viral and bacterial antigens (Eng et al., 2010a; McNeal et al., 1999; Mutwiri et al., 2008; Payne et al., 1995; Payne et al., 1998a). Recently, it has been shown that intranasal administration of influenza X:31 antigen with poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) resulted in significantly higher

antibody titers in nasal, lung and vaginal mucosal secretions in mice, suggesting that PCEP is an effective parenteral and mucosal adjuvant (Eng et al., 2010a; Shim et al., 2010). Although detailed mechanisms of action of polyphosphazenes are not known, excision of site of injection 24 h post-injection of PCPP with vaccine antigen had no detectable effects on antibody production, suggesting that formation of depot was not required for adjuvant activity (Payne et al., 1998a). However, polyphosphazenes have been shown to form water-soluble, non-covalent complex with protein antigens and this physical association with antigen might help to deliver antigens to antigen-presenting cells (APCs) (Andrianov et al., 2005).

Recently, the potent adjuvant activity of PCEP has been attributed to activation of innate immunity. *In vitro* studies by Mutwiri et al have shown that polyphosphazenes stimulate the production of innate cytokines in splenocytes, which may contribute to its adjuvant activity (Mutwiri et al., 2008). We recently reported that PCEP is a potent modulator of "adjuvant core response genes" at the site of injection that includes cytokines, chemokines, innate immune receptors and interferon-induced genes resulting in significant production of local cytokines and chemokines (Awate et al., 2012).

Several adjuvants including alum and MF59 induce cytokine and chemokine production at the injection site, recruit innate immune cells, increase antigen uptake by APCs and transport antigen to draining lymph nodes to initiate immune responses (Calabro et al., 2011; McKee et al., 2009; Seubert et al., 2008). Therefore, we hypothesized that PCEP induced cytokines and chemokines production at the site of injection influences local recruitment of various immune cells, which are involved in activation of immunity. In the present study, we show that intramuscular (i.m.) injection of PCEP promotes immune cell recruitment (including neutrophils, macrophages, monocytes, dendritic cells and lymphocytes) to the injection site and the draining lymph nodes. To further investigate the specific cellular targets and fate of PCEP after i.m. injection in mice, we labelled PCEP with fluorescence dye and examined whether recruited cells were associated with PCEP using FACS and confocal microscopy. Finally, we also examined the retention and distribution of PCEP after i.m. injection in muscle tissue.

4.2 Materials and methods

4.2.1 Animal experiments

Four to six week old female BALB/c mice (purchased from Charles River Laboratories, North Franklin, CT, USA) were used in this experiment. The animal experiments were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane use of animals.

4.2.2 Adjuvants

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). PCEP was dissolved in Dulbecco's phosphate buffered saline (PBS) (Gibco, NY, USA) by gentle agitation for 36 h at room temperature (RT). Imject alum (Thermo Fisher Scientific, IL, USA) used in these experiments is a mixture of aluminum hydroxide and magnesium hydroxide (40 mg/ml).

4.2.3 Injections

Mice were divided into three groups (n=5) and injected i.m. on both legs (quadriceps muscle) with 25 μl each of either phosphate-buffered saline (PBS) as control, 50 μg PCEP [This dose was selected based on previous experiments (Awate et al., 2012; Mutwiri et al., 2008; Mutwiri et al., 2007b)], or 1 mg of alum.

4.2.4 Isolation of recruited cells from muscle tissue

Muscle tissues were dissected from the site of injection and collected in a tube filled with ice-cold Hank's Balanced Salt Solution (HBSS) (Gibco). Muscle tissues were minced and incubated with digestion buffer (HBSS) supplemented with 0.1% type II collagenase D (Worthington Biochemical, NJ, USA), 0.2% BSA (Sigma-aldrich, MO, USA), 0.025% trypsin (Gibco) and 0.01% DNase I (Roche Diagnostics, Germany) for 45 min at 37°C under constant

agitation. The cell suspension was centrifuged at 300 x g for 10 min, reconstituted in RPMI (Gibco) supplemented with 10% FBS (Gibco) and filtered through 70 μm cell strainer to obtain single cell suspension. To further purify the cell suspension, it was layered on 25% percoll (GE healthcare, Sweden) and centrifuged at 2000 x g for 1 h. The cell pellets were washed twice and resuspended in RPMI (Gibco) with 10% FBS and used for fluorescent labeling for FACS analysis. Cell viability was estimated by Trypan Blue (Gibco) exclusion.

Similarly, draining lymph nodes were dissected, collected, minced and incubated with digestion buffer containing 2 mg/ml collagenase D (Roche Diagnostics, Germany) and 0.25 mg/ml DNase I in Hepes (Gibco) for 15 min at 37°C. It was then filtered through 70 μm cell strainer to obtain a single cell suspension, which was used for fluorescent labeling for FACS analysis.

4.2.5 Flow cytometry

For FACS staining, cells were incubated for 20 min at 4°C using the following antibodies: CD11b-FITC, Ly6C-APC, Ly6G-APC, F4/80-PE, CD11c-PE, CD3-APC, CD8-FITC, CD4-FITC, CD19-FITC (all from eBiosciences, CA, USA) and CD8-PerCP-Cy5.5, CD4-CD8-PerCP-Cy5.5 (all from BD Biosciences). The expression of surface markers was assessed using CellQuest analysis software on a FACSCalibure flow cytometer (BD Biosciences).

4.2.6 PCEP labeling

PCEP was reacted with 1,6-diaminohexane (Sigma-Aldrich) and a coupling reagent (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; Thermo Fisher Scientific) to obtain an amino-modified PCEP (Staros et al., 1986). A thiol group was attached to amino-modified PCEP, which was then labeled with Alexa-fluor 488 C_5-maleimide (Molecular probes, OR, USA) or 800CW licor IRDye (LI-COR Biosciences, NE, USA). Briefly, 1 mg of the thiol-modified PCEP was dissolved in 1 ml of PBS (pH 7.5) and mixed with 500 μl of 1 mg/ml of the dye solution in ultrapure water and reacted for 2 h at room temperature in the dark. The unlabelled dye was separated from the labelled products by size exclusion chromatography on

sephadex G-75 column (GE Healthcare, Sweden). The labelled PCEP was concentrated using Amicon ultra-centrifugal filter units with 10 kD molecular weight (EMD Millipore, MA, USA).

4.2.7 Confocal microscopy

The mice were injected with PCEP-labelled with Alexa fluor-488 and the muscle tissues were collected 24 h post-injection. Single cell suspensions obtained from the digested muscle tissues were incubated for 12 h using 2 well tissue culture chamber slides at 37°C and 5% CO_2. After 12 h, cells were fixed with 3.7% paraformaldehyde (RICCA chemicals, TX, USA) for 10 min and washed three times with 0.1 M PBS for 5 min each. For lysosomal localization, cells were incubated in 5 nM LysoTracker Red (Molecular probes, NY, USA) in RPMI media supplemented with 10% FBS for 30 min and again washed for three times with 0.1M PBS. Finally, the chambers were removed and slides were mounted with VECTASHIELD mounting medium containing DAPI (Vector Laboratories, CA, USA) to stain nucleus. The slides were allowed to cure for 24 h in the dark at RT before visualizing under Zeiss LSM 5 laser scanning confocal microscope (Carl Zeiss, Germany). The images were captured in RITC, FITC and bright field were over-laid to determine the localization of PCEP and lysosomal compartments.

4.2.8 *In vivo* imaging

At least one-week prior to injection, BALB/c mice were placed on AIN-93G Purified Diet (Harlan Teklad, WI, USA) to eliminate potential interference from fluorescence in standard pelleted diets. The injection sites were shaved prior to injection. Mice were injected i.m. with 10 μg PCEP labelled with 800CW IRDye. Control mice were injected with hydrolyzed 800CW IR Dye (carboxylate form) to act as a point of reference for background fluorescence with unconjugated dye. Mice were scanned at 3 h, 6 h, 24 h, 1 month and 3 month post-injection. To maintain sedation during the imaging process, the mice were anesthetized with 2% isoflurane (VET One, UK). Imaging was performed using the ODYSSEY® Imaging System and MousePOd® (LI-COR Biosciences, NE, USA), which allowed for administration of anesthesia and maintenance of localized temperature of 37 °C to reduce stress on the mice.

4.2.9 Statistical analysis

All the data for cell recruitment were analyzed using Graph-Pad Prism 5 software (GraphPad Software, San Diego, CA, USA). Differences in the cell numbers between the treatments were analyzed by two-way ANOVA by Ranks and the significant differences between the treatments were compared by Bonferroni multiple-comparison test where ***P<0.001, **P<0.01, *P<0.05.

4.3 Results

4.3.1 PCEP induces the recruitment of various immune cells at the injection site.

Our aim was to identify the cells recruited after injection of PCEP in mouse muscle tissues. FACS analysis was performed on the single cell suspensions obtained by enzymatic digestion of the muscle tissues taken from mice at 3 h, 24 h, one-week and two-weeks post i.m. injection. Recruited immune cells were identified based on specific or combinations of markers such as monocytes (CD11b, Ly6C), neutrophils (CD11b, Ly6G), macrophages (F4/80), DCs (CD11c), CD8 T cells (CD3/CD8a), CD4 T cells (CD3/CD4) and B cells (CD19).

Mice injected with PBS showed recruitment of few immune cells at the site of injection indicating tissue trauma due to injection alone (Fig. 4.1A). However, PCEP induced significantly higher recruitment of total cells compared to PBS- and alum-injected mice (Fig 4.1A). At 3 h post-injection, there was no significant difference in the recruitment of total immune cells in the PBS-, alum- and PCEP-injected mice. PCEP-induced cell recruitment was significantly higher at 24 h (P<0.001) post-injection relative to PBS-injected and declined thereafter, but was still higher relative to alum-injected and PBS control groups. Alum induced significantly higher recruitment at 24 h (P<0.001) relative to PBS-injected mice, but at one-week post-injection, the number of cells recruited to the injection site was similar to PBS and alum-injected mice (Fig 4.1A). Overall, we observed that the process of cell recruitment was transient as the cell numbers peaked at 24 h but decreased dramatically by one-week after injection of the adjuvant (Fig 4.1A).

Although lower relative to number of cells recruited after 24 h, significantly more cells were recruited to muscle two-week post-injection with PCEP ($P<0.001$) or alum ($P<0.001$) relative to PBS control mice (Fig 4.1A).

Kinetic studies on PCEP-induced myeloid cell recruitment revealed peak recruitment at one-week post-injection (Fig 4.1B). Relative to PBS-injected mice, PCEP recruited significantly higher neutrophils at 24 h ($P<0.001$), one-week ($P<0.001$) and two-week ($P<0.001$), post-injection. Compared to alum-injected mice, PCEP-induced significantly increased cells at 24 h ($P<0.05$) and two-week ($P<0.001$) post-injection. Recruitment kinetics of neutrophils observed in alum-injected muscle tissues was also significantly higher at 24 h ($P<0.001$), one-week ($P<0.01$) and two-week ($P<0.001$) post-injection relative to PBS-injected mice. PCEP induced highest number of neutrophils at one-week post-injection followed by macrophages, monocytes and DCs. The number of neutrophils recruited to both PCEP-injected and alum-injected muscle peaked after one-week post-injection and thereafter began to decline (Fig 4.1B).

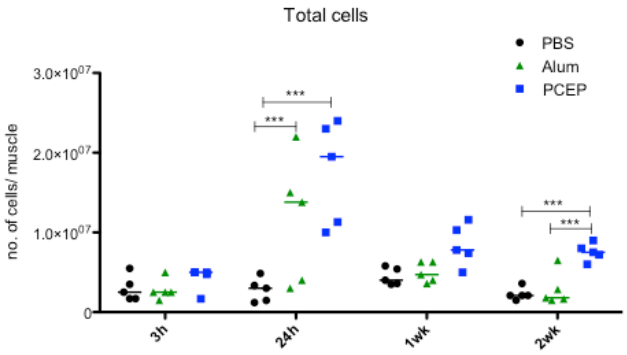
Compared to other myeloid cells, macrophages were the first cells to be recruited (3 h) in significant numbers ($P<0.05$) in PCEP-injected muscle and also highest in number at 24 h post-injection followed by neutrophils, monocytes and DCs (Fig 4.1B). PCEP induced significantly higher recruitment of macrophages ($P<0.001$) at 24 h, one-week and two-week post-injection relative to PBS-injected mice. However, compared to alum-injected mice, PCEP induced significantly higher recruitment of macrophages as early as 3 h ($P<0.05$), 24 h ($P<0.001$) and two-week ($P<0.001$) post-injection (Fig 4.1B).

PCEP-injected muscle tissues showed statistically similar monocyte and DC recruitment kinetics post-injection, with peak recruitment at one-week post-injection. PCEP induced significantly higher ($P<0.001$) recruitment of monocytes and DCs at 24 h, one-week and two-week post-injection compared to PBS-injected mice (Fig 4.1B). When compared to alum-injected mice, PCEP induced significantly higher recruitment of monocytes at 24 h ($P<0.05$) and two-week ($P<0.001$) post-injection. The number of monocytes recruited to alum-injected muscle

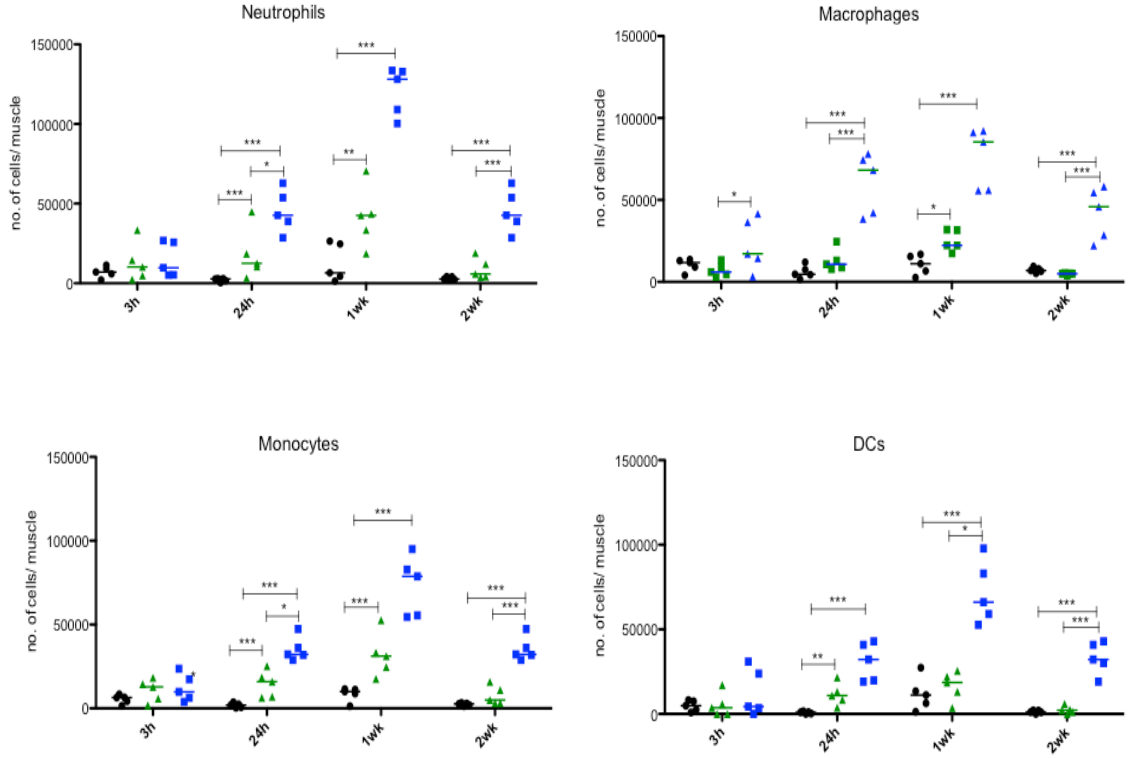
was significantly higher at 24 h ($P<0.001$) and one-week ($P<0.001$) relative to PBS control muscle. The number of DCs recuruited to PCEP-injected muscle was significantly higher at one-week ($P<0.05$) and two-week ($P<0.001$) relative to alum-injected muscle. Similarly, alum-injected muscle showed significantly higher recruitment of DCs at 24 h ($P<0.01$) post-injection compared to PBS-injected muscle (Fig 4.1B).

