In vitro and in vivo evaluation of novel biodegradable polymer adjuvants for vaccine delivery

by

Jennifer H. Wilson Welder

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Program of Study Committee:
Michael J. Wannemuehler, Major Professor
Balaji Narsimhan
F.C. Minion
Douglas E. Jones
Lisa K. Nolan

Iowa State University

Ames, Iowa

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DEDICATION

This dissertation is dedicated to the three men in my life:
my grandpa, Vern Amon
who always wanted me to be a doctor,
my mentor, Dr. Wannemuehler,
who gave me the opportunity
and
my husband, Jeff Welder,
who always believed I could.

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ABSTRACT

Infectious disease remains a constant threat to the health of man and his animals. Vaccination has been declared one of the medical triumphs of the twentieth century. For man or animal, vaccination remains the best and most cost effective means for the prevention of disease. Many novel vaccine antigens are rationally designed peptides and recombinant proteins which require the use of adjuvants or other immune enhancers to increase efficacy. Currently, there is a need not only for single dose vaccines (to improve patient compliance and improve animal welfare by reducing livestock handling) but also adjuvants that preserve the immunogenicity of the protein during encapsulation, storage and release and enhance the host's immune response to the antigen. Biodegradable polyanhydrides have shown many characteristics that fulfill these ideals but further study is needed. The studies presented in this dissertation were undertaken with the intent to define the interaction(s) between novel biodegradable polyanhydride microspheres and the host immune system. In order to address the role of polyanhydride chemistry on murine dendritic cells (DCs) in vitro, DC activation by polyanhydride microspheres was evaluated by surface marker expression and cytokine secretion. Several murine models, including a transgenic T cell transfer model, were used to evaluate the induction of antigen-specific immune response by immunizing mice with microsphere adjuvanted ovalbumin. The in vivo studies using ovalbumin encapsulated microspheres were carried out in three mouse strains to evaluate the memory or recall response induced by a single microsphere vaccination and to evaluate strain differences in response to the polyanhydride microspheres. Finally, microspheres loaded with the protease digested vaccine antigen derived from *Brachyspira hyodysenteriae* was used to vaccinate mice and pigs prior to disease challenge studies designed to evaluate the induction of protective immunity. Taken together, this body of work further adds to our knowledge of polyanhydride microspheres and their potential use as vaccine carriers.

CHAPTER 1

General Introduction and Literature Review

1.1 Dissertation Organization

This dissertation follows the new format of thesis organization wherein the middle chapters constitute manuscripts to be submitted to peer-reviewed journals. The introduction, literature review, general conclusion and all other manuscripts unless stated are referenced in the style of the journal *Vaccine*. The overall objective of this thesis was to evaluate novel biodegradable polyanhydrides as vaccine adjuvants. The introduction and literature review introduce current topics related to vaccination practices and key immune cells targeted by vaccination. Chapter 2 is a modified review article on adjuvants published in *Journal of Pharmaceutical Research* and is in the format of that journal. Jennifer Wilson-Welder and Maria Torres contributed equally to the writing of this article with Jennifer Wilson-Welder focusing on the introduction, immune response, and adjuvants with Maria Torres focusing on polymeric adjuvants and conclusions. Chapter 3 describes in vitro activation of dendritic cells (DCs) by polyanhydride microspheres (MS) and the use of transgenic murine models to elucidate MS-DC-T cell interactions. This work was a collaborative effort with Maria Torres fabricating microspheres and assisting in flow cytometery experiments, and assisting in manuscript preparation. Jennifer Wilson-Welder was responsible for designing, implementing and analyzing the DC activation, and in vivo and in vitro immune response of the transgenic T cells to ovalbumin and final manuscript preparation. Chapter 4 describes experiments evaluating the modulation of in vivo immune response by microspheres. For this series of experiments, Brenda Carrillo, Maria Torres, and Senja Lopac fabricated the microspheres, evaluated microsphere morphology, preformed protein release studies, and assisted with necropsies. Jennifer Wilson-Welder was responsible for experimental design, immunizations, blood collection, data collection and analysis and manuscript preparation. Chapter 5 is a short study evaluating long term storage of microspheres. Matt Kipper fabricated the microspheres. Jennifer Wilson-Welder performed the mouse immunizations, blood collections, data collection and analysis and manuscript

preparation. Chapter 6 is adapted from the final report of a grant written and executed by Jennifer Wilson-Welder funded by the National Pork Board. For these projects, Brenda Carrillo prepared the microspheres, preformed protein release analysis, and evaluated microsphere morphology where Jennifer Wilson-Welder was responsible for design, immunizations, data collection and analysis and manuscript preparation. The entire dissertation is tied together in General Discussion presented in Chapter 7.

1.2 Introduction

Man and his animals will always be challenged with infectious disease and thus the need to develop tools to combat infectious disease. A key tool in the fight against infectious disease had been the development and use of vaccines. Vaccination has often been touted as the most wide-reaching medical advance in the twentieth century. Through vaccination the devastating effects of six major human diseases have been controlled (diphtheria, tetanus, yellow fever, whooping cough and rabies), one disease had been globally eradicated (smallpox) and two diseases are on the verge of global eradication (measles and polio) [1]. On the animal side, foot-and-mouth, canine distemper, rabies, canine parvovirus, pseudorabies in swine, and furunculosis in fish are but a few examples where vaccination has had considerable impact on controlling disease [1, 2]. Despite advances in medical technology, development of new antimicrobials and changes in animal husbandry practices, it is not possible to cure or prevent every disease. The incidences of antibiotic resistant pathogenic bacteria are increasing. An about face in consumer acceptance of antimicrobials in food producing animals has curtailed their prophylactic use [3]. Thus, vaccination remains the most efficacious and cost effective means for the prevention of disease.

Most vaccines have been developed empirically, through trial and error, over time. These early vaccines usually consisted of killed or inactivated organisms. These preparations, in most cases provide protective immunity, also have considerable side effects. The use of whole cell pertussis vaccines have been discontinued in the United States due to swelling at the injection site and the induction of a high fever associated with vaccination. These adverse reactions are linked to the presence of bacterial cell

components (e.g., lipopolysaccharide, peptidoglycan, flagella) that induce host inflammatory responses. Knowledge of the mechanism(s) or basis of immune recognition for specific sequences or epitopes jump-started the development of subunit vaccines which eliminate the deleterious effects induced by bacterial components non-specifically activating pro-inflammatory responses. However, the success of these vaccines has proven to be limited. The addition of adjuvants to subunit vaccines has enhanced their efficacy [4].

First described by Ramon in 1925, adjuvants are substances that when added to an immunogen, enhance the subsequent immune response that may be measured as an increased antibody titer or as cell-mediated immunity [5, 6]. Charles Janeway referred to adjuvants as "the immunologist's dirty little secret" [7]: however, it is the development and application of adjuvants that will improve vaccination protocols in the 22nd century. They were termed "dirty" because it was known that less-purified vaccine preparations worked better and "secret" because the mechanism was unknown [5]. It is now well accepted that many of these whole organism vaccines contained pathogen-associated-molecular patterns (PAMPs) that are recognized by a variety of pattern recognition receptors (PRRs) on innate immune cells, including Toll-like receptors (TLRs). Activation of dendritic cells (DCs) and other antigen presenting cells (APCs) of the innate immune system induces increased MHC I or II expression (necessary for antigen presentation), CD40 and CD86 expression (T cell costimulation) and increased secretion of cytokines (IL-1α & β, IL-10, IL-12, IL-6 and TNF-α) depending on the TLR pathway stimulated [8]. These DC responses all have an effect on activation of antigen-specific T and B responses (i.e., adaptive immunity). More and more evidence is published showing the need for inclusion of TLR ligands in vaccine adjuvants in order to induce long term protective immunity [9, 10]. Indeed, vaccinology and the development of adjuvants have come into their own as crucial steps in linking innate and adaptive immunity for long-term protection.