Similar to what was observed in the myeloid cell populations, recruitment of $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells was significantly higher in the PCEP-injected muscle relative to both alum-injected and PBS-injected muscle tissues (Fig 4.1C). $CD4^+$ T cells were recruited in significant numbers within 3 h post-injection of PCEP compared to PBS and alum-injected mice, which thereafter declined. At 24 h ($P<0.001$) and two-week ($P<0.05$), PCEP-injected muscle tissue showed significantly higher recruitment of $CD4^+$ T cells compared to PBS-injected muscle. Similarly, PCEP induced significantly higher recruitment of $CD8^+$ T cells at 24 h ($P<0.001$), one-week ($P<0.01$) and two-week ($P<0.001$) post-injection relative to PBS-injected mice. Compared to alum, PCEP-injected muscle tissue showed significantly higher number of $CD8^+$ T cells at 24 h ($P<0.001$), one-week ($P<0.05$) and two-week ($P<0.001$) post-injection. Similarly, alum induced significantly higher recrutiment of $CD8^+$ T cells at 24 h ($P<0.05$) post-injection relative to PBS-injected mice (Fig 4.1C). Unlike T cells, PCEP-injected muscle tissue showed significantly higher recruitment of B cells only at 24 h post-injection compared to alum- and PBS-injected mice (Fig 4.1C). There was no significant difference in the recruitment of B cells at 3 h, one-week and two-week post-injection of PBS-, PCEP- and alum-injected mice (Fig 4.1C). At all time points, PCEP induced higher T cell recruitment compared to B cells (Fig 4.1C). PBS-injected muscle did not induce significant recruitment of lymphocytes at any time point (Fig 4.1C). In general, we observed that PBS-injected muscle had very low recruitment of myeloid and lymphoid cells in the muscle tissues.

4.1A


Total cells
PBS
Alum
PCEP
no. of cells/ muscle
3.0×10^07
2.0×10^07
1.0×10^07
0
3h
24h
1wk
2wk

4.1B


Neutrophils
Macrophages
Monocytes
DCs
no. of cells/ muscle
150000
100000
50000
0
3h
24h
1wk
2wk

4.1C

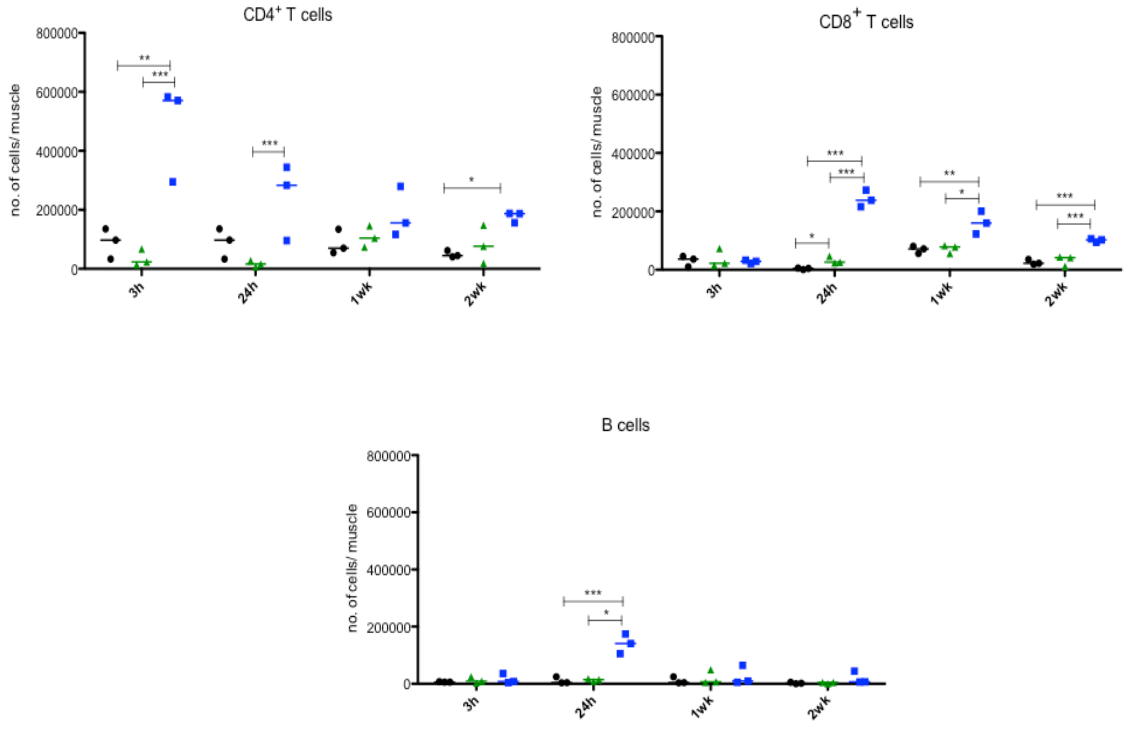


Fig 4.1 Cellular recruitment induced by adjuvants at the site of injection. Five mice per group were injected with 25 µl of PBS, PCEP (50 µg) or alum (0.5 mg) intramuscularly. Muscle tissues were collected at different time points and single cell suspensions were obtained by enzymatic digestion of muscle tissues. Cellular recruitment at the site of injection was analyzed by FACS analysis. (4.1A) Comparison of total cell recruitment at 3 h, 24 h, one-week and two-week in response to the PBS, PCEP or alum. (4.1B) Myeloid cell recruitment kinetics induced by PCEP and alum in the muscle tissue post-injection. (4.1C) Lymphoid cell recruitment kinetics induced by PCEP and alum at the injection site. Differences in the cell numbers between the treatments were analyzed by two-way ANOVA and the significant differences between the treatments were compared by Bonferroni multiple-comparison test where ***P<0.001, **P<0.01, *P<0.05.

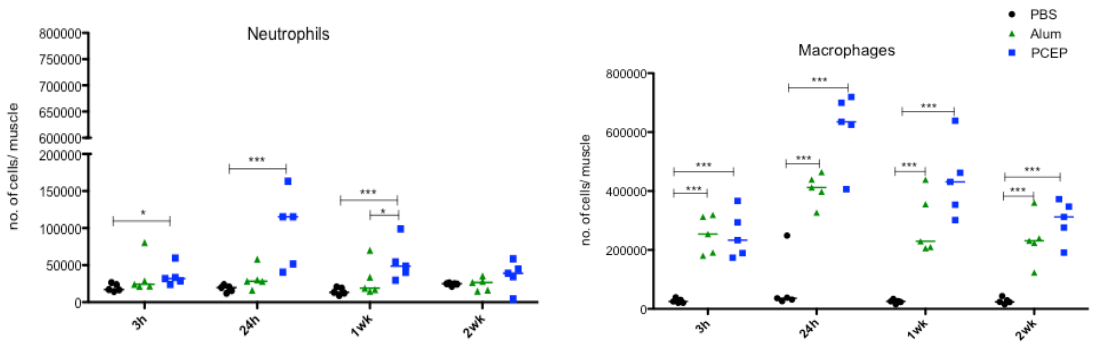
4.3.2 PCEP stimulates increased immune cell numbers in the draining lymph nodes

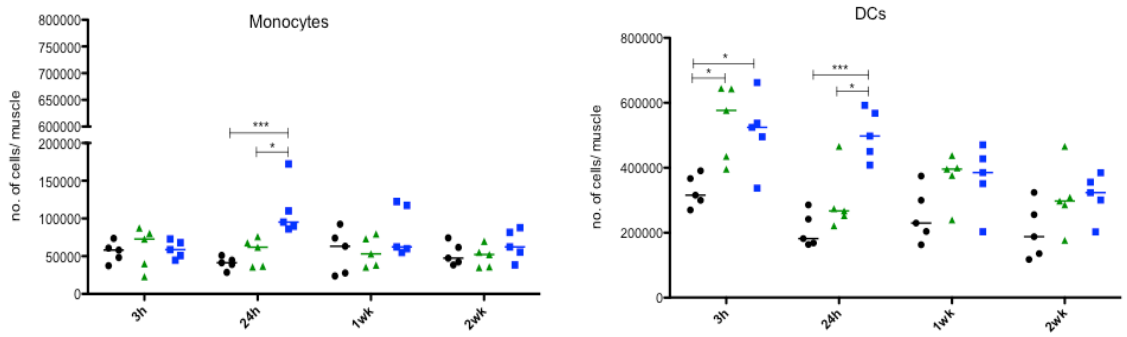
Draining lymph nodes are the primary inductive sites where immune responses are initiated. Hence, we examined the cell composition in the draining lymph nodes after i.m. injection of PBS, PCEP or alum. There were significantly higher numbers of myeloid and lymphoid cells detected in the draining lymph nodes after injection of PCEP compared to alum- and PBS- injected mice (Fig 4.2). This suggests that the increase in cell numbers in draining lymph nodes post-injection was attributed to PCEP.

Neutrophils were detected in the draining lymph nodes as early as 3 h ($P<0.05$) after i.m. injection of PCEP in mouse muscle (Fig 4.2A). Relative to PBS- and alum-injected mice, neutrophils were significantly higher ($P<0.001$) at 24 h and one-week responses after injection of PCEP (Fig 4.2A). Compared to PBS-injected mice, the number of macrophages in PCEP- and alum-injected mice were significantly higher ($P<0.001$) at all time points with peak at 24 h, which thereafter began to decline. Monocyte numbers were significantly increased at 24 h after injection of PCEP compared to PBS- and alum-injected mice (Fig 4.2A). However, there was no significant difference observed in monocyte numbers at 3 h, one-week and two-week post-injection in all groups. In comparison with other myeloid cells, DCs were detected in highest numbers at 3 h in the draining lymph nodes of PCEP-injected mice. Relative to PBS-injected mice, significantly higher numbers of DCs were detected at 3 h ($P<0.05$) and 24 h ($P<0.001$) in the draining lymph nodes of PCEP-injected mice. DCs and macrophages were increased in highest numbers in the draining lymph nodes while neutrophils, macrophages and DCs were earliest to be detected after injection of PCEP. Kinetic studies revealed peak in neutrophils, macrophages, monocytes and DCs numbers in the draining lymph nodes at 24 h post injection of PCEP relative to alum-injected and PBS control lymph nodes, which thereafter declined. For monocytes and DCs, there were no significant changes in cell numbers in any of the treatment groups in the lymph nodes at one-week and two-week post-injection (Fig 4.2A).

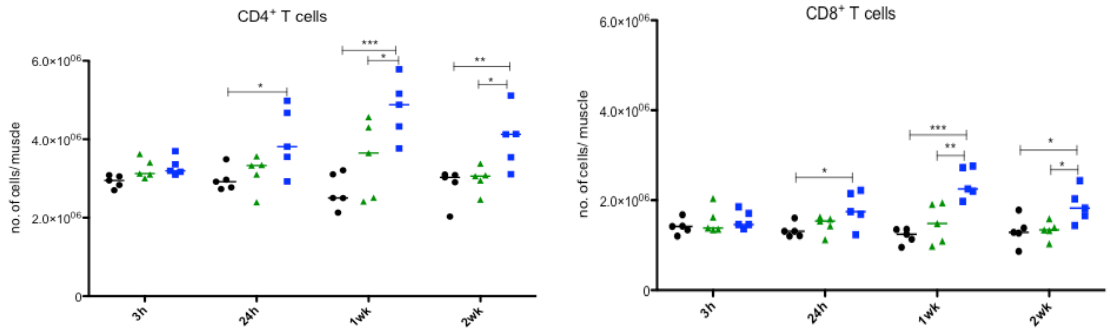
Lymphocytes were also increased in the draining lymph nodes of PCEP-injected mice compared to lymph nodes of alum- and PBS-injected mice (Fig 4.2B). The number of $CD4^+$ T cells were significantly higher at 24 h ($P<0.05$), one-week ($P<0.001$) and two-week ($P<0.01$) in the draining lymph nodes of PCEP-injected mice relative to PBS control mice. A similar kinetic pattern was observed for $CD8^+$ T cells in the draining lymph nodes of PCEP-injected mice. Compared to PBS-injected mice, the numbers of CD8+ T cells were significantly higher at 24 h ($P<0.05$), one-week ($P<0.001$) and two-week ($P<0.05$) in the draining lymph nodes of PCEP-injected mice. Unlike T cells, B cells were detected in significant numbers ($P<0.05$) only at one-week after injection of PCEP. Overall, PCEP and alum-injected lymph nodes showed no difference in lymphoid cell numbers at 3 h post-injection when compared to PBS-injected lymph nodes (Fig 4.2B). Among the lymphoid cells, $CD4^+$ T cells were most prominently increased in the draining lymph nodes of PCEP-injected mice (Fig 4.2B). The number of $CD8^+$ T cells ($P<0.001$), CD 4^+ T cells ($P<0.001$) and $CD19^+$ B cells ($P<0.05$) were significantly increased at one-week in the draining lymph nodes of PCEP-injected mice. There were no significant differences in lymphocyte numbers in the draining lymph nodes of alum and PBS-injected mice at any time point (Fig 4.2B).

4.2A





4.2B



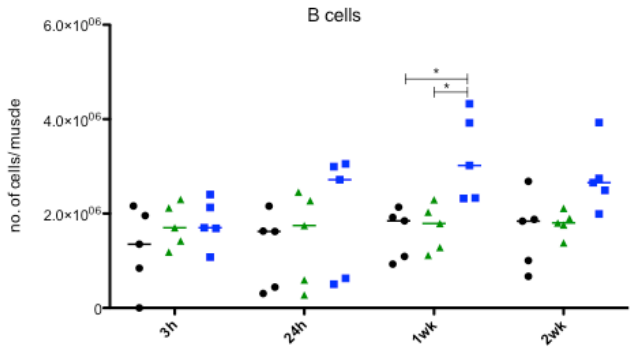


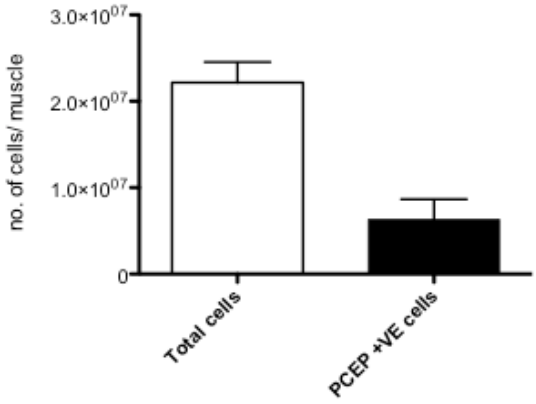
Fig 4.2 PCEP stimulates increased immune cell numbers in the draining lymph nodes. BALB/c mice (n=5 per group) were injected i.m. with either PBS, PCEP (50 ug) or alum (0.5 mg). Draining lymph nodes were collected, digested to obtain single cell suspensions and analyzed by flow cytometry. (4.2A) Neturophils, macrophages, monocytes and DCs kinetics in

the draining lymph nodes post injection of PCEP and alum. (4.2B) Kinetics of increase in lymphoid cells post-injection of adjuvants in the draining lymph nodes. Differences in the cell numbers were analyzed by two-way ANOVA and the significant differences between the treatments were compared by Bonferroni multiple-comparison test where ***P<0.001, **P<0.01, *P<0.05.

4.3.3 Uptake of PCEP by myeloid and lymphoid cells at the injection site

To determine whether PCEP is taken up by the cells recruited at the injection site, PCEP was labelled with Alex-fluor 488 fluorescent dye and mice were injected with dye labelled PCEP or unlabelled dye (served as control). PCEP was detected in approximately 28.55% of the total cells recruited to the site of injection (Fig 4.3A) but Alex-fluor 488 fluorescent dye alone was not detected in the recruited cells (data not shown). Approximately 90 percent of all recruited monocytes, macrophages and DCs had taken up PCEP, whereas 83 percent of neutrophils were positive for PCEP. There were large numbers of T cells recruited 24 h post-injection of PCEP, however only 9 percent of $CD8^+$ T cells and 14 percent of $CD4^+$ T cells were positive for PCEP (Fig 4.3B). Thus, the majority of myeloid cells take up PCEP within 24 h but only a minority of lymphocytes were PCEP-positive.

4.3A



4.3B

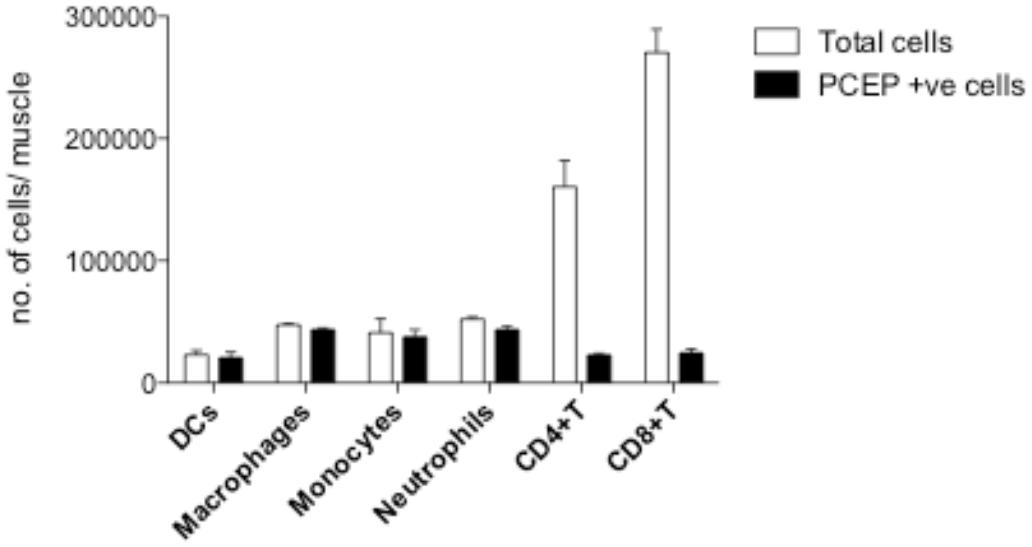
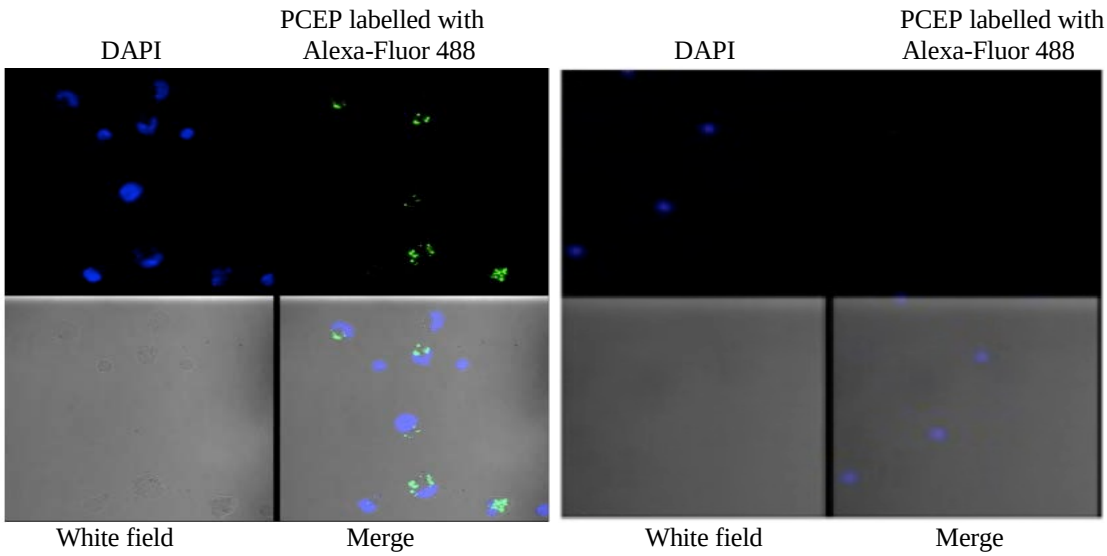


Fig 4.3 Cellular uptake of PCEP by recruited immune cells at the injection site. Three mice per group were injected i.m. with PCEP labelled with Alexa-fluor 488 fluorescent dye (50 ug). Muscle tissues were collected at 24 h post injection and single cell suspensions were obtained by enzymatic digestion of muscle tissues. (4.3A) PCEP was detected in the recruited cells 24 h post injection using FACS analysis. (4.3B) PCEP is taken up by various recruited immune cells at the injection site.

4.3.4 Intracellular uptake of PCEP at the site of injection

To identify the localization of PCEP in the recruited immune cells, mice were injected i.m. with Alex-fluor 488 labelled PCEP or unlabelled Alex-fluor 488 fluorescent dye (free dye). When we evaluated the merged confocal microscopic images of cells from mice injected with Alexa-fluor 488 labelled PCEP, we observed that the fluorescent dye was not in the nucleus or associated with plasma membrane. Instead, it appeared to be localized within a defined region in the cytosol of various recruited cell populations (Fig 4.4A and 4.4C-4.4E). Mice injected with free dye did not show dye localization or indeed dye uptake (Fig 4.4B).

4.4A **4.4B**



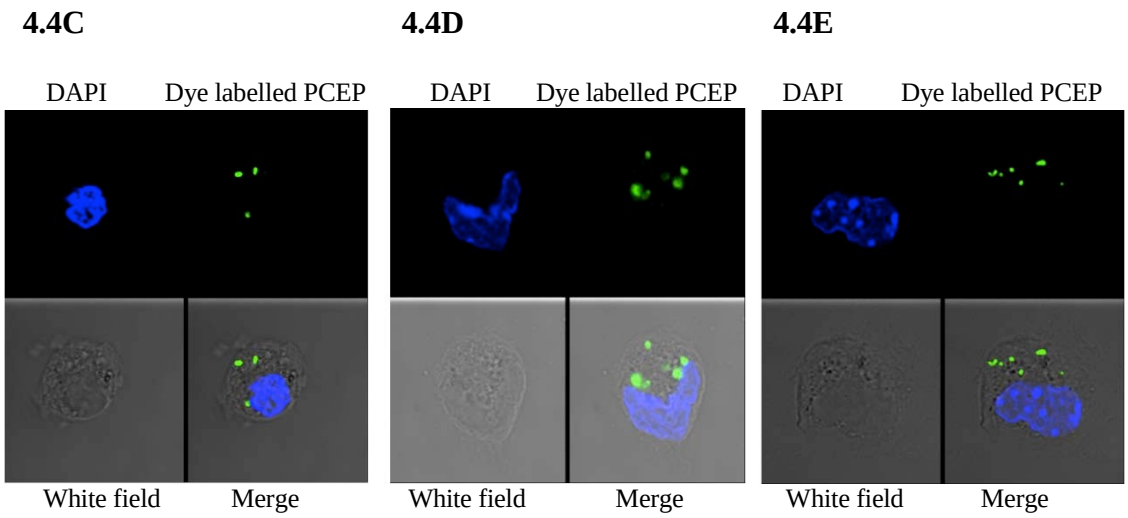
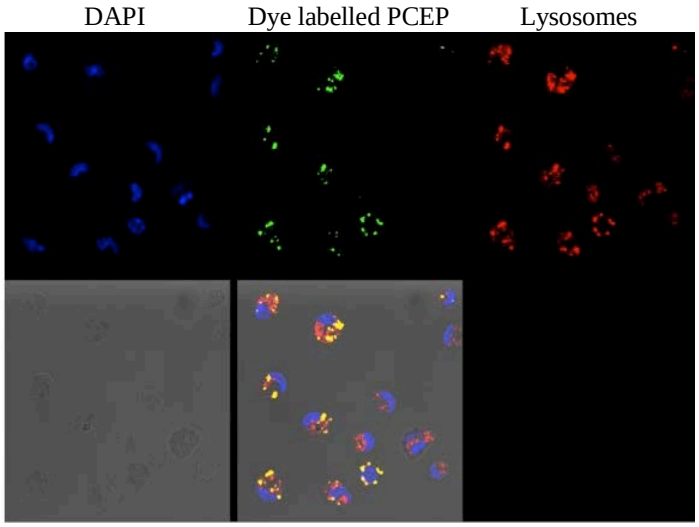


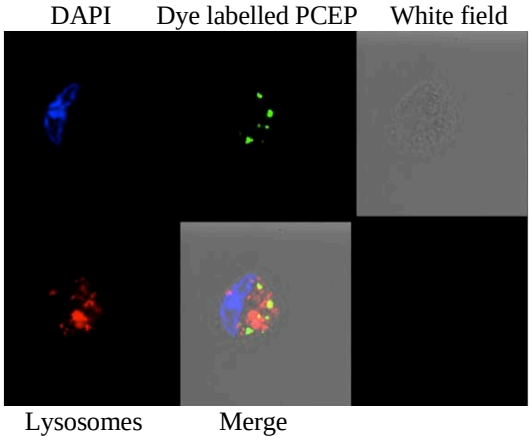
Fig 4.4 Intracellular uptake of PCEP at the injection site. Mice were injected i.m. with PCEP labelled with Alexa-fluor 488 fluorescent dye (50 ug) or free dye. Muscle tissues were collected 24 h post-injection and single cell suspensions were obtained by enzymatic digestion of muscle tissues. Intracellular localization of fluorescent-labelled PCEP was evaluated on representative cells using a confocal laser scanning microscope. Nuclei were stained with DAPI in each panel. (4.4A) Intracellular uptake of fluorescent labelled PCEP by various recruited cells at the site of injection. (4.4B) Cell suspension after injection of unlabelled Alex-flour 488 fluorescent dye in the muscle tissue. (4.4C-E) Higher magnification of various recruited cells that have taken up PCEP at the injection site. Merge images were shown for each figure. Green color indicates fluorescent labelled PCEP; blue color indicates the nucleus.

Single cell suspensions obtained from the site of injection were incubated with LysoTracker Red dye (to track acidic organelle like lysosomes in live cells). LysoTracker Red dye appeared to be overlaid on Alexa-Fluor 488 labelled-PCEP (yellow: green and red combined i.e., in the same location) suggesting that dye labelled PCEP was localized within the lysosomes of the recruited immune cells (Fig 4.5A and 4.5B).

4.5A



4.5B



4.5C

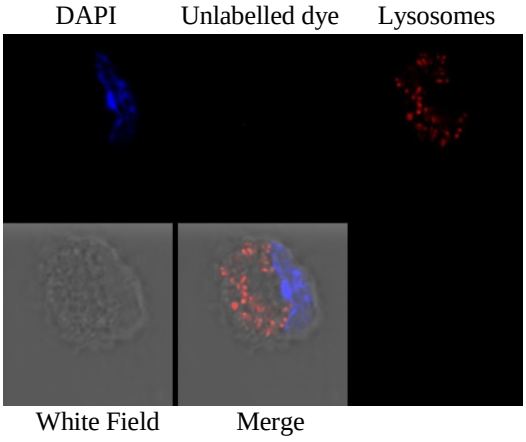
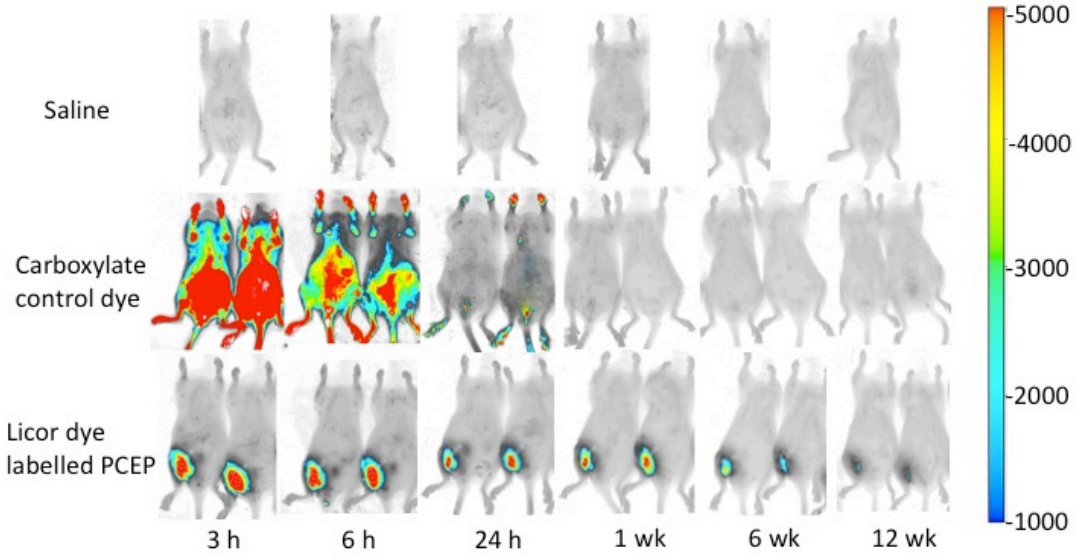


Fig 4.5 Lysosomal localization of PCEP. (4.5A) Mice were injected i.m. with PCEP labelled with Alexa-flour 488 fluorescent dye (50 ug). Muscle tissues were collected at 24 h post-injection and single cell suspensions were obtained by enzymatic digestion of muscle tissues. Microscopic evaluation of PCEP localization was performed on representative cells using a confocal microscope. Nuclei were stained with DAPI in each panel. Lysosomes were detected using LysoTracker Red. Images captured in RITC, FITC and bright field were overlaid to determine the colocalization of fluorescent labelled PCEP in the lysosomes of recruited cells. (4.5B) Higher magnification of the representative cell. (4.5C) mice injected with unlabelled Alex-flour 488 fluorescent dye . Green color indicates intracellular distribution of fluorescent labelled PCEP in the various recruited cell populations; blue color indicates the nucleus (blue); red color indicates acidic lysosomal compartments; yellow color indicates fluorescent labelled PCEP in lysosomal compartment.

4.3.5 Retention of PCEP in muscle tissue after injection

To study the retention of PCEP at the injection site, we performed whole animal imaging on BALB/c mice. Mice were injected i.m. with either unlabelled PCEP, non-reactive carboxylate 800CW IRDye or PCEP labelled with 800CW IRDye and scanned at 3 h, 6 h, 24 h, 1 wk, 6 wk and 12 wk using the ODYSSEY imaging system. Light intensities were static to allow comparisons across the treatment groups. Control mice injected with non-reactive carboxylate 800CW IRDye (control dye) showed high fluorescent intensity throughout the body within 3 h post-injection (Fig 4.6A). The non-reactive control dye was rapidly cleared from the body within 24 h post-injection. The unlabelled PCEP-injected mice (negative control) did not show any fluorescence. Mice injected with PCEP labelled with 800CW IRDye showed localized distribution at the site of injection from 3 h to 12 wk post-injection (Fig 4.6A). Average fluorescent intensity of PCEP labelled 800CW IRDye measured at the site of injection was compared with non-reactive carboxylate 800CW IRDye-injected control mice using a consistent surface area. The average fluorescent intensity of PCEP labelled 800CW IRDye was highest 3 h to 6 h post-injection and was reduced approximately two-third after 24 h (Fig 4.6B). The average fluorescent intensity remained relatively stable with a consistent decline and labelled PCEP was still detectable 12 wk post-injection (Fig 4.6B).

4.6A


Saline
Carboxylate control dye
Licor dye labelled PCEP
3 h
6 h
24 h
1 wk
6 wk
12 wk
5000
4000
3000
2000
1000

4.6B

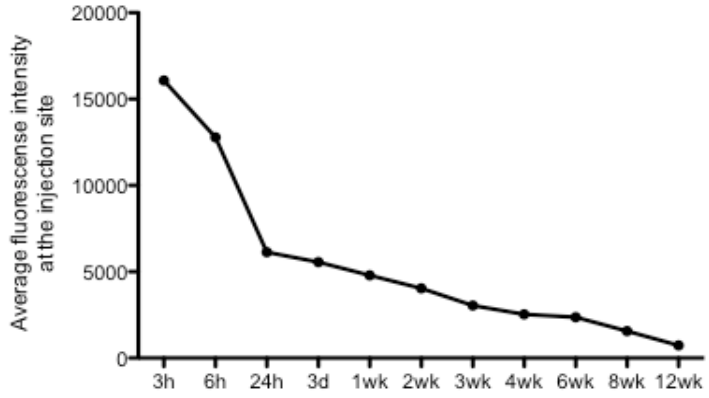

Average fluorescense intensity at the injection site
20000
15000
10000
5000
0
3h
6h
24h
3d
1wk
2wk
3wk
4wk
6wk
8wk
12wk

Fig 4.6 ***In vivo*** **imaging: near-infrared fluorescence dye labelled PCEP kinetics at the injection site.** (4.6A) BALB/c mice were injected i.m. with either PBS, carboxylated free dye or licor 800CW fluorescent dye labelled-PCEP (10 μg). Control animals were injected with either PBS (as a point of reference for background fluorescence) or hydrolyzed carboxylate 800CW IRDye (as a reference for uncounjugated dye). Real-time fluorescent images were obtained at 3 h, 6 h, 24 h, 1 wk, 6 wk and 12 wk post-injection using ODYSSEY Imaging System under identical imaging conditions. Intensity and sensitivity settings were held constant for each image. The mice were scanned at 800 nm (green fluorescence) and shown here with pseudo-color image representation of the 800 nm channels. The pseudo-color images map the intensity of fluorescence to a color pallet that is shown as a legend on the extreme right of the image. Red color indicates the highest intensity, while the blue represents the lowest intensity. Since the 800 IRDye was scanned at 800 nm, there was negligible background fluorescence from body tissues or animal feed. (4.6B) Quantification of average fluorescent intensity at the site of injection post-injection at various time points. Average intensity in pixels was obtained from site of injection post-licor 800CW fluorescent dye labelled-PCEP injection and was compared with carboxylate 800CW IRDye-injected control mice using a consistent surface area.