While successful vaccines do exist, there is a need to develop vaccines for complex polymicrobial diseases, diseases requiring cytotoxic T cells, parasite infections (i.e., helminthes or malaria), chronic infections (mycoplasma and mycobacteria), and

pathogens that target immune cells (HIV) [11, 12]. By understanding the complex interactions between innate and adaptive immunity, improved and efficacious vaccines can be developed for these complex disease problems.

One of the most promising areas in adjuvant technology is the adaptation of controlled release from biodegradable polymers. Biodegradable polymers have been studied for several decades for use in various therapeutic applications including vaccination [13, 14]. Microspheres based on biodegradable polymers offer the advantage of replacing traditional multiple dose vaccine regimens resulting in greater patient compliance (receiving full regimen), reducing animal handling and stress, reducing injection site reactivity, and, in the end, improving not only vaccine efficacy, but in our livestock species, producer profits as well [2, 3, 15].

The studies presented in this dissertation were undertaken with the intent to develop an understanding of the interaction between novel biodegradable polyanhydride microspheres and the host immune system. Activation of murine DCs in vitro by polyanhydride microspheres was evaluated by surface marker expression and cytokine secretion in order to understand the role of chemistry in DC activation. Several murine models were used to evaluate the induction of antigen-specific immune response by immunizing mice with microsphere adjuvanted ovalbumin. The in vivo studies were carried out in three mouse strains to evaluate the memory or recall response induced by a single microsphere vaccination and to evaluate strain differences in response to the polyanhydride microspheres. Finally, microspheres loaded with the protease digested vaccine antigen derived from *Brachyspira hyodysenteriae* whole cells were used to vaccinate mice and pigs prior to disease challenge studies designed to evaluate the induction of protective immunity. Taken together, this body of work further adds to our knowledge of polyanhydride microspheres and their potential use as vaccine carriers.

2 Literature Review

2.1 Host-Microbe Recognition

It is estimated that 500 - 1,000 species of microorganisms, numbering up to 1014, colonize humans and rodents [16, 17]. Many scientists think this number may be a gross

underestimate, as many species are unable to be grown in culture. Most of these organisms reside in host niches in a commensal relationship. However, even these helpful organisms can become pathogenic if allowed out of defined niches. Thus, multi-cellular organisms have defense mechanisms to make their internal environments hostile to would be invaders [18]. These mechanisms are generally induced (antimicrobial peptides, reactive nitrogen and oxygen free radicals) or exist in a resting state (macrophages, DCs and adaptive immunity B and/or T cells) until triggered. Therefore, there must be key features in the infection process that triggers immune responses [18]. The key sentinels and defenders of the body include neutrophils, mast cells, macrophages and dendritic cells [19]. The latter two possess not only phagocytic capability but can also present phagocytized antigen to the cells of the adaptive immune system. These cells, along with B cells are often termed antigen presenting cells (APCs), are able to internalize antigen through cell mediated processes including phagocytosis, receptor mediated endocytosis, and macropinocytosis [20].

Janeway predicted APCs possess germ-line encoded receptors, pathogen recognition receptors (PRRs), that are conserved and recognize evolutionarily conserved molecules essential for pathogen function [18]. Many different cells aside from innate immunity cells express PRRs and produce inflammatory mediator when PRRs are triggered (e.g., interferon by virally infected cells and IL-8 from damaged epithelial cells) [18]. These receptors recognize “non-self” molecules that represent a “stranger” to the APCs. An alternative, but not necessarily contradictory, view to Janeway’s “stranger” hypothesis is a model proposed by Polly Matzinger. The Matzinger “danger” hypothesis says that PRRs are not necessarily pathogen recognition receptors but pattern recognition receptors that bind to self molecules normally sequestered in intracellular compartments of healthy cells and are released as “danger” signals when cells are stressed, damaged, or die of non-apoptotic means [18]. There is an element of truth to both of these theories. As an example, the ligands that will bind to and signal through TLR4 include: lipopolysaccharide of Gram negative bacteria (LPS), fusion protein of respiratory syncytial virus, heat-shock protein 60, fibronectin, breakdown products of hyaluronan, β-defensin, taxol, and bacterial fimbrial proteins [21]. PRRs include TLR, nucleotide-

binding oligomerization domain (NOD), CD14, LPS binding protein, lipoprotein receptors, and mannose receptors [21, 22]. Expression of mannose receptors, in addition to other PRR, provides selectivity in binding of specific ligands and identifying the nature of the danger or damage signal [20].

2.2 Toll-Like Receptors

TLRs were discovered in 1997 and are the, mammalian homolog of *Drosophila* Toll and 18-wheeler [18]. Currently, there are 11 identified TLR within the human genome [23], and their ligands and activities are summarized in Table 1. TLRs bind both pathogen associated molecular patterns (PAMPs) and host derived "danger" signals [18]. TLRs are transmembrane signaling proteins consisting of lucine rich repeat binding domains and an intracellular TIR-domain (Toll-IL-1 receptor) [24]. Signaling is controlled through accessory adaptor proteins leading to MAPKK (mitogen activated protein kinase kinase) and/or NFκB signaling pathways resulting in NFκB and/or AP-1 gene transcriptions [24]. The two key adaptor proteins in TLR signaling are MyD88 and TRIF. Mice deficient in both MyD88 and TRIF are unable to initiate signal transduction through TLRs [25]. Not all TLRs or even TLR ligands are equal in the magnitude of intracellular signaling. This differential activation among TLRs provides both specificity and discriminatory powers to APC activation. For example, TLR4 but not TLR9 ligands are able to signal through NFκB through a MyD88 independent pathway that results in costimulatory molecule expression on DCs but not cytokine secretion resulting in a skewed Th2 T cell profile (e.g., IL-4 production) [26]. Furthermore, DCs production of IL-12 and TNF-α are MyD88 dependent, but DC phenotypic maturation (including CD40 expression) is not [26].

TLRs are also expressed on other cell types such as neutrophils, mast cells, basophils, eosinophils, epithelial cells, endothelial cells [27]. Third-party recognition (healthy neighbor cells) of TLR-ligands increases non-classical MHC molecules (MICA & MICB) which could activate γδ-T cells, natural killer (NK) cell or mast cells, to produce cytokines to activate DCs [18]. Signaling through TLRs seems to have importance not just in pathogen recognition and vaccine induced immunity [28] but also

in upregulating host repair mechanisms [29]. In many animal models of autoimmune disease, inclusion of a TLR ligand with the triggering immunogen was needed for development of the autoimmune phenotype [27].