4.4 Discussion

In this study, the effects of PCEP on cell recruitment at the site of injection in mouse muscle were investigated in detail. We choose i.m. injection because it is the preferred route of administration for majority of vaccines. We show that PCEP is a potent inducer of cell recruitment at the injection site with lymphocytes, neutrophils and macrophages being the most abundant cells, followed by monocytes and DCs. The neutrophil, macrophages, monocyte and DCs were also increased in the draining lymph nodes after PCEP injection. Interestingly, PCEP injection also led to robust local infiltration by T and B lymphocytes but only a proportion of these cells took up the labelled PCEP, whereas most of the recruited myeloid cells did take up the adjuvant. Further, PCEP was localized to the intra-cytoplasmic lysosomal compartments of various recruited immune cells. Overall, these data suggest that PCEP induces recruitment of APCs to the injection site, which then take up adjuvant and presumably traffick to the draining lymph nodes.

Macrophages and DCs have been shown to be present in resting muscle tissue (Pimorady-Esfahani et al., 1997). We observed few myeloid and lymphoid cells at the site of injection of PBS, which might be consequent to the trauma caused by injecting liquid into the muscle tissue. These data are consistent with our previous studies where we have reported that a few genes were induced at the site of PBS-injection in muscle tissues (Awate et al., 2012). However, both PCEP and alum induced significantly higher total cell recruitment into the injected muscle tissue compared to PBS-injected mice. Various studies have shown that both alum and MF59-induced secretion of chemokines that recruit monocytes and macrophages, and in addition MF59-induced recruitment of granulocytes (Seubert et al., 2008). MF59-induced granulocyte and monocyte recruitment are CCR2-dependent, which is a receptor for CCL2 and CCL12 chemokines (Dupuis et al., 2001; Seubert et al., 2008). Similarly, alum has been shown to secrete various cytokines including IL-1β and IL-5 and chemokines including CCL2 and keratinocyte-derived chemokine (KC) at the injection site, which led to influx of eosinophils, monocytes, neutrophils and DCs (McKee et al., 2009). Amongst other cells, mast cells have been shown to be one of the major sensors of alum (McKee et al., 2009). In addition to cell recruitment, alum and MF59 augment the

differentiation of monocytes into DCs (Seubert et al., 2008). Previously, we have also shown that PCEP is a strong inducer of cytokines and chemokines at the site of injection including CCL2 and CCL12 both of which are potent chemotactic proteins (Awate et al., 2012). PCEP has also been shown to stimulate production of innate cytokines IL-12 and IFN-γ in mouse splenocytes (Mutwiri et al., 2008). These PCEP-induced cytokines and chemokines are potent cell activators and chemoattractants. This may explain why in the present study we observed increased cellular recruitment of neutrophils, macrophages, monocytes, DCs and lymphocytes after injection of PCEP.

Neutrophils were one of the most abundant myeloid cell population recruited after injection of PCEP. Neutrophils provide the first line of defense during infections and inflammation. Many studies have shown that granulocytes can also act as professional APCs under specific conditions (Sokol et al., 2009; Wang et al., 2007). In addition, neutrophils have been shown to transport antigens to the draining lymph nodes (Maletto et al., 2006). Neutrophils are recruited in high numbers at the injection site following administration of the vaccine adjuvants alum, MF59 or Complete Freund Adjuvant (CFA), which then traffick to the draining lymph nodes (Calabro et al., 2011; Maletto et al., 2006; Seubert et al., 2008). MF59 has also been shown to be internalized by DCs expressing high MHC class II and special marker DEC205 in the muscle tissue (Dupuis et al., 1998). Likewise, we observed that the recruited immune cells took up PCEP in the muscle tissue. Recruited myeloid cells took up PCEP in higher amounts compared to lymphoid cells, which may be due to higher phagocytic abilities of APCs.

Similar to what was observed with MF59 and CFA, neutrophils were the most abundant cell population recruited in muscle tissue after PCEP injection. In addition, the neutrophil numbers were significantly increased in the draining lymph nodes suggesting that neutrophils might play a role in PCEP-induced adaptive immunity. The role of neutrophils in innate immune responses is well known however; its role in activating adaptive immunity has not been studied in detail. Neutrophils might activate adaptive immunity by releasing cytokines and chemokines, which chemoattract various innate immune cells including DCs and transport antigen to the draining

lymph nodes (Abadie et al., 2005; Bennouna et al., 2003; Bonneau et al., 2006). Studies have shown that granulocytes can upregulate the expression of MHC class II and co-stimulatory molecules and can present MHC class II restricted antigen to activate adaptive immunity (Culshaw et al., 2008; Kim et al., 2009; Wang et al., 2007). Contrary to the role of neutrophils in activating adaptive immunity, Wang et al. have shown that neutrophils play an important negative role in CD4 T and B cell responses induced following immunization with antigen and alum (Yang et al., 2010). One of the reasons for the negative effect could be that the neutrophils compete for antigens with professional APCs at the injection site. Another reason for the neutrophil-mediated negative effect on adaptive immunity could be interaction between neutrophils and APCs in the lymph nodes. Depletion of neutrophils in mice immunized with antigen and incomplete freund's adjuvant (IFA) has been shown to drastically improve the DC-T cell interactions in the lymph nodes suggesting indirect role of neutrophils in modulating adaptive immune responses (Yang et al., 2010). Neutrophils are recruited in high numbers at the injection site following administration of vaccine adjuvants including alum and MF59, which then traffick to draining lymph nodes (Calabro et al., 2011; Seubert et al., 2008), similar to what was observed in our studies after injection of PCEP. Significant numbers of neutrophils were recruited within 24 h of PCEP injection both at the injection site and in the draining lymph nodes.

We also observed an increase in numbers of lymphocytes within 24 h post-injection of PCEP in the muscle tissues. MF59 and alum did not induce recruitment of lymphocytes to the injection site (Calabro et al., 2011). However, intraperitonial injection of ISCOMs led to recruitment of lymphocytes at the injection site (Smith et al., 1999). Recently, Vitoriano-Souza et al showed that injection of saponin, IFA and monophosphoryl lipid A (MPL) into the skin of mice induced selective recruitment of neutrophils, macrophages and lymphocytes (Vitoriano-Souza et al., 2012). Innate immune cells recruited to the injection site might have non-specific effector functions, however lymphocytes have capacity to either initiate or regulate antigen-specific immune responses. Hence, the lymphocyte recruitment to the injection site might have two explanations. One is that they come to injection site as effector cells to amplify the immune responses or regulate/terminate these responses. Another possibility is that APCs mainly activate

lymphocytes in the lymph nodes but their trafficking to injection site might suggest their ability to interact with APCs in anatomically different environment (Drayton et al., 2006; Singh and O'Hagan, 1999). However, detailed studies are required to understand the role of lymphocytes in the adjuvant activity of PCEP especially at the injection site.

Most studies on mechanisms of action of adjuvants report cellular recruitment at the site of injection, but whether this cellular recruitment is critical in enhancing antigen-specific immune responses is not known. Depletion studies suggest that the role of recruited innate immune cells at the injection site is redundant in the generation of adaptive immune responses (Calabro et al., 2011; McKee et al., 2009). Mast cell and neutrophil depletion did not affect the adjuvanticity of alum and MF59 respectively (Calabro et al., 2011; McKee et al., 2009). Interestingly, these studies were performed by depleting single cell populations. Identifying the role of a specific cell population *in vivo* is even more challenging due to complex environment at the injection site. Injection of adjuvants often leads to recruitment of a variety of cell populations and due to high redundancy in the immune system, other recruited cells may compensate for the depleted single cell population. In this regard, mice whose specific cell populations have been depleted were shown to produce cytokines and chemokines to recruit innate immune cells and activate T cells (Calabro et al., 2011; Seubert et al., 2008).

Until recently, depot effect was considered as a classic mechanism of action of many adjuvants including alum, CFA, biodegradable microparticles and cationic liposomes (Henriksen-Lacey et al., 2010; Herbert, 1968; Kreuter, 1988; Osebold, 1982). In depot effect antigens adsorbed onto adjuvants are retained at the injection site and are slowly released to ensure prolonged or sustained stimulation of the immune system. Various studies have shown that surgical removal of the antigen-alum depot at the injection site 14 days after immunization had no effect on the immune responses (Schijns, 2000). Apparently, even the adsorption of antigen to alum was not required for alum adjuvant activity (De Gregorio et al., 2008; Iyer et al., 2003). A recent report by Hutchison et al. clearly showed that alum adjuvant activity does not depend on depot effect (Hutchison et al., 2012). Removal of the injection site 2 h after antigen and alum administration

had no effect on humoral or cell-mediated immunity (Hutchison et al., 2012). Similar studies were performed with dye labelled MF59, which revealed that within 6 h of i.m. injection, 90% of MF59 was cleared from the injection site suggesting that MF59 does not form long-term depot at the site of injection (Dupuis et al., 1999). Likewise, ISCOMs tends to be rapidly transported to the draining lymph nodes after administration (Morein and Bengtsson, 1999). Therefore, these studies clearly indicate that depot effect may not be required for adjuvant activity of many particulate adjuvants. Similar to particulate adjuvants, the adjuvant activity of polyphosphazene may not depend on formation of depot at the site of injection (Payne et al., 1998a). Excision of site of injection 24 h post-injection of poly[di(carboxylatophenoxy)-phosphazene] (PCPP) with vaccine antigen had no detectable effects on antibody production (Payne et al., 1998a). In our study, we have used *in vivo* whole body imaging of mice injected with PCEP labelled with infra-red dye to detect PCEP distribution and retention at the site of injection. PCEP was localized strongly at the site of injection. However, 70% of PCEP was rapidly cleared from the site of injection 24 h post-injection. Further studies are required to confirm whether depot effect plays any significant role in the adjuvant activity of PCEP.

In conclusion, PCEP injection causes recruitment of various immune cells to the site of injection and these cells presumably traffick to the draining lymph node. Most of these innate immune cells recruited to injection site internalize the PCEP. However, the role of these recruited cells in the enhancement of antigen-specific immune responses remains to be determined. Further studies are required to investigate detailed relationship between recruited immune cells and adjuvant activity of PCEP.

CHAPTER 5. CASPASE-1 DEPENDENT IL-1β SECRETION AND T CELL ACTIVATION BY THE NOVEL ADJUVANT PCEP

Relationship of this study to the dissertation

In our studies in chapter 3, we have shown that PCEP upregulates NLRP3 gene and pro-inflammatory cytokines IL-1β and IL-18 at the injection site. Since caspase-1 is a critical component of NLRP3 inflammasome, in this study we examined the role of caspase-1 in PCEP-mediated secretion of IL-1β and IL-18 by splenic dendritic cells and PCEP-mediated maturation of dendritic cells. We have also showed PCEP-mediated lymphocyte recruitment (Chapter 4) at the injection site. Hence, in this study we examined the potential of PCEP to directly activate lymphocytes and induce antigen-specific T cell responses in mice. These studies suggest that PCEP activates innate immunity leading to increased antigen-specific cellular responses.

5.1.1 Introduction

Modern vaccines with highly purified antigens require addition of adjuvants to enhance the immune responses. Although how adjuvants enhance immune responses is largely unknown, a few mechanisms have been proposed including, depot formation, increase in cytokine and chemokine production, immune cell recruitment, enhanced antigen uptake and presentation by antigen presenting cells (APCs) and increase in T cell and B cell priming (Brewer et al., 1999; Mannhalter et al., 1985; Osebold, 1982). Generally, adjuvants utilize a combination of these various mechanisms to promote antigen-specific immune responses.

Dendritic cells (DCs) are professional APCs and specialize in antigen uptake, processing and presentation to T and B lymphocytes resulting in activation of adaptive immune responses (Steinman, 2012). Maturation of DCs is essential for inducing T cell activation and differentiation (Coyle and Gutierrez-Ramos, 2001). Liposomes and monophosphoryl lipid A (MPL) adjuvants have been shown to induce maturation of DCs by upregulating the expression of MHC class II and

co-stimulatory molecules CD86 and CD80 (Copland et al., 2003; De Becker et al., 2000; Shah et al., 2003; Sokolovska et al., 2007). Further, *in vitro* studies have shown that alum increases antigen uptake by DCs and alters the magnitude and duration of antigen presentation (Mannhalter et al., 1985; Morefield et al., 2005), however; both alum and MF59 failed to directly activate or induce maturation of DCs (Seubert et al., 2008; Sun et al., 2003).

Recognition of various pathogens through pattern recognizing receptors (PRRs) expressed by DC leads to DC maturation and subsequently enhances adaptive immune responses. Various studies have shown that TLR dependent signalling is not required by alum and MF59 to induce antigen-specific antibody responses (Gavin et al., 2006; Seubert et al., 2011). Studies with MyD88-deficient mice have shown that MF59-induced Th1 and Th2 responses are dependent on MyD88 (Seubert et al., 2011). However, antigen-specific T cell responses induced by alum depends on MyD88 and uric acid (Kool et al., 2008b). Uric acid is released as a result of cell damage and necrosis induced by alum at the injection site, which act as danger signals for activation of NOD-like receptor family, pyrin-domain-containing 3 (NLRP3). The NLRP3 inflammasome is an intra-cytoplasmic multi-protein complex containing NLRP3 receptor, apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1 (Schroder and Tschopp, 2010). Activation of NLRP3 inflammasome induces caspase-1 activation, which in turn cleaves proforms of IL-1β and IL-18 to their bioactive forms (Martinon et al., 2009). IL-1β is a potent pro-inflammatory cytokine that plays an important role in regulation of immune responses and promotes Th17 differentiation (Lalor et al., 2011). NLRP3 inflammasome can be activated by various stimuli including damage-associated molecular patterns (DAMPs), monosodium urate (MSU), alum, bacterial and viral nucleic acids, bacterial toxins, muramyl dipeptide, environmental irritants such as asbestos and silica, metabolic stress and UVB irradiation (Schroder and Tschopp, 2010). *In vitro* studies by various investigators have showed that activation of NLRP3 is required for alum-induced IL-1β and IL-18 secretion (Eisenbarth et al., 2008; Franchi and Nùñez, 2008; Hornung et al., 2008; Kool et al., 2008a). Alum-induced secretion of IL-1β and IL-18 was shown to be caspase-1 dependent (Li et al., 2007; Sokolovska et al., 2007). However, the role of NLRP3 in adjuvant activity of alum *in vivo* is not clear.

Polyphosphazenes are a novel class of adjuvants that have been shown to be effective as parenteral and mucosal adjuvants in small as well as large animals (Eng et al., 2010a; Shim et al., 2010). In particular, the new generation polyphosphazene polyelectrolyte, poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) promotes enhanced and long-lasting immune responses with a variety of viral and bacterial antigens (Eng et al., 2010a; McNeal et al., 1999; Mutwiri et al., 2008; Payne et al., 1995; Payne et al., 1998a). In addition, PCEP induces a mixed Th1/Th2 type responses giving broad-spectrum immunity (Dar et al., 2012; Mutwiri et al., 2008; Mutwiri et al., 2007b). However, the mechanisms by which PCEP induces higher immune responses are poorly understood.

We have previously shown that *in vitro*, PCEP stimulates the production of innate cytokines in mouse splenocytes, which might contribute to its adjuvant activity and that intramuscular (i.m.) injection of PCEP resulted in induction of adjuvant core response genes and production of various cytokines and chemokines at the site of injection (Awate et al., 2012; Mutwiri et al., 2008). PCEP enhanced the expression of NLRP3 gene and induced local production of pro-inflammatory cytokines IL-1β and IL-18 (Awate et al., 2012). In the present study, we investigated the role of caspase-1 in PCEP-mediated pro-inflammatory cytokine production, the potential of PCEP to directly activate DCs and lymphocytes and the capacity of PCEP to induce antigen-specific cellular responses in mice.

5.2 Materials and methods

5.2.1 Animals

Four to six weeks old female BALB/c and C57BL/6 mice purchased from Charles River Laboratories (North Franklin, CT, USA) were used in all the experiments. The animal experiments were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane use of animals.

5.2.2 Adjuvants

PCEP was synthesized by Idaho National Laboratories (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). PCEP was dissolved in Dulbecco's Phosphate buffered saline (PBS) (Sigma-Aldrich, MO, USA) by gentle agitation for 36 h at room temperature (RT). Imject alum (Thermo Fisher Scientific, IL, USA) used in these experiments was a mixture of aluminium hydroxide and magnesium hydroxide (40 mg/ml). Lipopolysaccharide (LPS) was purchased from InvivoGen, CA, USA.