Table 1: Summary of TLR receptors, ligands and key features[a]

TLR	LIGAND	KEY FEATURES
TLR2 functions as a dimer with TLR1 or TLR6	peptidoglycan, lipoproteins, lipoarabinomannan, LPS[b] from *Leptospira*, zymosan, necrotic cells	Induces Th2 & IL-10
TLR3	dsRNA (viral)	Located in phagolysosome
TLR4	LPS, lipotecholic acids, Hsp60, β-defensin, fusion protein of RSV, hyaluronan, taxol, MPLA	Induces IL-1β, nitric oxide, & TNFα
TLR5	flagellin	Located on the basolateral surface of epithelium, induces Th1, can override Tregs
TLR7 & TLR8	ssRNA, imidazoquinolines (synthetic)	Intracellular, induces IL-12 & IFN-γ
TLR9	Unmethlyated CpG motif containing oglionucleotides	Intracellular, induces Th1
TLR10	No known ligand	On human Treg
TLR11	profilin on uropathogenic bacteria	

[a] *taken from* [21, 24, 27, 30, 31].
[b] LPS, lipopolysaccharide; ds, double stranded; Hsp, heat-shock protein; RSV, respiratory syncytial virus; MPLA, monophosphoryl lipid-A; ss, single stranded; Treg, regulatory T cell.

2.3 Dendritic Cell Phenotype

Dendritic cells are not endowed with much of the pathogen destroying machinery of macrophages or the antibody producing capability of B cells limiting them to the unique role of damage assessor and communicator to T cells [18]. The antigen presentation and naïve T cell priming ability of DCs provides a bridge between innate immunity and adaptive immunity [32]. Additionally, DCs can uptake and retain antigen

for long periods of time and efficiently present or transfer the antigen to naïve B cells to initiate antibody production [33].

Dendritic cells do not exist as a homogenous population and can vary greatly in phenotypic markers, expression of CD8α, level of mannose receptors, transcription levels of IL-12 and IL-10, ability to present antigen in the context of major histocompatability complex class I or II (MHC I or MHC II) [34]. First, DCs are characterized as either myeloid or plasmacytoid owing to the progenitor cell from which they originated. Myeloid DCs in humans have been described as expressing TLR2, TLR3, TLR4, produce IL-12, and can trigger Th17 T cells [27]. In contrast, plasmacytoid DCs on the other hand, express TLR7 and TLR9, and produce type 1 interferons making them more suited for detection of viral pathogens. Secondly, DCs are characterized by expression of CD11c and CD8α. In the mouse, two main DC populations have been described. The first express $CD8\alpha^{+}CD11c^{+}$ and are found in the T cell region of the spleen, thymus, skin draining and mesenteric lymph nodes and are further characterized by their ability to produce high amounts of IL-12 [26]. The second population of DCs are $CD8\alpha^{-}CD11c^{+}$ and are found in the marginal zone of lymph nodes and migrate into the T cell areas only after microbial challenge [26]. The literature shows that each of the subsets of DCs ($CD8\alpha^{+}$ or $^{-}$, plasmacytoid vs. myeloid) and/or the nature of microbial stimuli (regardless of subset) can trigger cytokines necessary for naïve T cell induction towards a Th1 or Th2 bias showing plasticity in their makeup [18].

2.4 Dendritic Cell Activation

The complexities of DC biology as expanded over the years from a simple immature-mature paradigm to terminology that not only describes the phenotype of the DCs but also the underlying mechanisms associated with effector functions [32]. In peripheral tissues, DCs are generally accepted to be immature, actively sampling antigens from their environment. Upon encounter with microbial ligands or other stimuli, DCs undergo phenotypic changes that allow for rapid uptake of antigen and enhanced phagocytic capability followed by a rapid decrease in this ability [32, 35]. Along with this phenotypic change, DCs typically increase expression of lymphocyte function

associated molecules (LFA1) an integrin used in migration leading to the erroneous belief that maturation was equated with migration [32]. In truth, DCs with a mature phenotype can be found in the periphery and DCs in immature states can be found resident in immune tissues [18]. While the terms maturation and activation are often used somewhat interchangeably, the scientists in this field have yet to adopt a single terminology making it unclear if maturation and activation describe the same cellular events. The term activation can mean simply change in resting state, therefore implying many routes of activation with varying consequences [18]. Figure 1 illustrates some of the key features of DC maturation/activation. DC maturation/activation include enhanced expression of phenotypic markers (CD11a, CD11c), antigen presentation molecules (MHC I and MHC II) and costimulatory molecules (CD40, CD80, CD86,CD83) necessary for T cell synapse formation [36]. In this context, DC activation as such has been observed after in vivo following the injection of LPS, bacterial DNA, double-stranded RNA, or extracts of microorganisms; in addition, in vitro DC activation has been demonstrated following the addition of inflammatory cytokines, heat-shock proteins (HSP), and other stimuli to cell culture medium [18]. The pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 downregulate DC phagocytic activity, but have been shown to increase expression of CD80, CD83, CD86, MHC I, MHC II, CCR-7 and enhance DC sensitivity to chemokines such as CCL19 and CCL21 [35]. Acidic extracellular conditions (pH 6.5), as found in infected or necrotic tissue, enhance the ability of DCs to increase endocytosis, surface marker expression (CD11c, MHC II, CD40, CD86), and present antigen for either CD8 stimulation or antibody generation [33]. However, not all microbial stimuli or PRR-ligands induce DC activation. For example, binding of specific microbial ligands to mannose receptors, DEC-205, or other DC lectin-receptors by microorganisms enhances endocytosis but does not induce activation [18]. Signals secreted by other innate immune cells can also effect DC maturation.

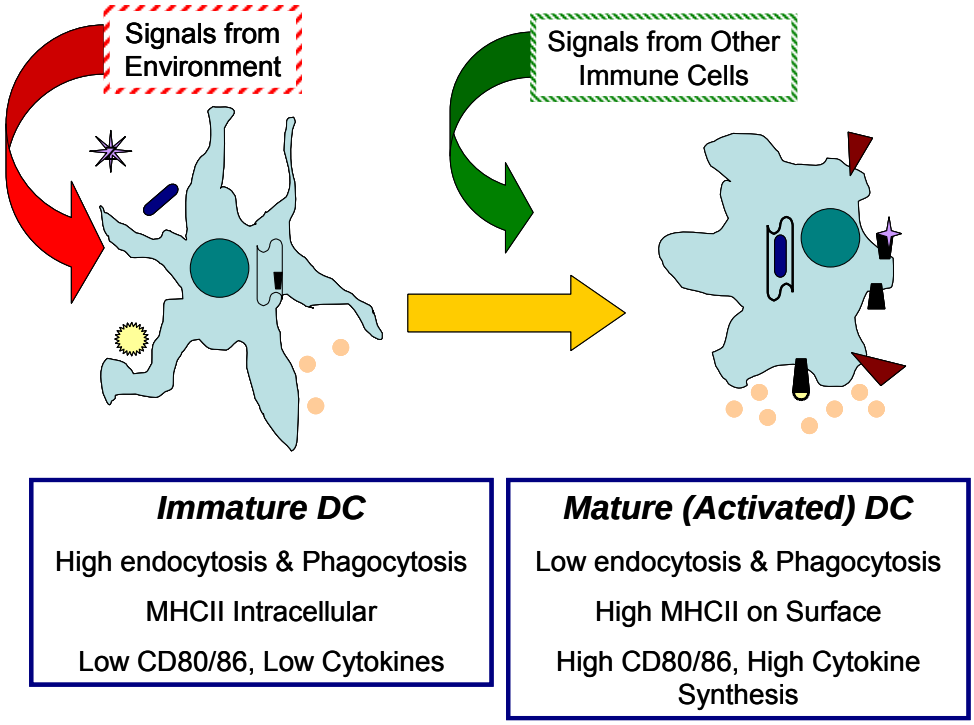

Figure 1: Illustration of key differences in immature and mature (activated) dendritic cells. Microbial components, products from apoptotic or necrotic cells, and chemokines from epithelial cells all constitute environmental stimuli. Activation signals from immune cells could include pro-inflammatory cytokines from NK or γδ-T cells and binding of CD40 to CD40L/CD154. Activated DCs express MHC II on their surface (black trapezoid) along with costimulatory molecules (brown triangles) and secrete cytokines (tan circles).

2.4.1 CD40

CD40 is expressed by monocytes and is up-regulated when DCs migrate from periphery to lymph nodes [26]. CD40 is a transmembrane protein and a member of the TNF receptor family. Ligation of CD40 on macrophages is responsible for macrophage activation for intracellular killing of pathogens and inducible nitric oxide production [19]. The ligand for CD40, CD40L or CD154 is expressed on many cells: mast cells, basophils, platelets, and activated T cells [18]. CD40 binding can activate DCs but not in the absence of other microbial-derived stimuli [18]. This being said, $CD11c^{+}$ DCs expressing high or low levels of CD40 can be found in the lymph nodes which may represent a nascent population of DCs that continually migrate to draining lymph nodes from the tissue sites in the absence of microbial stimuli [26].

CD40 represents a positive feed-back stimulation where ligation of CD40 upregulates more CD40 surface expression. CD40 expression is regulated by the transcription factor NFκB [26]. Signaling through CD40 also increases expression of MHC, costimulatory and adhesion molecules, and IL-12 production [26]. IL-12 production in this instance, is dependent on transcription factor NFκB, which also triggers production of interferon-α/β which then favors the development of antigen-specific $CD8^+$ T cells [37]. Upregulation of CD80/86 upon ligation of CD40 on the DC provides necessary threshold stimuli for CTL cross priming [26]. Signaling through CD28 (on T cell) is critical for induction of CTL responses as shown by impaired CTL in $CD80/86^-$ or $CD28^{-/-}$ mice [26].

A number of organisms can inhibit CD40 expression, NFκB translocation, and/or the ability of APCs to respond to a variety of pathogenic signals including LPS, cytomegalovirus, *Toxoplasma* tachyzoites, or *Plasmodium* infected erythrocytes each of which may contribute to persistence of pathogen within the host [26]. Furthermore, the inflammatory inhibitor aspirin prevents CD40 up-regulation and IL-12 production from DC in cell culture [26]. Blocking of CD40 and CD40 upregulation may inhibit subsequent immune activation.

2.4.2 CD209/DC-SIGN

Dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN or CIRE in mice) or CD209 is a member of the C-type lectin family of receptors [38-41]. CD209 appears to have three main functions: 1) CD209 binds to high mannose oligosaccharides on pathogen or parts of pathogen for antigen uptake into MHC II loading compartments; 2) CD209 binds to ICAM-2 on vascular endothelium to facilitate DC migration and translocation; 3) CD209 binds to ICAM-3 on T cells enhancing and stabilizing the APC-T cell synapse [39]. Expression of CD209 is enhanced by the cytokine mediators GM-CSF and IL-4 [39].

Binding of CD209 leads to efficient endocytosis of materials, enabling immature DCs to bind and internalize a number of pathogen derived antigens [38]. CD209 forms clusters in plasma membrane in absence of ligation, and is also concentrated on the

leading edge of the migratory DC [38]. In this respect, CD209 may provide a high affinity and avidity interaction for the binding of pathogens that have a limited binding surface with which to interact with receptors on the DC [38]. For example, HIV binding to CD209 leads to non-fusion uptake of virions for later presentation to $CD4^+$ T cell [38].

CD209 also expresses a very high affinity for adhesion molecule ICAM-3 which is expressed on resting T cells [39]. CD209-ICAM-3 may mediate the initial scanning of antigen loaded MHC II by the TCR [39].

2.5 $CD4^+$ T Cells

The adaptive immune response involves three main cell types. B cells produce antibody upon encounter with antigen and undergo differentiation into plasma cells when provided with differentiation signals (IL-4, IL-5, and IL-6) from CD4 cells. $CD8^+$ or cytotoxic T cells are responsible for killing virally infected or tumor cells in a cell-cell contact manner. $CD4^+$ T cells, or helper T cells, provide activation signals to B cells, $CD8^+$ T cells and macrophages. $CD4^+$ T cells consist of four main subpopulations, summarized in Table 2, including Th1, Th2, Th17 and Treg cells.

The Th1-Th2 paradigm has dominated immunological literature for the last 15 years. It has survived in part because of its simplicity and predictability [42]. Activation of either Th1 T cells and secretion of Th1 associated cytokines have been shown to inhibit Th2 T cell responses and vice versa [43]. A Th0 response is sometimes described as a response in which the $CD4^+$ T cells do not produce a polarized cytokine response. These Th0 cells may represent an intermediate state of activation and will complete polarization upon further activation and differentiation induced by specific stimuli. However, not every immunological outcome was well described by Th1-Th2. A new set of $CD4^+$ T cells producing IL-17 was described in 2005, and are referred to as Th17 cells [27].

2.5.1 Th1

Th1 $CD4^+$ T cells are a key component of cell-mediated immunity and these cells or their cytokines also contribute to pro-inflammatory pathways. Th1 cells are classically

assigned the capacity to activate macrophages via secretion of IFN-γ for enhanced intracellular killing, activation of $CD8^+$ T cells, and activation of NK cells. Cytokines secreted or induced by Th1 cells include IL-2, IL-3, IFN-γ, TNF-α, TNF-β, GM-CSF and these cytokine responses are regulated by the transcription factors STAT-4 and T-bet [19, 44, 45]. Th1 cells induce class switching to the antibody isotype IgG2(a/c) by differentiating B cells. Aberrant Th1 responses have been linked to host-mediated pathological lesions as seen in tuberculosis, sarcoidosis, collagen induced arthritis, and inflammatory bowel disease [46-49].

2.5.2 Th2

Th2 $CD4^+$ T cells are classically associated with the induction of humoral immunity as they induce B cell activation, differentiation and antibody class switching to IgG1 and IgE isotypes [44]. Th2 cells are necessary for the clearance of extracellular bacteria and parasites [50]. Th2 responses are characterized by the production of cytokines IL-4, IL-5, IL-6, IL-10, IL-13, TNF-β and these responses are regulated by the transcription factor STAT6 and GATA-3 [19, 51, 52]. Dysregulation of Th2 responses have been implicated in asthma, atopic dermatitis, and certain cancers (basal cell carcinomas and gastric cancers) [53-57].