5.2.3 PCEP labeling

PCEP was reacted with 1,6-diaminohexane (Sigma-Aldrich) and a coupling reagent (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; Thermo Fisher Scientific) to obtain an amino-modified PCEP (Staros et al., 1986). A thiol group was attached to amino-modified PCEP and now the thiol modified PCEP was labelled with Alexa-fluor 488 C_5-maleimide (Molecular probes, OR, USA) with the following modifications. Briefly, 1 mg of the thiol-modified PCEP was dissolved in 1 ml of PBS (pH 7.5) and mixed with 500 µl of 1 mg/ml of the dye solution in ultrapure water and reacted for 2 h at room temperature in the dark. The unlabelled dye was separated from the labelled products by size exclusion chromatography on sephadex G-75 column (GE Healthcare, Sweden). Later, the labelled PCEP was concentrated using Amicon ultra-centrifugal filter units with 10 kD molecular weight (EMD Millipore, MA, USA).

5.3.4 Isolation of splenic-derived DCs

Spleens were aseptically removed from naïve/untreated mice and placed in cold HEPES (Gibco, NY, USA) with collagenase D (Roche Diagnostics, Germany) solution. Cells were disrupted by injecting HEPES with collagenase D solution into the spleen with syringe, later cut into smaller pieces, and incubated at 37°C for 30min. The spleen tissues were teased with syringe plunger through the nylon mesh. The cell suspension obtained was resuspended with autoMACS rinsing buffer with 0.5% BSA (Miltenyi Biotec, CA, USA). Total DCs (conventional and

plasmacytoid DCs) from mouse spleen cell suspension were positively selected using Pan DC microbeads (Miltenyi Biotec), according to the manufacturer's instructions. Isolated DCs were stained with CD11c-PE (eBiosciences, CA, USA) to check for purity using flow cytometry (the purity of the isolated splenic DCs were >80%).

5.2.4 Splenic DC culture

MACS isolated splenic DCs were cultured (1 X 10^6 cells/well) with media, PCEP (50 ug/ml), alum (0.5 mg/ml), LPS (0.1 ug/ml) alone or PCEP and LPS or alum and LPS combinations at 37°C. In some experiments DCs were incubated with caspase-inhibitor YVAD-fmk (R&D Systems, MN, USA) along with vaccine adjuvants. After 12 h of stimulation, culture supernatants and cells were collected for cytokine measurement and immunoblot respectively.

5.2.6 Measurement of cytokines in culture supernatants

IL-1β concentration was assayed in culture supernatants using DuoSet ELISA development system (R&D Systems) following manufacturer's protocol. For IL-18, Immunol II microtitre plates (Dynex Technology Inc., VA, USA) were coated overnight with anti-mouse IL-18 antibody (MBL, Japan) at 2 μg/ml concentration at room temperature (RT). The Microtitre plates were washed 3 times with Tris-buffered saline (pH 7.3) containing 0.05% Tween 20 (TBST) and 100 μl of diluted sera or tissue samples were added to the wells and incubated for 2 hrs. The wells were washed again 3 times with TBST and biotinylated rat anti-mouse IL-18 antibody (MBL, 1/1000) was added to the wells in a 100 μl volume and incubated for 2 h at RT. Wells were washed and horseradish-peroxidase conjugated streptavidin was added to each well followed by incubation for 20 min at RT. Wells were washed 3 times with TBST before addition of 3,3',5,5'-Tetramethylbenzidine (TMB; Sigma-Aldrich). The microtitre plates were incubated for 20 min at RT before stopping the reaction with 2 N sulphuric acid. For IL-1β and IL-18, the absorbance was read as optical density (OD) at 570 nm in a Microplate Reader (Molecular Devices, CA, USA). The samples were assayed in triplicate, and the cytokine concentration was determined by extrapolation from a standard curve generated by serial dilution of the respective appropriate recombinant murine cytokines.

5.2.7 Immunoblotting

After 12 h of culture, splenic DCs were washed with ice-cold PBS and lysed with RIPA buffer (0.5 M Tris [pH 8.0], 0.15 M NaCl, 0.1% SDS, 1% NP-40, 1% deoxycholic acid) containing protease inhibitors (1×Complete Protease Inhibitor; Roche Diagnostics). The lysates were homogenized by passing through a syringe with 22-gauge needle several times and incubated on ice for 15-20 min. The lysates were centrifuged at 12000 g for 5 min and the supernatant was analyzed for total protein content by using BCA protein assay kit (Thermo Fisher Scientific). Thirty micrograms of total protein from each lysate was subjected to 12.5 % SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membrane (Bio-Rad, Germany). Membranes were blocked for non-specific binding with Tris-buffered saline (TBS) (0.1 M Tris [pH 7.6], 0.9% NaCl containing 0.1% Tween 20 and 5% skim milk) for 1 h at room temperature. The membrane was probed by antibody to pro-IL-1β (sc-7884; Santa Cruz Biotechnology, CA, USA) diluted 1:40 and procaspase-1 (sc-514; Santa Cruz Biotechnology) diluted 1:200, followed by incubation with IRDye secondary antibodies (LI-COR Biosciences, NE, USA) diluted 1:5000. Finally, the infrared signals of immunoblots were detected by Odyssey infrared imager (LI-COR Biotechnology). Immunoblotting for β-actin (Sigma-Aldrich) served as a loading control.

5.2.8 *In vitro* PCEP uptake

For PCEP uptake studies, splenic DCs were incubated with PCEP-labelled with Alexa-fluor 488 fluorescent dye (50 μg/ml) for 12 h on 2 well tissue culture chamber glass slides (Thermo Fisher Scientific) at 37°C and 5% CO_2. After 12 h, cells were fixed with 3.7% paraformaldehyde for 10 min and washed thrice with 0.1M PBS for 5 min each. Finally, the chambers were removed and slides were mounted with VECTASHIELD mounting medium containing DAPI (Vector Laboratories, CA, USA) to stain the nucleus. The slides were allowed to cure for 24 h in dark at RT before visualizing under Zeiss LSM 5 laser scanning confocal microscope (Carl Zeiss, Germany).

5.2.9 Generation and culture of BMDCs

Legs (femurs and tibias) were aseptically removed from the mice and flushed with ice-cold PBS to isolate bone marrow (BM) cells. Isolated BM cells were centrifuged at 350 g for 10 min at 4 °C and resuspended with 5 ml of 0.84% ammonium chloride solution for 5 min at RT to lyse the red blood cells. The cells were washed two times in PBS, centrifuged at 350 g for 10 min at 4 °C and then resuspended in complete RPMI 1640 media (containing 10% fetal calf serum (FBS), Hepes, non-essential amino acids, sodium pyruvate, antibiotic/antimycotic [all from Gibco] and β-mercaptoethanol [Sigma-Adlrich]) supplemented with 100 ng/ml recombinant mouse Fms-related tyrosine kinase 3 ligand (Flt3L; PeproTech, NJ, USA). BM cells were cultured in complete RPMI media supplemented with murine Flt3L at 1 X 10^6 cells/ml in a 6-well plate (Costar Corning, NY, USA) in a humidified incubator containing 5% CO_2 at 37°C for 7 days. On 7^{th} day, immature DCs (iDCs) were harvested from the culture plates by pipetting and washing with PBS at RT. Harvested iDCs were again resuspended in complete RPMI supplemented with murine Flt3L and cultured for 24 h with media, PCEP (50 μg/ml) or LPS (100 ng/ml) at 1 X 10^6 cells/ml in a 24-well plate at 37°C. After 24 h, BMDCs were harvested and stained with mAbs directed against maturation (MHC class II) and co-stimulatory molecules CD86 and CD40 (all from eBiosciences).

5.2.10 Allogeneic mixed leukocyte reaction (MLRs)

The stimulatory capacity of DCs was assessed in an allogenic MLR. BMDCs were generated from BALB/c mice and then stimulated with media, PCEP (50 μg/ml) or LPS (1 μg/ml) at 37°C and 5% CO2 in a humidified incubator. After 24 h, BMDCs (stimulator cells) were harvested, washed with PBS and co-cultured with $CD4^+$ T cells in complete RPMI medium. $CD4^+$ T cells (responder cells) were obtained from C57BL/6 or BALB/c mice spleen cell suspension using $CD4^+$ T cell microbeads (Miltenyi Biotec), according to the manufacturer's instructions. Isolated $CD4^+$ T cells were stained with CD4-PE (eBiosciences) to check for purity using flow cytometry. The positively selected $CD4^+$ T cells were washed and resuspended in complete RPMI medium. Triplicate wells of 2 X 10^5 $CD4^+$ T cells from C57BL/6 or BALB/c mice (responder cells) were seeded in a 96 well round-bottom plate, and titrated numbers of BMDCs (stimulator cells) were added (DC and T cell ratio; 1:10, 1:40, 1:160 and 1:640). Cells were co-cultured for

five days and pulsed with 0.4 μCi/well of titrated thymidine (ARC, MO, USA) during the last 18 h of culture. Thymidine incorporation was measured using a liquid scintillation counter (Wallac-Perkin-Elmer,Wallac, Finland). Results were expressed as mean counts per minute (cpm). The stimulation index represents the ratio of cpm obtained in the stimulated cultures to cpm obtained in controls (media). A stimulation index of ≥3 indicates a positive proliferation response.

5.2.11 Splenic B and T cell isolation

Spleens were aseptically removed from naïve/untreated mice and placed in cold HEPES (Gibco) with collagenase D (Roche Diagnostics) solution. Spleen cells were disrupted by injecting HEPES with collagenase D solution into the spleen with syringe, later cut into smaller pieces, and incubated at 37°C for 30 min. The spleen tissues were teased with syringe plunger through the nylon mesh. The cell suspension obtained was resuspended with autoMACS rinsing buffer with 0.5% BSA (Miltenyi Biotec). $CD4^+$ T cells and B cells were positively selected from mouse spleen cell suspension using $CD4^+$ (L3T4) and CD45R (B220) microbeads respectively (Miltenyi Biotec), according to the manufacturer's insructions. Isolated $CD4^+$ T and B cells were stained with CD4-PE and CD19-FITC respectively (both from eBiosciences) to check for purity using flow cytometry.

5.2.12 T cell culture and proliferation assay

MACS isolated splenic $CD4^+$ T cells (1×10^6 cells/well) were cultured in the presence of media, PCEP (10 μg/ml) or Con A (2 μg/ml) in a humidified incubator containing 5% CO_2 at 37°C. Culture supernatants were collected after 24 h for quantification of IL-2, IL-4 and IFN-γ using DuoSet ELISA development system (R&D Systems). For proliferation assay, triplicate wells of naïve CD4+ T cells (2×10^5 cells/well) were cultured in the presence of medium, PCEP (5 μg/ml, 10 μg/ml and 25 μg/ml) and 2 μg/ml of Con A (Sigma-Aldrich) into 96-well round bottom plates for 5 days. The cells were pulsed with 0.4 μCi/well of titrated thymidine (ARC) during the last 18 h of culture. Thymidine incorporation was measured using a liquid scintillation counter (Wallac-Perkin-Elmer). Results were expressed as mean counts per minute (cpm). The stimulation

index represents the ratio of cpm obtained in the stimulated cultures to cpm obtained in controls (media). A stimulation index of ≥3 indicates a positive proliferation response.

5.2.13 B cell culture and proliferation assay

MACS isolated splenic B cells (2 X 10^6 cells/well) were cultured in the presence of media, PCEP (10 μg/ml) or LPS (0.1 μg/ml) in a humidified incubator containing 5% CO_2 at 37°C. Culture supernatants were collected after 48 h for quantification of IL-6 (DuoSet ELISA development system; R&D Systems) and IgM (mouse IgM Ready-SET-Go kit; eBioscience) by ELISA. For proliferation assay, triplicate wells of naïve B cells (2 X 10^5 cells/well) were cultured in the presence of medium, PCEP (5 μg/ml, 10 μg/ml and 25 μg/ml) and LPS (0.1 μg/ml) into 96-well round bottom plates for 5 days. The cells were pulsed with 0.4 μCi/well of titrated thymidine (ARC) during the last 18 h of culture. Thymidine incorporation was measured using a liquid scintillation counter (Wallac-Perkin-Elmer). Results were expressed as stimulation index. The stimulation index represents the ratio of cpm obtained in the stimulated cultures to cpm obtained in controls (media). A stimulation index of ≥3 indicates a positive proliferation response.

5.2.14 Immunization of mice

Mice were divided into groups and immunized with 25 μl each of either phosphate-buffered saline (PBS) as control, 10 μg OVA (Hyglos GmbH, Germany) or 50 μg of PCEP co-delivered with 10 μg OVA. Endotoxin concentration in OVA used in immunization studies was <1EU/mg. Half of mice in each group were euthanized 9 days after immunization to collect spleens. The remaining mice were given a secondary immunization on day 14 and euthanized 21 day after the first immunization to collect spleens.

5.2.15 Intracellular IFN-γ staining

Spleens were digested with collagenase solution to get single cell suspension. To investigate IFN-γ production, splenocytes (1 X 10^6 cells/well) were cultured in 96-well round bottom culture plates and restimulated with 10 μg/ml OVA and incubated at 37°C and 5% CO_2 in a humidified incubator. Intracellular staining for IFN-γ was performed after 12 h of incubation. Cells

were fixed with 4 % paraformaldehyde (RICCA chemicals, TX, USA) and stained with CD4 and CD8a T cell markers listed previously. Subsequently, cells were permeabilized with cytofix/cytoperm (BD Biosciences, CA, USA) and stained for intracellular IFN-γ (BD Biosciences) in PBS. Enumeration of IFN-γ responses by $CD4^+$ and $CD8a^+$ T cells were done by flow cytometric analysis.

5.2.16 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). The differences between groups were analyzed by one-way ANOVA and statistical significance between the treatments were compared with Dunn's and Tukey's multiple comparison test; ***P<0.0001, **P<0.001, *P<0.05.

5.3 Results

5.3.1 PCEP induces robust secretion of IL-1β and IL-18 in splenic DCs

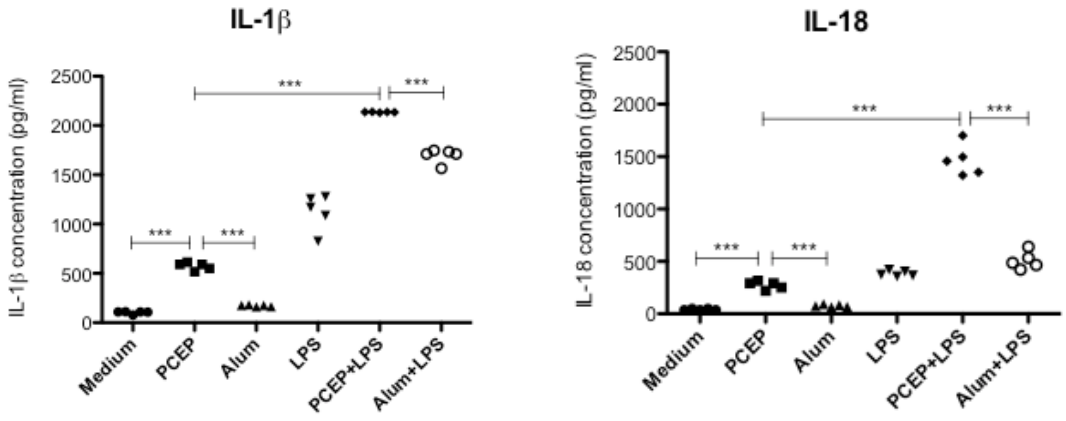
In our previous studies we showed that PCEP up-regulates the expression of NLRP3 gene and induces production of pro-inflammatory cytokines IL-1β and IL-18 at the site of injection (Awate et al., 2012). In various *in vitro* studies, alum-induced IL-1β secretion has been shown to involve activation of inflammasomes (Eisenbarth et al., 2008; Franchi and Nùñez, 2008; Harris et al., 2010; Kool et al., 2008a; Li et al., 2007). Hence, we decided to test the ability of PCEP to induce secretion of IL-1β and IL-18 in splenic DCs and further confirm the role of caspase-1 in PCEP-mediated IL-1β and IL-18 secretion.

Splenic DCs from BALB/c mice were stimulated for 12 h with PCEP or alum in the presence or absence of TLR4 agonist LPS, and subsequently assayed for mature IL-1β and IL-18 secretion in the culture supernatants (Fig 5.1A). Stimulation of splenic DCs with PCEP induced significantly higher IL-1β and IL-18 secretion relative to media and alum. In addition, stimulation with PCEP in the presence of LPS triggered significantly higher secretion of IL-1β and IL-18

compared to PCEP or LPS alone (Fig 5.1A). Further, in the presence of LPS, PCEP induced significantly higher secretion of IL-1β and IL-18 compared to alum and LPS combination (Fig 5.1A).