Table 2: Summary and key features of $CD4^+$ T cell subpopulations[a].

	Th1	**Th2**	**Th17**	Treg
INDUCED BY	IL-12[b] (p35/p40), IFN-γ	IL-10, IL-4	IL-23 (p19/p40) Simulation of NOD2	TGF-β, IL-10
HALLMARK CYTOKINE (PRODUCED BY T CELL)	IFN-γ, IL-12, TNF-α	IL-4, IL-5, IL-6, IL-10	IL-17 Can trigger IL-6, IL-8	IL-27
EFFECTOR FUNCTION	DTH, activation of NK cells, $CD8^+$, and MO, IgG2a	IgG1 & IgE esoinophils, mast cells	Activation of MO, neutrophil recruitment, Secretion of GM-CSF, G-CSF by stromal cells & chondrocytes	Suppresses APCs, Th17, Th1 and/or Th2
PROTECTIVE AGAINST	Intracellular pathogens, tumor growth	Extracellular pathogens, expulsion of parasites from gut	Extracellular bacteria	Peripheral tolerance, suppression of Th1 & infection induced immuno-pathology
ABBARENT RESPONSE	Collagen induced arthritis, IBD, sarcidosis, pathogenesis in TB	Allergy, asthma, atopic dermatitis, some cancers	Autoimmune disorders, EAE	Immune mediated pathology

[a]Adapted from: 19, 22, 37, 42, 44-47, 50, 52-54, 57, 58, 60, 62, 63 [b]IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; DTH, delayed-type hypersensitivity; NK, natural killer; MO, macrophage; Ig, immunoglobulin; IBD, inflammatory bowel disease; TB, tuberculosis; NOD, nucleotide-binding oligomerization domain; GM-CSF, granulocyte macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; EAE, experimental allergic encephalomyelitis; APC, antigen presenting cell

2.5.3 Th17

Recently, IL-17 secreting $CD4^+$ T cells or Th17 cells have been described and are associated with pro-inflammatory responses needed for the clearance of extracellular pathogens [58]. The main role of Th17 cells seems to be to induce recruitment of neutrophils and other innate immune cells to the site of infections. Thus, Th17 have been shown to be necessary for clearance of several extracellular pathogens including *Bordetella pertussis* [59] and *Klebsiella pneumoniae* [37]. Th17 cells are characterized by their production of IL-6 and IL-17, and the responses are regulated by the activation of the transcription factor RORγT [19]. Most IL-17-mediated immune responses result in the induction of pro-inflammatory cytokines (IL-1β, TNF-α) or neutrophil-attractive chemokines [37]. One way in which mouse and human immune systems differ is in the induction of Th17 cells. In mice, naïve Th17 cells are triggered by TGF-β and IL-6 production from APCs [42]. In humans, these same cytokines activated memory but not naïve Th17 cells [42]. It was found that IL-6, IL-1, IL-23, and NOD2 activation were all necessary to induce the induction of Th17 cells from naïve $CD4^+$ T cells in human lymphocyte populations [42]. As a mediator of cellular infiltration and tissue inflammation, Th17 cells are often described as a "double-edged sword". IL-17 is over-expressed in autoimmune diseases such as inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, and psoriasis, [16, 27, 58], and Th17 cells may contribute to the inflammation and arthritis associated with *Borrelia burgdorferi* (Lyme disease) [60].

2.5.4 Treg

Regulatory T cells have been recognized as a separate effector cell over the past 10 years, even though a suppressive population was described more than 30 years ago [61]. These cells constitutively express the IL-2 receptor alpha chain (CD25) and can be induced in the thymus to self antigens or in the periphery to both self (natural Tregs) and non-self (pathogen derived) antigens (adaptive Tregs) [62]. The transcription factor, FoxP3, is a critical component of Treg development both for natural and adaptive Tregs, and is detectable in these cell subsets in both mouse and humans [19]. Tregs can suppress

effector cell function by both cell-cell contact dependent and independent mechanisms. Tregs inhibit effector cell function by inhibition of IL-2 transcription. This may be partially mediated through engaging the glucocorticoid-induced TNF receptor family-related protein (GITR) [62]. Tregs also secrete cytokines IL-10, TGF-β, and IL-27 which all have immuno-regulatory effects. IL-27 is suppressive to macrophages and granulocytes, but enhances the proliferation of Th1 $CD4^+$ T cells [37]. Furthermore, Tregs have a suppressive effect on the APCs themselves, by enhancing secretion of IL-10, and by direct killing of APCs mediated by granzyme B [63]. Tregs function to limit immune responses to self antigens and prevent immune-mediated pathological changes within infected tissues. Elimination of Treg cells may induce wide-spread autoimmunity and unregulated, chronic inflammatory responses [62, 63].

2.6 T Cell Activation by Dendritic Cells

T cell activation by DCs can best be described by a process involving three separate signaling molecules [19]. Illustrated in Figure 2, the first signal is antigen derived from host or pathogen presented in the context of MHC class I or class II molecules (i.e., pMHC-TCR). The second signal includes co-stimulatory molecules such as CD80, CD86 (CD83 in humans, the B7 molecules under older nomenclature), LFA1 and CD40 that stabilize the T cell synapse and/or provide secondary intracellular signaling. The third signal involves the binding of cytokines secreted from DCs and other innate immune cells (NK cells) to their receptor on the T cells in order to facilitate the polarization of the T cell response into Th1 (IL-12), Th2 (IL-4), Th17 (IL-23) or Treg (TGF-β) phenotypes. In vivo, tolerogenic DCs only produce signal 1 resulting in the incomplete activation of T cells. In contrast, DCs that are capable of activating effector T cells produce signals 1, 2, and polarizing signal 3 [18]. T cell polarization is dependent on type and concentration of antigen, costimulatory molecules on DCs, phenotype of DC, local cytokine milieu, as well as the frequency of antigen specific T cells and density of APCs [64, 65]. For example, $CD8\alpha^+$ DCs induced a Th1 like response whereas $CD8\alpha^-$ DCs induced a Th0 or Th2 like response [64]. One current hypothesis related to the polarization of activated T cell is that the prolonged interaction between T cell and DC

polarizes the T cell and creates unequal planes of cell division, giving rise to two slightly different daughter cells that may have differing functions (central memory, efffector,Th1, Th17 or Th2)[65]. Corthay proposed a 3 cell model of T cell activation. Experimental evidence shows that IFN-γ and IL-4 are necessary for the development of $CD4^+$ Th1 or Th2 cells respectively, but neither DCs nor naïve T cells make IL-4 or IFN-γ to mediate the observed responses [36]. NK, NK-T cells, γδ-T cells, mast cells, eosinophils, and basophils all can produce either IL-4 or IFN-γ [36]. Once activated, an autocrine feedback loop enhances functional differentiation and the polarization of Th1 or Th2 responses. Signals provided by a third cell type allows DCs to gather more information about the nature of the pathogenic invaders or degree of tissue damage in order to properly modulate the desired immune response [36].

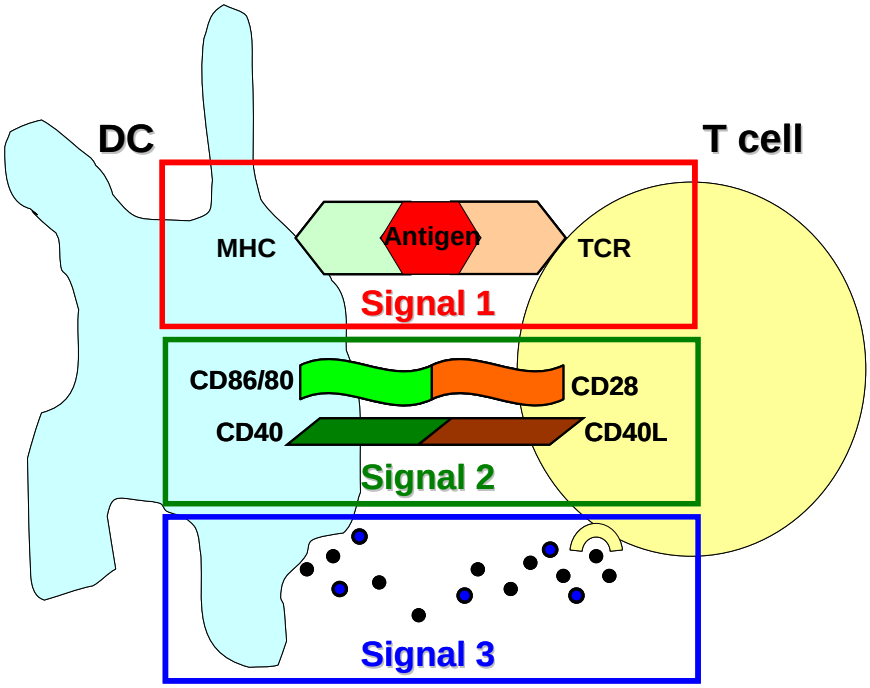

Figure 2: Three signal activation of the T cell by a dendritic cell. Signal 1 is comprised of the major histocompatability proteins (MHC) that presents the processed antigen to the T cell receptor (TCR). The second signal is derived from the ligation of costimulatory molecules CD80/CD86 and CD40 on the DC with CD28 and CD40L/CD154 on the T cell respectively. Signal 3 is provided by cytokines released from the DC or surrounding cells that initiate separate but complementary signaling cascades that enhance the induction and amplification of the antigen specific T cells.

Signal 1 (antigen presented in the context of MHC) is going to cause the antigen specific naïve T cell to pause as it scans DC in the T cell zone of the lymphoid tissues. If a match of MHC-antigen-T cell receptor (TCR) is made, costimulatory molecules CD86 binding to CD28, CD209 binding to ICAM-3 and CD40 binding to CD40L/CD154 stabilize the interaction and the T cell synapse is formed, with more surface molecules moving into the synapse formation. The grouping of these molecules allows for the intracellular grouping of signaling molecules, providing a threshold-like signal for gene activation. The third signal, cytokines present in the milieu, provides the final signal for subpopulation polarization. Th1/Th2 polarization is controlled by phosphorylation MAP kinases (ERK, c-Fos) and activation of pathway/subpopulation specific transcription factors [64]. In vitro studies have led to a model of progressive T cell differentiation where T cell fitness and survival is dependent on strength of antigen stimulus to the DC and the signals provided to the T cell [19, 66].

Cytokines are key regulators in the immune system. Cytokines regulate antigen presentation, migration and function of APCs such as DCs [37]. Furthermore, the cytokine milieu surrounding the T cell and APC as they interact significantly contributes to the polarizing of the T cell response [37]. Cytokines can be used to functionally identify the T cell population being analyzed (e.g., Th1 vs. Th2 vs. Th17) and cytokine secretion (e.g., IFN-γ TNF-α and IL-2) along with surface marker expression (CD44 and CD62L) can be used to define the memory/effector phenotype of differentiated T cells [66]. All of this demonstrates the importance of the role of cytokines during interactions between DCs and T cells.

IL-12, which is secreted by DCs, provides the third signal for induction of Th1 cells. IL-12p40 forms a dimer with IL-12p35 to form bioactive IL-12p70, which induces the active IL-12 receptor on naïve T cells [67]. IL-12p40 can also form a dimer with IL-12p19 to form IL-23, which triggers the activation of Th17 cells [67]. In contrast, the IL-12p40 homodimer (IL-12p80), produced in excess by activated DCs, binds to the IL-12 receptor but does not induce a biological response. This appears to be one mechanism that protects mice from septic shock [67]. However, further studies show a role for IL-

12p80 in $CD8^+$ activation and $CD4^+$ dependent responses such as DTH and resistance to mycobacterial infection [37, 67].

IL-6 has many functions including B cell differentiation in acute phase responses [28]. IL-6 is observed often in conjunction with TNF-α and is considered inflammatory in nature. Other roles of IL-6 include the need for IL-6 to inhibit the regulatory functions of Treg cells [28]. This was shown by an impaired immune response to ovalbumin delivered with LPS in IL-6 knockout mice [28]. The response could be recovered by depleting Treg cells with anti-CD25, or blocking the function of Treg cells [28].

IL-10 produced by other immune cells or by T cells has a suppressive effect on DCs, and actually enhances their ability to activate/induce Tregs [26]. This is yet another example of slight differences in mouse and human immunology. In human peripheral mononuclear cells, the secretion of large amount of IL-10 indicates a regulatory cell population whereas in mice, IL-10 is associated with a Th2-type response [64].

Not all classes of microbial stimuli result in the same polarization of Th1-Th2 T cell responses. Differential activation of the DCs and production of IL-12(p70) can result in either a Th1 or Th2 response. *Toxoplasma* extracts and *E. coli* LPS stimulate IL-12(p70) production by $CD8^+$ DCs that primes for a Th1 response [64]. Some viruses induce IFN-α from plasmacytoid DCs and stimulate Th1 responses [64]. In contrast, schistosome egg antigens, filarial worm antigens, cholera toxin, lipotoxins stimulated by *Toxoplasma*, some forms of *Candida albicans*, or highly purified preparations of *Porphyromonas gingivalis* LPS, do not stimulate IL-12(p70), and favor Th2-like responses [64]. Furthermore, a single pathogen may contain components that induce both Th1 and Th2 T cell responses. For example *Vibrio cholerae* contains unmethylated CpG DNA that triggers Th1-like immune responses, while the B subunit of cholera toxin elicits a Th2-like immune response [64]. From an evolutionary standpoint, the immune bias resulting from microbial infection (i.e., Th1-Th2-Th17-Treg) may represent the eternal struggle between immune system and pathogen with the result being the development of an immune response that is not totally suited to the host's needs or elimination of the pathogen but may represent a truce between the two [64]. Thus, Th1 and Th2 immune responses may be polar ends of a continuum of responses that might be

induced following a single infection [36]. Responses in vivo may be more or less plastic that is proposed by in vitro models.