Since PCEP alone was able to induce significant IL-1β secretion, we assessed induction of pro-IL-1β and pro-caspase-1 by PCEP. Splenic DCs were stimulated with PCEP or alum in the presence or absence of LPS for 12 h and cell extracts were analyzed for pro-IL-1β and pro-caspase-1 by Western blot. We observed that PCEP alone induced intracellular production of pro-IL-1β in splenic DCs (Fig 5.1B). However, pro-IL-1β induction was not as strong as induction by the PCEP and LPS combination. LPS induced pro-IL-1β production regardless of presence or absence of PCEP or alum. Similarly, PCEP alone induces higher secretion of pro-IL-1β compared to alum alone (Fig 5.1B). Pro-caspase-1 was produced in all the splenic DC treatments including media control. β-actin was used as loading control in all Western blot experiments.

5.1A



5.1B

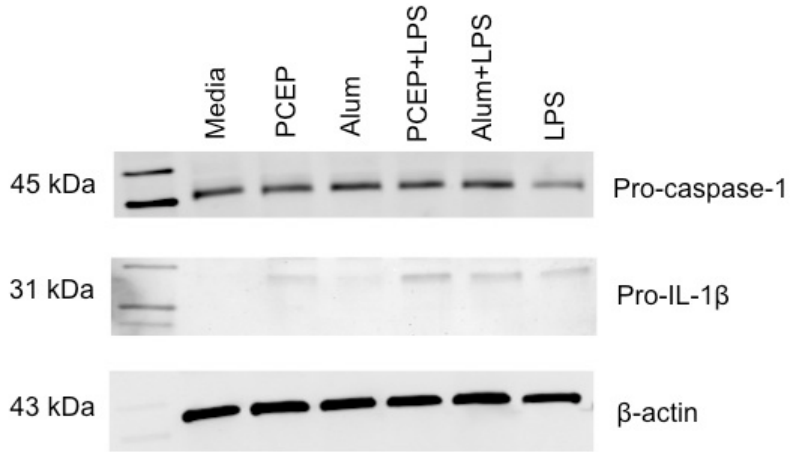


Fig 5.1 PCEP induces robust secretion of IL-1β and IL-18 in splenic DCs. Splenic DCs from BALB/c mice were incubated for 12 h with media, PCEP (50 ug/ml), alum (40 mg/ml), LPS (0.1 ug/ml) alone or PCEP+LPS or alum+LPS combinations. Supernatants were collected for measuring IL-1β and IL-18 by ELISA and the cell extracts were analyzed for pro-IL-1β by western blotting. **5.1A,** PCEP significantly enhanced the secretion of IL-1β and IL-18 in splenic DCs. **5.1B,** Pro-caspase-1 (45 kDa), pro-IL-1β (31 kDa) and β-actin (43 kDa) detection in cell lysates by Western blot analysis. PCEP alone induced pro-IL-1β production in splenic DCs. Data was analyzed by one-way ANNOVA and the comparison between the treatments was done by Tukey's multiple comparison test: ***P<0.0001.

5.3.2 PCEP-mediated IL-1β secretion is caspase-1 dependent

Caspase-1 is a critical component of NLRP3 inflammasome, which cleaves the pro-form of IL-1β and IL-18 into mature forms. Hence, we examined the role of caspase-1 in secretion of IL-1β and IL-18 by splenic DCs. Splenic DCs were treated with or without the caspase-1 inhibitor (CI) YVAD-fmk and then stimulated with media, PCEP, alum, LPS alone or PCEP and LPS or alum and LPS combinations for 12 h. Secretion of IL-1β and IL-18 was analyzed in culture supernatants. Pre-treatment with YVAD-fmk significantly inhibited IL-1β and IL-18 secretion in response to adjuvants alone and in adjuvants given in combination with LPS (Fig 5.2). The most significant reduction in IL-1β and IL-18 secretion was observed in YVAD-fmk-treated DCs that were stimulated with PCEP+LPS (Fig 5.2). The same was observed with alum+LPS-treated splenic DCs. These results suggest that PCEP- and alum-mediated secretion of IL-1β and IL-18 in splenic DCs was caspase-1 dependent.

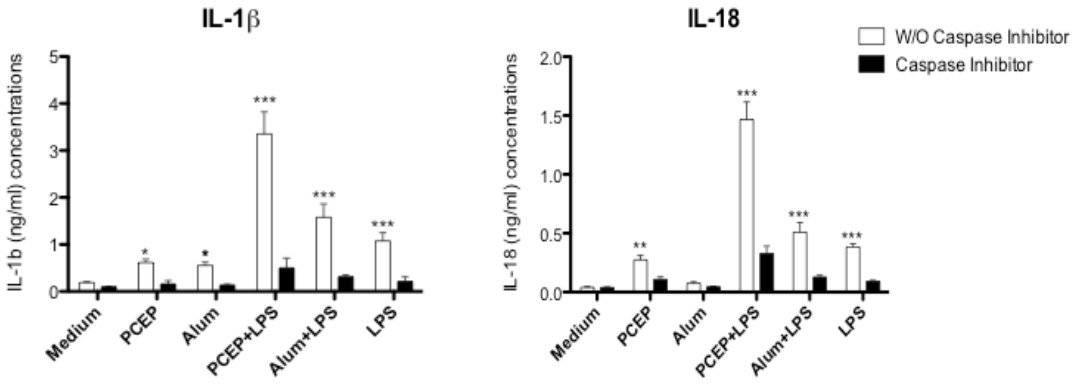
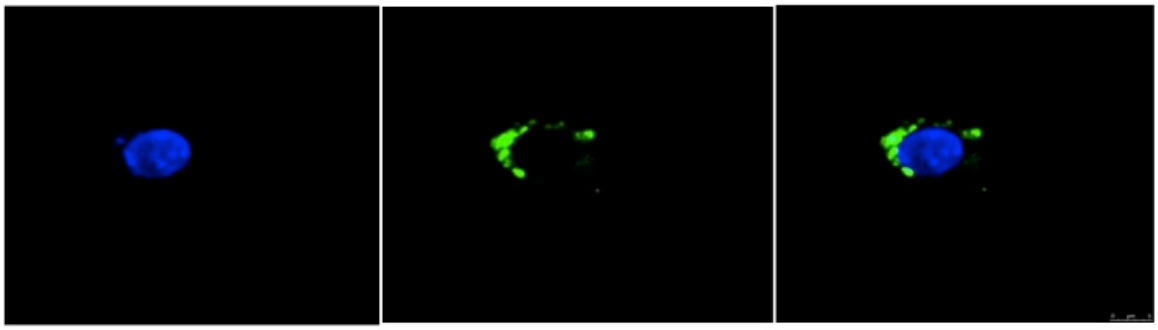


Fig 5.2 Role of caspase-1 in PCEP stimulated IL-1β and IL-18 secretion. Splenic DCs were treated with or without the caspase-1 inhibitor (CI) YVAD-fmk (40μM) and then incubated with media, PCEP (50 ug/ml), alum (40 mg/ml), LPS (0.1 ug/ml) alone or PCEP+LPS or alum+LPS combination. Supernatants were collected after 12 h of stimulation, and were analysed for IL-1β and IL-18 by ELISA. Data was analyzed by one-way ANNOVA and the comparison between the treatments was done by Tukey's multiple comparison test: ***P<0.0001, **P<0.001, *P<0.05.

5.3.3 Intracellular uptake of PCEP by DCs

PCEP was labelled with Alexa-fluor 488 fluorescent dye to identify its uptake by splenic DCs. Microscopic evaluation of splenic DCs incubated with fluorescent dye labelled PCEP revealed that PCEP was readily taken up and are localized within the cytoplasm of the splenic DCs (Fig 5.3).



DAPI | PCEP labelled with Alexa-fluor 488 dye | Merge

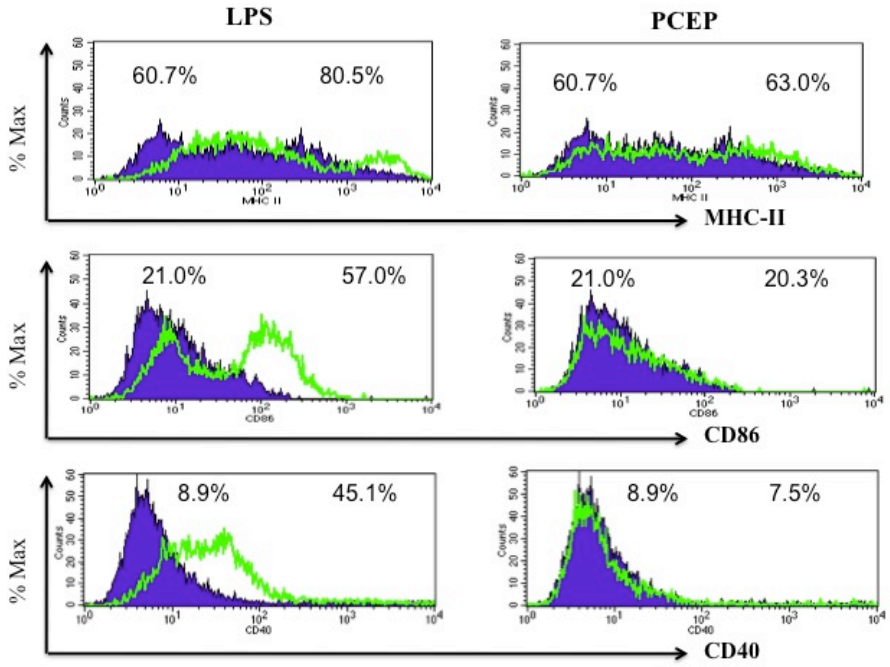
Fig 5.3 Intracellular uptake of PCEP by splenic DCs *in vitro*. Splenic DCs were incubated with PCEP labelled with Alexa-fluor 488 fluorescent dye (50 μg/ml) or free dye for 12 h. Intracellular localization of fluorescent-labelled PCEP was evaluated on representative cells using a confocal laser scanning microscope. Nuclei were stained with DAPI in each panel. Green color indicates fluorescent labelled PCEP; blue color indicates the nucleus.

5.3.4 PCEP does not induce MHC class II and co-stimulatory molecules expression in vitro

To investigate PCEP-mediated maturation and activation of DCs, we generated DCs from mouse bone marrow. PCEP did not induce significant MHC class II and co-stimulatory molecules CD86 and CD40 expression in BMDCs compared to negative control (Fig 5.4A). In contrast, MHC class II, CD86 and CD40 molecules were highly expressed in LPS-treated BMDCs (Fig 5.4A).

The functional capacity of DC to stimulate T cell responses is assessed by mixed leukocyte reaction (MLR). One of the factors affecting the stimulatory capacity includes the state of maturity of DCs. Hence we assessed the functional capacity of DCs stimulated with PCEP to induce T cell proliferation. BMDCs generated from BALB/c mice were stimulated with PCEP or LPS and then co-cultured with $CD4^+$ T cells isolated from C57BL/6 (allogeneic MLR) and BALB/c (syngenic MLR) mouse splenocytes in various DC:T cell ratios. PCEP-induced T cell proliferative responses were significantly higher than negative controls both in allogeneic and syngenic responses suggesting that PCEP did induce maturation of DCs (Fig 5.4B). However, LPS-induced T cell proliferative responses were higher than both negative control and PCEP-treated cells (Fig 5.4B).

5.4A


LPS
PCEP
60.7%
80.5%
60.7%
63.0%
% Max
MHC-II
21.0%
57.0%
21.0%
20.3%
CD86
8.9%
45.1%
8.9%
7.5%
CD40

5.4B

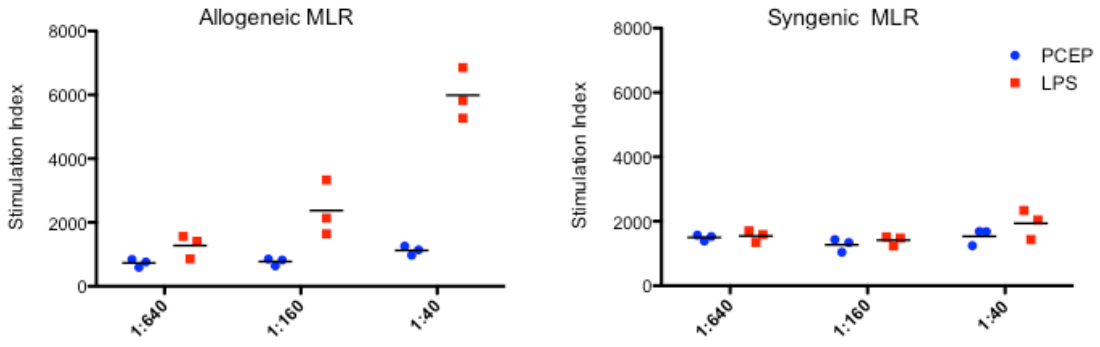

Allogeneic MLR
Syngenic MLR
Stimulation Index
8000
6000
4000
2000
0
1:640
1:160
1:40
PCEP
LPS

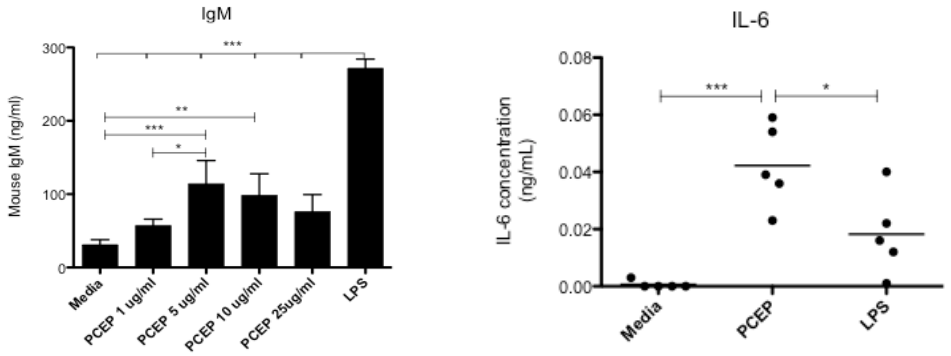
Fig 5.4 PCEP does not induce MHC class II and co-stimulatory molecules expression *in vitro*. 5.4A, BMDCs (1 X 10^6 cells/ml) were incubated with media, PCEP (50 μg/ml) or LPS (100 ηg/ml) for 24 h. Cells were stained with MHC class II, CD 86 and CD40 antibodies and analyzed by flow cytometry. The overlay histograms show the % of maximum cells positive for MHC class II, CD86 or CD40 in PCEP- and LPS-treated BMDCs. Blue shaded area represents media control and the green overlay line represents LPS- and PCEP-treated BMDCs. **5.4B,** Functional capacity of DCs to stimulate T cells was tested using MLR. BMDCs generated from BALB/c mice were cultured in media stimulated with PCEP (50 μg/ml) or LPS (1 μg/ml) for 24 h, irradiated and then co-cultured with $CD4^+$ T cells isolated from C57BL/6 (allogenic) and BALB/c (syngenic) mice splenocytes for 4 days. PCEP- or LPS-induced lymphocyte proliferative responses were measured by 3H-thymidine incorporation, shown at different DC-to-responder cell ratios (1:10, 1:40 and 1: 160). Results are expressed as stimulation index (cpm in the stimulated cultures/cpm in the controls). Tests were carried out in triplicates. A stimulation index of ≥3 was considered positive for proliferative responses.

5.3.5 PCEP induces direct activation of naïve B but not naïve T cells in vitro.

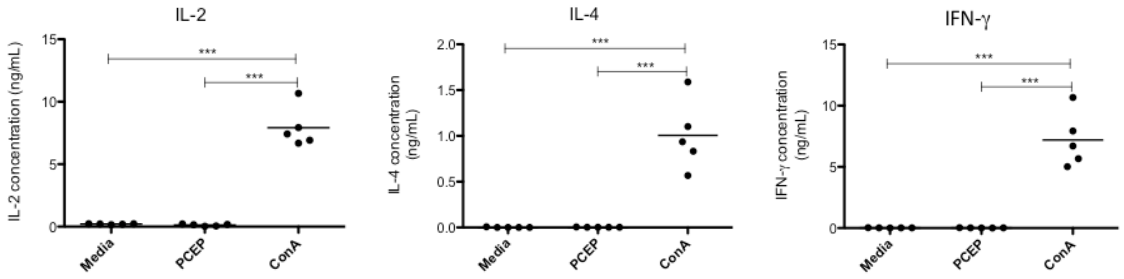
In our previous study, PCEP induced significant recruitment of T and B lymphocytes to the site of injection, and some of these cells also took up PCEP in moderate amounts. Hence we investigated the capacity of PCEP to induce direct activation of T or B lymphocytes *in vitro*. MACS isolated $CD4^+$ T cells and $CD19^+$ B cells were cultured in the presence of PCEP and culture supernatants were analyzed for cytokine and IgM responses. PCEP stimulated significant production of IgM in a dose-dependent manner with highest production when used at 5 μg/ml suggests direct activation of naïve B cells (Fig 5.5A). In addition, PCEP induced significant production of IL-6, however the amounts of IL-6 produced was low (Fig 5.5A). Further, we observed that PCEP did not induce secretion of IL-2, IL-4 and IFN-γ in naïve $CD4^+$ T cells culture supernatants (Fig 5.5B). Additionally, PCEP did not induce IL-10 and IL-12 production by naïve B cells or T cells (data not shown).