2.7 Swine Dysentery

Swine dysentery (SD) is severe diarrheal disease of swine. At any age, pigs can be affected; however the most severe economic losses are at the grower/finisher stage where sudden death can occur [68]. The etiologic agent is an anaerobic spirochete termed *Brachyspira hyodysenteriae* which colonizes the ceca and colon of infected pigs without any systemic spread [69-71]. The disease can be identified by the clinical signs of mucohemorrhagic diarrhea, general poor condition, and shedding of spirochetes in the stool. The acute phase of the disease appears to be driven by leukocytes responding to the translocation of luminal and commensal bacteria into the lamina propria following epithelial erosion due pathogenic hemolysin and other factors secreted by *B. hyodysenteriae* [72]. The chronic phase is mediated by the infiltration of $CD4^+$ T cells into the colonic mucosa [72]. Histological evaluation of colonic tissue following infection reveals colonic crypt elongation, superficial epithelial erosion, submucosal edema, inflammatory cell infiltrate and mucosal hyperplasia [73]. The disease is endemic in most pig producing countries, where infection prevalence can be as high as 35% of the swine herds [74, 75]. Efforts to maintain herds free of SD are difficult, as wild rodents, dogs and waterfowl are natural hosts of the bacterium [76, 77]. Control measures include antibiotic therapy, however, recently, antibiotic resistant strains have emerged [78]. Currently in the United States, there is no available licensed vaccine for SD. Efforts to produce both recombinant and whole cell vaccines have met with varying success [79-82]. Outer membrane preparations and other recombinant vaccines elicit only partial protection [79-82]. Using a squalene/pluronic acid adjuvant, protection was conferred by a pepsin digest preparation of whole cell *B. hyodysenteriae* [82-84].

Exact pathogenic factors of *B. hyodysenteriae* are poorly defined. *B. hyodysenteriae* produces a hemolysin that induces some of the early morphological changes seen in mucosal epithelia in mice [85]. Another key virulence factor appears to be a modified lipoogliosaccharide. Butanol/water extracts of endotoxin-like material

induced IL-1 and TNF-α from murine peritoneal extrudate cells at 5- to 50- fold higher concentrations than was required for *E. coli* endotoxin but in an LAL assay showed similar endotoxic activity [81]. However, it was noted that the number of endotoxin units per nanogram of endotoxin possessed similar endotoxic activity. Further tests showed that LPS preparations (phenol-water extraction) of *B. hyodysenteriae* were non-mitogenic to murine splenocytes and non-pyrogenic in rabbits [81]. Finally there appears to be a synergistic role between *B. hyodysenteriae and* members of the normal microbiota. Previous studies using germ-free piglets or mice do not develop lesions or clinical disease despite the fact that *B. hyodysenteriae* did colonize the cecum and colon of the inoculated animals [86-88]. Co-infection with *B. hyodysenteriae* and *Bacteroides vulgatus* is sufficient to induce clinical disease phenotype in germ-free animals [86-88].

Mice are an accepted model for studying the disease caused by *B hyodysenteriae* infection in swine [85, 89-92]. In contrast to pigs, the lesions induced by *B. hyodysenteriae* infection are largely limited to the ceca of infected mice. Previous studies published by Wannemuehler et. al., showed that neutrophil infiltration was important for the development of the acute inflammation and development of disease [91]. Antibody depletion of neutrophils or blocking neurotphil translocation into mucosal tissues significantly reduced lesions in the acute phase of disease [91]. Acute colitis, in the mouse model, upregulates IL-1β, IL-6, TNF-α, and MIP-2, cytokines that are part of pro-inflammatory pathway (unpublished data).

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CHAPTER 2

Vaccine Adjuvants: Current Challenges and Future Approaches

Jennifer H. Wilson Welder[1], Maria P. Torres[2], Matt J. Kipper[3], Surya K. Mallapragada[2], Michael J. Wannemuehler[1], Balaji Narasimhan[2]*

[1] Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011. [2]Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011. [3]Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, CO 80523-1370

*Corresponding Author, nbalaji@iastate.edu

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Abstract:

For humans, companion animals, and food producing animals, vaccination has been touted as the most successful medical intervention for the prevention of disease in the twentieth century. However, vaccination is not without problems. With the development of new and less reactogenic vaccine antigens, which take advantage of molecular recombinant technologies, also comes the need for more effective adjuvants that will facilitate the induction of adaptive immune responses. Furthermore, current vaccine adjuvants are successful at generating humoral or antibody mediated protection but many diseases currently plaguing us, such as tuberculosis and malaria, require cell mediated immunity for adequate protection. A comprehensive discussion is presented of current vaccine adjuvants, their effects on the induction of immune responses, and vaccine adjuvants that have shown promise in recent literature.

Keywords:
vaccine adjuvants, vaccines, immunology, mucosal immunization, biodegradable polymers, alum, liposomes, TLR ligands, polymeric biomaterials, controlled release/delivery

Introduction

Over the last 200 years, the use of vaccines has proven to be one of the most successful medical interventions in the reduction of disease caused by infectious agents[1]. For example, through vaccination, disease caused by the smallpox virus was eradicated worldwide. Europe, the Western Pacific and the United States have been declared free of polio and have discontinued using the Sabin (oral-live) vaccine, now including the killed version (Salk vaccine) as part of the childhood vaccination schedule[2]. In veterinary medicine, control and eradication of diseases such as swine cholera, parvovirus-induced enteritis, distemper virus, and pseudorabies virus have all been achieved through intervention strategies employing vaccination programs[3]. Vaccination has been touted as the greatest medical achievement in the 20th century.

Despite advancements and improvements in vaccine efficacy and implementation over the past several decades, infectious disease still remains the largest cause of death world-wide. Unfortunately, many of these deaths occur in children and infants caused by diseases that are preventable by vaccination [4,5]. According to the WHO, 14% of the global childhood mortality is caused by vaccine preventable diseases such as measles, disease caused by *Haemophilus influenzae* type b (Hib), pertussis (whooping cough) and Tetanus [6]. Many challenges still remain with regard to fully realizing the health benefits of active immunization programs. Some of these obstacles include the development of single-dose vaccines, methods to overcome the poor immunogenicity of recombinant and subunit immunogens, and the ability to rapidly and rationally develop vaccines against emerging pathogens. One promising strategy for addressing these challenges is the development of new vaccine adjuvants, or carriers that enhance the effectiveness of vaccines.

Current immunization practices often require multiple doses to achieve protective immunity. Health care workers have observed that dropout rates in a vaccination programs can reach as high as 70% in some developing countries [7]. Recent failures of the human chicken pox vaccine demonstrated that current recommended single dose is not protective in an outbreak situation [8]. Many patients whom contracted mumps in Canada could only document a single immunization [9]. The World Health Organization (WHO) listed the development of a single-dose vaccine as number one in their "Grand Challenges" for human health in 2005 [10]. While not receiving the full regimen of a multi-dose vaccine may have dire consequences in human health, in most livestock systems, it is often impractical in terms of cost, labor and stress on the animal to immunize more than once [11]. Vaccination still remains a cost effective way to combat disease [12]. Prophylactic administration of an efficacious vaccine can be more cost effective than therapeutic treatment, more ecologically friendly than the use of antimicrobial agents (i.e., less chance of antibiotic resistant bacteria in the environment) and offers greater flexibility in management options. It is estimated that for each $1 spent on vaccines, $5 to $10 are saved in what would have been lost to disease [13]. It is estimated that 30 to 50% of the antibiotics produced are used in agriculture, many at sub-therapeutic levels in feed to promote growth by suppressing bacterial growth [14]. Emerging antibiotic resistance, changes in consumer acceptance of antimicrobial use in food producing animals, and high cost of treatment as compared to prevention dictates that novel biologics for disease prevention must be developed [15]. Vaccination against infectious agents has greatly improved the health of humans, companion animals, and livestock species worldwide. A single-dose vaccine, whether for humans or animals, would greatly increase patient compliance, thus improving the efficacy of many vaccines (i.e., a full dosing regimen received at once), and reduce the costs associated with vaccination programs.