We have previously shown that PCEP is taken up by splenic DCs but also induces maturation *in vitro*. To determine whether PCEP can induce lymphocyte proliferation directly, we performed *in vitro* B and T cell proliferation assays using LPS and Con A as positive controls, respectively. Naïve B and T cells were directly stimulated with various concentrations of PCEP *in vitro*. PCEP did not induce positive proliferation responses in B cells (Fig 5.5C) and T cells (Fig 5.5D) at any concentrations. The data suggests that PCEP induces direct activation of naïve B cells (but does not induce B cell proliferative responses) and does not activate T cells.

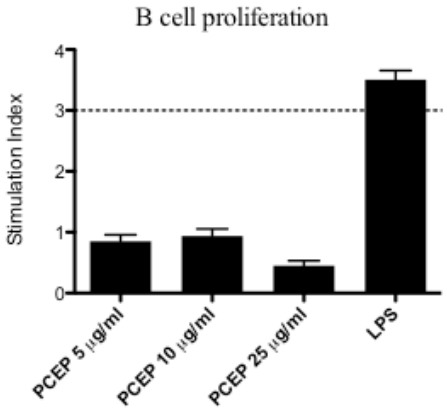
5.5A



5.5B



5.5C



5.5D

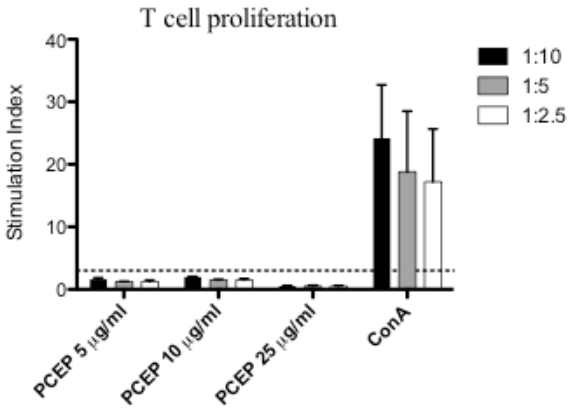
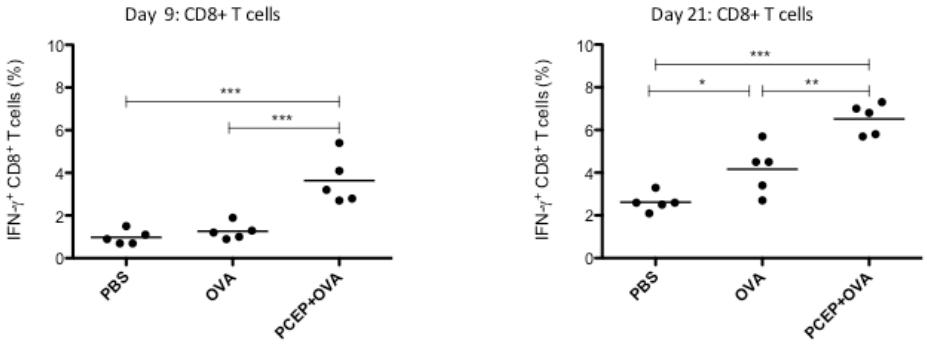


Fig 5.5 PCEP induces direct activation of naïve B but not naïve T cells *in vitro*. 5.5A, Splenic $CD19^+$ B cells (2×10^6) were cultured in the presence of media, PCEP (10 μg/ml) or LPS (0.1 μg/ml) and culture supernatants were collected after 48 h for quantification of IL-6 and IgM by ELISA. **5.5B,** Splenic $CD4^+$ T cells (1×10^6) from BALB/c mice were cultured in the presence of media, PCEP (10 μg/ml) or Con A (2 μg/ml) and culture supernatants were collected after 24 h for quantification of IL-2, IL-4 and IFN-γ by ELISA**. 5.5C,** Naïve B cells (2×10^5 cells/well) were cultured in the presence of medium, PCEP (5 μg/ml, 10 μg/ml and 25 μg/ml) and LPS (0.1 μg/ml) for 5 days. **5.5D,** Naïve CD4+ T cells (2×10^5 cells/well) were cultured in the presence of medium, PCEP (5 μg/ml, 10 μg/ml and 25 μg/ml) and 2 μg/ml of Con A for 5 days. PCEP specific lymphocyte proliferative response was measured by 3H-thymidine incorporation. Results are expressed as stimulation indexes (cpm in the stimulated cultures/cpm in the controls). Tests were carried out in triplicates. A stimulation index of ≥3 was considered positive for proliferative responses (above dashed lines). All the ELISA data was statistically analyzed by one-way ANOVA and the differences between the treatments were compared by Tukey's multiple-comparison test for T cell and B cells, where ***P<0.0001, **P<0.001, *P<0.05.

5.3.6 PCEP induces increased IFN-γ production in $CD4^+$ and $CD8^+$ T cells.

To evaluate antigen-specific T cell responses induced by PCEP, BALB/c mice were immunized with PCEP co-delivered with OVA. Mice immunized with PBS or OVA served as controls. Half of the mice received a secondary immunization on day 14. Mice were sacrificed and spleens were collected on day 9 and 21 post-immunization. To investigate IFN-γ production by T cells, we restimulated splenocytes with OVA and the frequency of IFN-γ^+ $CD8^+$ and $CD4^+$ T cells was analyzed by flow cytometry. As shown in Fig 5.6A, intracellular IFN-γ production was increased in mice immunized with PCEP+OVA when compared with OVA alone. Frequencies of IFN-γ^+ $CD8^+$ T cells on day 9 (3.6% vs 1.3%) and day 21 (6.5% vs 4.2%) were significantly higher in mice immunized with PCEP+OVA than in mice immunized with OVA alone (Fig 5.6A). Similarly, frequencies of IFN-γ^+ $CD4^+$ T cells on day 9 (9.0% vs 3.8%) and day 21 (7.3% vs 5.3%) were significantly higher in mice immunized with PCEP+OVA than in mice immunized with OVA alone (Fig 5.6B). These results indicate that PCEP induces antigen-specific activation of $CD8^+$ and $CD4^+$ T cells.

5.6A



5.6B

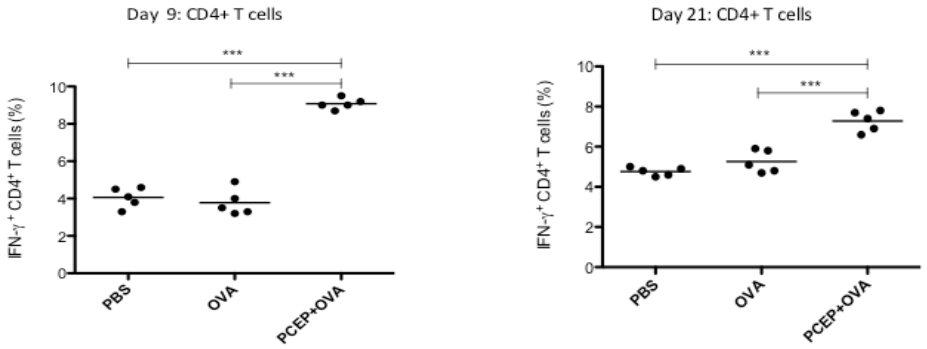


Fig 5.6 PCEP induces antigen-specific IFN-γ production in $CD4^+$ and $CD8^+$ T cells. BALB/c mice were immunized with 25 μl each of either phosphate-buffered saline (PBS) as control, 10 μg OVA or 50 μg of PCEP co-delivered with 10 μg OVA. Booster immunization was given on day 14 to half of the mice in each group. Mice were euthanized on day 9 and 21 after first immunization to collect spleens. **5.6A-B,** Splenocytes (1 X 10^6 cells) were restimulated with OVA (10 μg/ml) for 12 h and intracellular production of IFN-γ by $CD8^+$ and $CD4^+$ T cells was analyzed by flow cytometry. Statistical analysis was done by one-way ANOVA and the differences between the treatments were compared by Tukey's multiple-comparison test, where ***P<0.0001, **P<0.001, *P<0.05.

5.4 Discussion

Polyphosphazenes have shown great potential as vaccine adjuvants. However, the mechanisms by which they induce these immune responses are largely unknown. Hence we performed the present investigations to understand the mechanisms of action of PCEP *in vitro.* Stimulation of splenic DCs with PCEP led to secretion of pro-inflammatory cytokines IL-1β and IL-18 in a caspase-1 dependent manner. PCEP did not induce MHC class II and co-stimulatory molecule expression in DCs. However, these DCs did induce allogenic MLR at relatively low levels. Further, we observed that PCEP directly activates B cells but not T cells. However, PCEP does induce antigen-specific IFN-γ in both $CD8^+$ and $CD4^+$ T cells.

In vivo, PCEP induces local production of various cytokines and chemokines at the site of injection (Awate et al., 2012). In particular, PCEP induced significant production of pro-inflammatory cytokines IL-1β and IL-18 in muscle tissue. Additionally, PCEP also significantly upregulated the gene expression of NLRP3 in PCEP-injected muscle tissues. Hence, we further explored the mechanisms of PCEP-mediated IL-1β and IL-18 release in splenic DCs. The secretion of pro-inflammatory cytokine IL-1β requires two signals; 1) synthesis of pro-IL-1β-mediated via TLR agonists and 2) activation of the inflammasome complex (NLRP3) leading to activation of caspase-1, which in turn cleaves pro-IL-1β and allowing release of mature IL-1β (Schroder and Tschopp, 2010).

Li and his colleagues reported for the first time that alum-induced secretion of IL-1β and IL-18 was caspase-1 dependent (Li et al., 2007). In subsequent *in vitro* studies, various investigators have showed that activation of NLRP3 is required for alum-induced IL-1β and IL-18 secretion (Eisenbarth et al., 2008; Franchi and Nùñez, 2008; Hornung et al., 2008; Kool et al., 2008a; Sokolovska et al., 2007). Similar to alum, PCEP-induced IL-1β and IL-18 secretion was caspase-1 dependent strongly suggesting involvement of NLRP3 in PCEP adjuvant activity. In addition, PCEP alone was able to induce pro-IL-1β in splenic DCs. Interestingly, the production of pro-IL-1β was increased by addition of LPS. Our data is in agreement with activity of alum *in vitro* where LPS priming to induce pro-IL-1β prior to alum stimulation was a pre-requisite for secretion

of IL-1β (Eisenbarth et al., 2008; Kool et al., 2008a). Contrary to *in vitro* studies, the role of inflammasomes in the adjuvant activity of alum *in vivo* has yielded conflicting results. Using NLRP3, ASC and caspase-1 knockout mice, Eisenbarth et al. showed that NLRP3 inflammasome is a crucial component in the adjuvant activity of alum. NLRP3, ASC and caspase-1 knockout mice immunized with OVA adsorbed on alum, failed to induce antigen-specific antibody responses (Eisenbarth et al., 2008). Another study by Kool et al showed that alum induced lower influx of inflammatory cells in the peritoneal cavity of NLRP3-deficient mice. They also showed that alum-mediated activation of adaptive immune responses was NLRP3-dependent (Kool et al., 2008a). Similar studies done by Li et al. showed that NLRP3-deficient mice injected with alum adsorbed diphtheria toxoid or OVA vaccine elicited impaired levels of antigen-specific antibody responses (Li et al., 2008). All these studies indicate that NLRP3 inflammasome is critical in the adjuvant activity of alum *in vivo*. In contrast, Franchi and Núñez clearly showed that antigen-specific IgG production was not impaired in NLRP3-deficient mice following intraperitoneal injection of human serum albumin (HSA), a T cell dependent antigen in the presence of alum (Franchi and Nùñez, 2008). The conflicting results with regard to the role of inflammasome in adjuvant activity of alum have been attributed to the differences in the nature of alum used in different studies, immunization protocols and the mouse strains used (De Gregorio et al., 2008; Marrack et al., 2009). However, NLRP3 did affect alum-mediated cellular recruitment suggesting that inflammasomes might play an important role in activating innate immunity, but the contribution of inflammasome in activation of adaptive immunity remains elusive.

Two independent studies have demonstrated that NLRP3 is not required for the adjuvant activity of MF59 (Ellebedy et al., 2011; Seubert et al., 2011). However, an adaptor molecule required for the assembly of inflammasome, ASC is crucial for MF59 adjuvanticity (Ellebedy et al., 2011). A recent study by Embry et al., showed that MPL failed to induce intra-cytoplasmic assembly of NLRP3 inflammasome leading to failure of caspase-1 activation and maturation of pro-inflammatory cytokines IL-1β and IL-18 (Embry et al., 2011).

Some theories proposed for alum-mediated activation of NLRP3 includes phagosomal destabilization and release of cathepsin B, low intracellular potassium (K+) concentrations and generation of reactive oxygen species (ROS) (Hornung et al., 2008; Kool et al., 2008a; Petrilli et al., 2007). Phagocytosis of crystalline particles such as MSU or alum results in phagosomal destabilization and lysosomal rupture releasing protease cathepsin B in the cytosol (Hornung et al., 2008). The released cathepsin B led to activation of NLRP3 and secretion of pro-inflammatory cytokines IL-1β and IL-18. We report here that PCEP is taken up by the splenic DCs *in vitro* and it induces release of IL-1β and IL-18 in a caspase-1-dependent manner. PCEP was detected in the intracellular lysosomal compartments of the recruited immune cells at the injection site (unpublished data). Taken together, these results suggest that PCEP might induce lysosomal rupture and protease release to activate NLRP3, which in turn release active caspase-1 in the cytosol.

Activation of innate immunity is essential to induce adaptive immune responses. Increased expression of MHC class II, activation marker CD86 and maturation marker CD83 leads to enhanced ability of APCs to induce T lymphocyte activation and differentiation (Coyle and Gutierrez-Ramos, 2001). Previously, we have shown that PCEP upregulates the production of various chemokines including CCL2, CCL4, CCL12 and CXCL10 at the injection site (Awate et al., 2012). Due to chemotactic potential of these chemokines, we observed increased recruitment of various myeloid and lymphoid cells to the PCEP-injected muscle tissue (unpublished data). In addition, we also observed increase in number of APCs in the draining lymph nodes. DCs were first to be recruited and increased in highest number in the draining lymph nodes within 3 h post-injection of PCEP (unpublished data). Given the fact that DCs are the only professional APCs involved in antigen processing and presentation to induce adaptive immune responses, it will be important to evaluate the potential of PCEP to activate DCs. Splenic DCs were partially matured and showed increased expression of MHC class II molecules (data not shown) and therefore, we used BMDCs for DC activation studies. Complete Freund's adjuvant, LPS, liposomes, CpG, MF59, AS04 and α-galactosylceramide (α-GAL) have been shown to induce DC maturation to enhance adaptive immunity (Copland et al., 2003; De Becker et al., 2000; De Smedt et al., 1996;

Fujii et al., 2003; Shah et al., 2003). However, *in vitro* studies on human cells have shown that alum and MF59 failed to directly activate DCs but enhanced the surface expression of MHC class II and co-stimulatory molecules (CD83 and CD86) on monocytes, macrophages and granulocytes that resulted in increased T cell proliferation (Seubert et al., 2008; Sun et al., 2003). Similar to alum and MF59, PCEP failed to induce direct maturation of DC *in vitro*. Alum does not enter DCs directly but rather interacts with DC membrane lipids to delivers the antigen via abortive phagocytosis (Flach et al., 2011). This activates DCs, induces expression of co-stimulatory molecules (CD80 and CD86) and adhesion molecules [intracellular adhesion molecule-1 (ICAM-1)] leading to strong contact with $CD4^+$ T cells, which subsequently leads to B cell responses (Flach et al., 2011). Although, PCEP failed to induce DC maturation, we observed that B cells were directly activated by PCEP *in vitro.*

Exogenous antigens are presented on MHC class I molecules via cross-presentation pathways (Bevan, 2006). Alum is a known inducer of T helper (Th) 2 type responses (Marrack et al., 2009). However, mice primed with OVA plus alum have been shown to induce $CD8^+$ T cell responses via cross-presentation by specialized $CD8\alpha^+$ DCs (MacLeod et al., 2011). Further, alum plus OVA primed CD8 T cells differentiated into IFN-γ producing cells, whereas CD4 T cells differentiated into IL-4 producing cells (MacLeod et al., 2011). In comparison with alum, PCEP promotes antigen-specific mixed Th1 and Th2 type immune responses (Mutwiri et al., 2007a). Both *in vivo and in vitro* studies have shown that PCEP induced significant production of proinflammatory and Th1 type cytokines including IL-1β, IL-12, IL-18, IFN-γ and TNF-α (Awate et al., 2012; Mutwiri et al., 2008). In agreement with our previous results, we observed that immunization of mice with OVA plus PCEP induced antigen-specific IFN-γ production by splenic $CD8^+$ and $CD4^+$ T cells.

In conclusion, we have shown that PCEP-mediated secretion of pro-inflammatory cytokines IL-1β and IL-18 is caspase-1 dependent. Understanding the role of these pro-inflammatory cytokines in adjuvant activity of PCEP will provide critical information on how innate immunity influences the activation of adaptive immune responses.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

Vaccination continues to be an important public health tool to decrease mortality and morbidity caused by infectious diseases. Modern vaccines with purified antigens are often poorly immunogenic and require addition of adjuvants to be effective. An ideal adjuvant should have a good safety record, induce both cellular and humoral responses that are sustained for a long duration, and reduce the dose of antigen to lower the cost of production. Many adjuvants do not fulfill many of these criteria and therefore, for almost a century, alum-based mineral salts were the only widely used adjuvants for human use. In the last decade, MF59, AS04, AS03 and virosomes were approved in Europe whereas only AS04 got the regulatory approval for human use in USA in 2009, and AS03 in Canada in the same year. There are many new adjuvants that are in various stages of research and development.

Polyphosphazene polyelectrolytes are a novel class of synthetic, water-soluble and biodegradable polymers that have shown great potential as vaccine adjuvants. Several studies in laboratory and large animals have revealed that polyphosphazenes dramatically enhance the magnitude, quality and duration of immune responses to a variety of bacterial and viral antigens with PCEP being the most powerful (McNeal et al., 1999; Mutwiri et al., 2008; Mutwiri et al., 2007a; Payne et al., 1998a; Wu et al., 2001b).

Although adjuvants are widely used in vaccine formulations, their mechanisms of action are poorly understood. This is well captured in a famous quote by Janeway that adjuvants are “the immunologist’s dirty little secret” (Janeway, 1989). Studies from the past decade on adjuvant mechanisms are slowly unfolding the complexicity of adjuvant activity. These studies suggest that adjuvants employ one or more of the following mechanisms to elicit immune responses: 1) sustained release of antigen at the site of injection (depot effect), 2) up-regulation of cytokines and chemokines, 3) cellular recruitment at the site of injection, 4) increase antigen uptake and presentation to antigen presenting cells (APC), 5) activation and maturation of APC (increased MHC class II and co-stimulatory molecules expression) and migration to the draining lymph

nodes, 6) activation of inflammsomes, and 7) immunomodulation/priming of T cells or B cells (Cox and Coulter, 1997; Fraser et al., 2007; Hoebe et al., 2004).

Similar to other adjuvants, the mechanisms of action of PCEP are poorly understood. Previously, it was reported that the adjuvant activity of the polyphosphazene PCPP does not depend on depot formation at the site of injection. Excision of site of injection 24 h post-injection of PCPP with vaccine antigen had no detectable effect on antibody production suggesting that depot formation was not required for adjuvant activity (Payne et al., 1998a). However, polyphosphazenes have been shown to form water-soluble, non-covalent complexes with protein antigens which may help "direct" antigens to APCs (Andrianov et al., 2005). *In vitro* studies by Mutwiri et al have shown that polyphosphazenes stimulates the production of innate cytokines, which might contribute to its adjuvant activity (Mutwiri et al., 2008). However, the mechanisms by which polyphosphazenes enhance immune responses have not been systematically investigated. Hence we embarked on investigations of the cellular and molecular mechanisms of action of PCEP.

It was recently reported that MF59, alum and CpG stimulated a set of common genes referred to as "adjuvant core response genes" that included cytokines, chemokines, innate immune receptors, interferon-induced genes and adhesion molecules (Mosca et al., 2008). We first sought to determine if PCEP induced expression of these adjuvant core response genes at the injection site. We found that PCEP was a strong inducer of adjuvant core response genes in mouse muscle. In addition to adjuvant core response genes, PCEP also upregulated the gene expression of inflammasome receptor NLRP3 and various pro-inflammatory cytokines including IL-1β and IL-18 (Awate et al., 2012). In addition, we found significantly increased production of cytokines and chemokines in muscles injected with PCEP but we did not observe any systemic responses (Awate et al., 2012). Thus, PCEP induces local but not systemic responses suggesting that this adjuvant may be safe to use. However, further systematic safety studies are required to confirm the same. Similarly, clinically approved adjuvants MF59 and alum have also been shown to induce local cytokines and chemokines production in muscle following injection (Calabro et al., 2011;

Didierlaurent et al., 2009; McKee et al., 2009). Systemically, alum does not induce any cytokines, while MF59 upregulates the expression of IL5 (Mosca et al., 2008).

Since PCEP enhances local production of cytokines and chemokines that have potent chemotactic potential, we examined the ability of PCEP to recruit immune cells to the injection site and identify the specific cellular targets of PCEP. PCEP induced increased recruitment of various myeloid and lymphoid cells to the injection site that included neutrophils, macrophages, monocytes and DCs relative to alum- and PBS-injected muscle. Lymphocytes, neutrophils and macrophages were recruited in highest numbers followed by monocytes and DCs. Kinetic studies revealed that cell recruitment declined two-weeks post-injection suggesting that it is transient in nature. Neutrophils were recruited in high numbers at the injection site following administration of the vaccine adjuvants alum, MF59 or Complete Freund Adjuvant (CFA), which then traffick to the draining lymph nodes (Calabro et al., 2011; Maletto et al., 2006; Seubert et al., 2008). Similarly, i.m. injection of PCEP resulted in significant increase in number of DCs, monocytes, neutrophils and macrophages in the draining lymph nodes. DCs and macrophages were increased in highest numbers in the draining lymph nodes while DCs, macrophages and neutrophils were earliest to be detected after injection of PCEP. This is a significant finding given that DCs are the predominant APCs in initiation of immune responses in the lymph nodes. Surprisingly, we also observed an increase in number of T and B lymphocytes in muscle tissues and the draining lymph nodes after injection of PCEP. MF59 and alum do not induce recruitment of lymphocytes at the injection site (Calabro et al., 2011). Innate immune cells recruited to the injection site might have non-specific effector functions, however lymphocytes have capacity to either initiate or regulate antigen-specific immune responses.

We further examined the specific cellular targets of PCEP at the injection site. For this experiment, we labelled PCEP with Alexa-fluor 488 fluorescent dye and injected intramuscularly in muscle tissue. We observed that most of the recruited immune cells took up PCEP in the muscle tissue. Most of the APCs recruited to the injection site took up PCEP in higher amounts compared to lymphoid cells. This could be due to higher phagocytic abilities of APCs. Similarly, MF59 has

been shown to be internalized by DCs expressing high MHC class II and special marker DEC205 in the muscle tissue (Dupuis et al., 1998). Further, confocal studies revealed that PCEP was localized in the intra-cytoplasmic lysosomal compartments of various recruited immune cells. Overall, these data suggest that PCEP induce recruitment of APCs to the injection site, which then take up the adjuvant and then presumably migrate to the draining lymph nodes.

Studies with labelled MF59 revealed that 90% of MF59 was cleared from the injection site within 6 h post-injection suggesting that MF59 does not form long-lived depot at the site of injection (Dupuis et al., 1999). Similar studies with alum have clearly showed that alum adjuvant activity does not depend on antigen depot (Hutchison et al., 2012). Excision of the injection site 24 h post-injection of polyphosphazene PCPP with vaccine antigen had no detectable effects on antibody production (Payne et al., 1998a). Hence, we used whole body imaging to detect the *in vivo* PCEP distribution and retention of PCEP at the site of injection. For this experiment, PCEP was labelled with infra-red dye and injected into the mouse muscle. We observed that PCEP was localized strongly to the site of injection. Although 70% of PCEP was rapidly cleared from the site of injection within 24 h, we still observed traces of PCEP 12 wk post-injection at the site of injection. Our observations suggests that depot formation may not be a primary mechanism which mediates the adjuvant activity of PCEP, but further studies are required to confirm the same.

Since, we observed upregulation of NLRP3 gene and local production of IL-1β and IL-18 after injection of PCEP, we investigated the role of the inflammasome in adjuvant activity of PCEP *in vitro*. NLRP3 is an intra-cytoplasmic multi-protein complex that play an important role in the activation of caspase-1, which in turn cleaves proforms of IL-1β and IL-18 to their bioactive forms (Martinon et al., 2009). Caspase-1 is a critical component of NLRP3 inflammasome; therefore, we examined the role of caspase-1 in PCEP-mediated secretion of IL-1β and IL-18 by splenic DCs. Pre-treatment of splenic DCs with caspase-inhibitor YVAD-fmk significantly inhibited IL-1β and IL-18 secretion in response to PCEP. NLRP3 inflammasome is activated by various stimuli including the particulate adjuvant alum (Eisenbarth et al., 2008; Franchi and Nùñez, 2008; Hornung et al., 2008; Kool et al., 2008a). However, the role of inflammasomes in adjuvant activity

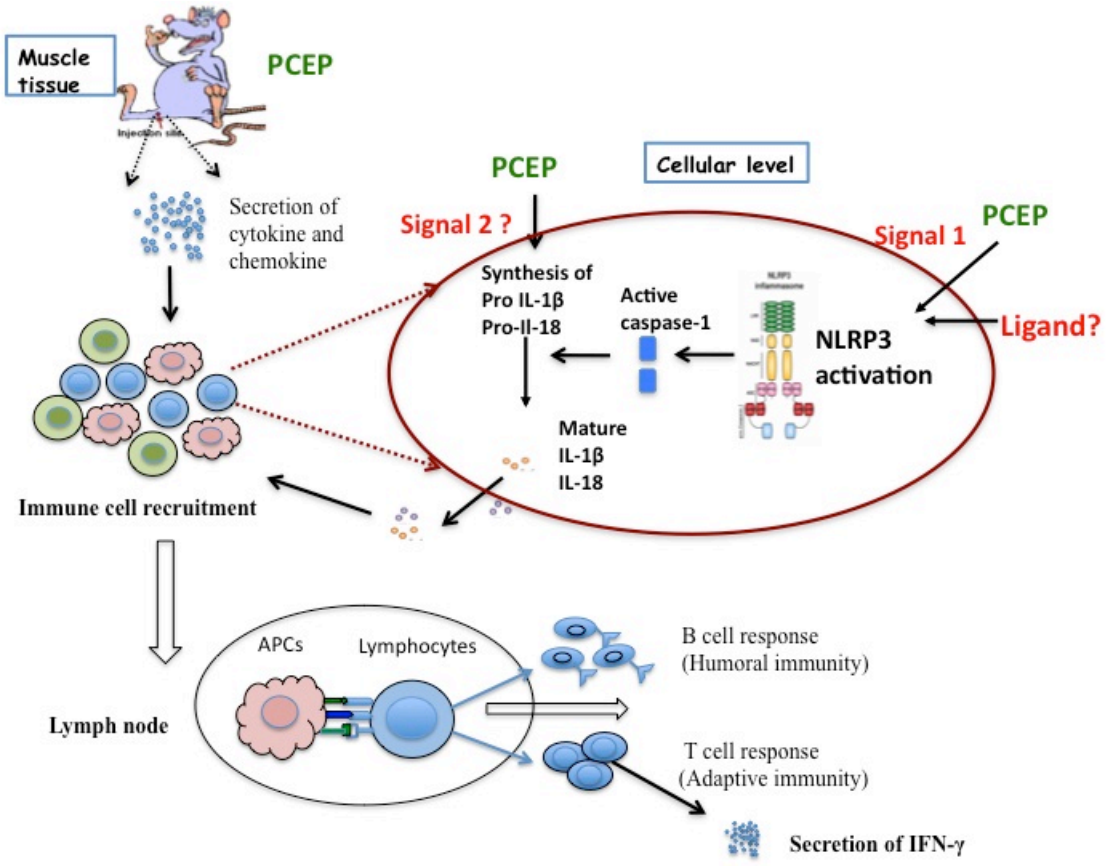
of alum *in vivo* has yielded conflicting results (De Gregorio et al., 2008; Franchi and Nùñez, 2008). One of the mechanisms proposed for alum-mediated activation of NLRP3 involves phagosomal destabilization and release of cathepsin B. Phagocytosis of crystalline particles such as MSU or alum results in phagosomal destabilization and lysosomal rupture releasing the protease cathepsin B in the cytosol that leads to activation of NLRP3 and subsequent secretion of IL-1β and IL-18 (Hornung et al., 2008). Our *in vivo* studies revealed intracellular lysosomal localization of PCEP in the recruited immune cells at the injection site suggesting that one of the mechanisms of PCEP-mediated NLRP3 activation might be via lysosomal rupture and release of protease enzymes in the cytosol.

Maturation of DCs is important to induce effective immune responses. Mature DCs increase the expression of MHC class II and co-stimulatory molecules expression, which enhances their ability to induce T cell activation and differentiation (Coyle and Gutierrez-Ramos, 2001). Since there was significant increase in DCs both at the site of injection and in the draining lymph nodes after injection of PCEP, we examined the effect of PCEP on activation and maturation of DCs. Since splenic DCs are partially mature, we generated BMDCs from mouse bone marrow. FACS analysis revealed that PCEP did not induce MHC class II and co-stimulatory molecules (CD86 and CD40) expression in BMDCs. In addition, PCEP-stimulated BMDCs induced low allogeneic T cell proliferation compared to LPS-stimulated BMDCs. Similar to our observations, alum and MF59 also failed to directly activate DCs *in vitro* (Seubert et al., 2008; Sun et al., 2003). However, alum has been shown to interact with DC membranes and deliver antigen via abortive phagocytosis, which leads to DC activation and strong contact with $CD4^+$ T cells, subsequently resulting in enhanced B cell responses (Flach et al., 2011). PCEP induced recruitment of B and T cells in mouse muscle, which took up PCEP in minimal quantities. Hence, we examined the ability of PCEP to induce direct activation of B and T cells *in vitro*. We observed that although PCEP induces direct activation of naïve B cells, it does not induce B cell proliferative responses and does not activate or induce proliferation of naïve T cells *in vitro.*

Finally, we examined the ability of PCEP to induce antigen-specific $CD8^+$ T cell responses. We observed that mice immunized with OVA plus PCEP induced significant production of antigen-specific IFN-γ by splenic $CD8^+$ and $CD4^+$ T cells. This is in agreement with our previous *in vivo and in vitro* studies indicating that PCEP induced significant production of Th1 type cytokines including IFN-γ (Awate et al., 2012; Mutwiri et al., 2008).

Taken together our investigations suggest that PCEP directly activates innate immunity and B cell responses but not T cells. However, PCEP does induce antigen-specific T cell responses. PCEP-induced activation of innate immune responses at the injection site involves activation of adjuvant core response genes, production of cytokines and chemokines, recruitment of various immune cells and presumably activation of the NLRP3 inflammasome. All these events promote a strong immunocompetent environment at the injection site that may significantly contribute to the adjuvant activity of PCEP. Understanding the mechanisms of action of adjuvants will provide critical information on how innate immunity influences the development of adaptive immunity and help in rational design of vaccines against various diseases.

Fig 6.1 Proposed mechanisms of action of PCEP.



FUTURE STUDIES

We have shown that PCEP induces production of cytokines and chemokines, which in turn recruit various immune cells to the injection site. Further studies are required to understand the different contribution of each of these mechanisms in the activation of optimal adaptive immune responses. PCEP have been shown to recruit myeloid and lymphoid cells; however, the relationship between these recruited cells and induction of immune responses remains to be determined. Further, it will be interesting to determine whether this cellular recruitment is critical for the adjuvant activity of PCEP by depletion of single or multiple cell populations.

The role of inflammsomes in adjuvant activity remains unclear. We have shown that PCEP-mediated secretion of pro-inflammatory cytokines IL-1β and IL-18 is caspase-1 dependent. Additional studies are required to confirm the role of the inflammsome and understand the caspase-1 regulation in adjuvant activity of PCEP. There are many questions that need to be addressed such as to determine if the inflammasome contributes to the adjuvant activity of PCEP. How do PCEP activate the inflammasome? Does inflammasome or its activated products IL-1β and IL-18 have any role in induction of adaptive immune responses? Are there other inflammasomes involved in the adjuvant activity of PCEP?

Finally, we have shown that PCEP activates innate immune responses at the injection site. Future studies examining the receptors targeted by PCEP will provide important information on mechanisms of action of polyphosphazenes.

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