Recent developments in both synthetic and naturally derived adjuvants suggest that single-dose vaccines for a variety of pathogens may be realized in the near future. However, no single adjuvant will be effective for all vaccine applications. Developing new adjuvants for improved immunotherapy requires the development of complementary strategies that address all the complex variables involved in immune surveillance [16].

Thus, before discussing recent developments in vaccine adjuvants, we briefly discuss innate and adaptive immunity and the various types of vaccines currently used to confer protective immunity.

Innate and Adaptive Immunity

Innate and adaptive immune systems work together as a complex integrated system [17]. When cells from innate defenses recognize foreign structures or pathogens, a cascade of events ensues which functions to eliminate or contain the threat. The innate immune system is involved in surveillance and detection of foreign invaders and, as such, is a key target for activation by vaccine adjuvants. Innate immunity comprised of a variety of hematopoietic and cellular factors including the complement system, phagocytic cells, NK cells, naturally occurring antibodies, γδ T cells, and antimicrobial peptides [18,19]. The innate immune system uses relatively few molecules to recognize these foreign invaders described by Janeway and Mezhitov as pathogen-associated-molecular-patterns (PAMPs) [18]. Depending on the vigor of the innate immune response, the adaptive immune response may or may not be actively engaged. In contrast to innate immunity, adaptive immunity recognizes antigen-specific epitopes via specialized cell surface receptors (antibody or T cell receptor) resulting in a specific or more directed immune response [18]. It has been shown that a combination of innate immunity and prolonged presence of the pathogen-derived immunogens significantly influence the induction of a robust immune response[20]. To enhance immune activation, adjuvants can be tailored to specifically activate the type of immune response needed against a particular disease (antibody, cell-mediated, or mucosal immunity) without the need to suffer the consequences of an active infection [21].

A critical innate immune cell that is involved with induction of immune responses is the dendritic cell (DC). DCs are found in all body tissues and, as such, are effectively distributed to play a central role in stimulation and regulation of adaptive immunity (cell mediated and humoral immunity) [22]. In the blood and tissues, DCs are in an "immature" state, capable of phagocytosis, and express low levels of costimulatory molecules and molecules associated with cellular migration (CCR7, DC-SIGN, and DEC-205) [23]. In the

basal and suprabasal epidermis, resident DCs or Langerhan's cells are the first cells to encounter microbes or injected immunogens. These cells provide innate immune surveillance and are continually replenished form special progenitors that reside in the dermis [24]. Dendritic-like cells are also resident in the lungs where they discriminate between pathogenic and harmless inhaled particles [24]. In fact, pulmonary DCs are key producers of IL-10 and, as such, are suppressors of airway inflammation. Within the gut mucosa, DCs extend their pseudopodia between epithelial barriers to sample luminal contents [25]. Among the many different PAMPs on DCs, Toll-like receptors (TLRs) allow DCs to recognize specific microbial ligands (CpG DNA, lipoteichoic acid. Lipopolysaccharide, flagellin) [17]. TLRs are type I transmembrane proteins that mediate the initial recognition of microbial pathogens and as such are likely targets for stimulation by vaccine adjuvants [26,27]. Stimulation of TLR and other pattern recognition receptors result in the activation of specific intracellular signaling pathways (e.g., MyD88-dependent and –independent) leading to activation of transcription factors (NFκB and/or AP-1) necessary for cellular migration, maturation and antigen presentation. DCs acquire antigen by three main mechanisms: 1) phagocytosis or energy-dependent engulfment of bacteria, particulate matter or cellular debris; 2) macropinocytosis uptake of soluble antigens; 3) receptor mediated uptake triggered by mannose receptors, complement receptors or Fc receptors. Upon activation via TLRs and/or other environmental cues, such as IL-8, DCs undergo maturation and migration to the draining lymph node. Following maturation, DCs lose much of their phagocytic capacity while increasing surface expression of migratory and co-stimulatory molecules, such as MHC I/II, CD80, CD86, and CD40. This process is accompanied by migration to the draining lymph node(s). Within the lymph node, DCs continue maturation and serve as potent antigen presenting cells (APC) to naïve $CD4^+$ and $CD8^+$ T cells.

There are two antigenic processing pathways within DCs that lead to the major histocompatibility complex (MHC) molecules, whose function is to bind peptide fragments derived from pathogens and display them on the cell surface for T cell recognition [28,29]. Antigens taken up by DC via phagocytosis are contained within a phagosome or early endosome. The phagosome fuses with a lysosome generating a

phagolysosome. Following changes in the pH of the phagolysosome, proteolytic enzymes are activated and the antigen is degraded into small peptide fragments (9-13 amino acids in length) in order to facilitate their presentation to T cells and B cells. Antigens contained within phagolysosomes representing exogenous antigens are loaded into MHC II and then presented on the cellular surface for stimulation of $CD4^+$ T cells. A diagrammatic representation of a mature DC presenting antigen via MHC II, the exogenous pathway, is shown in Figure 1a.

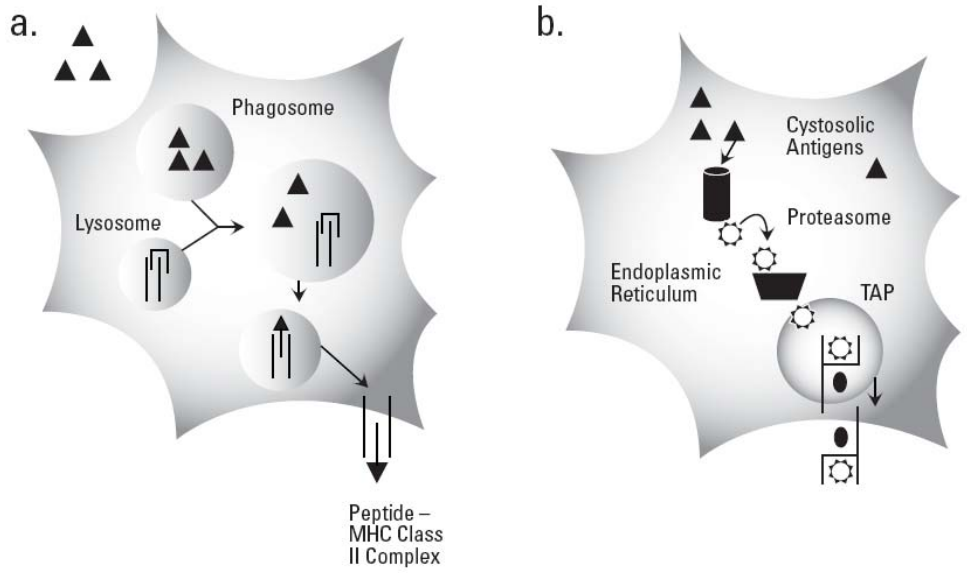
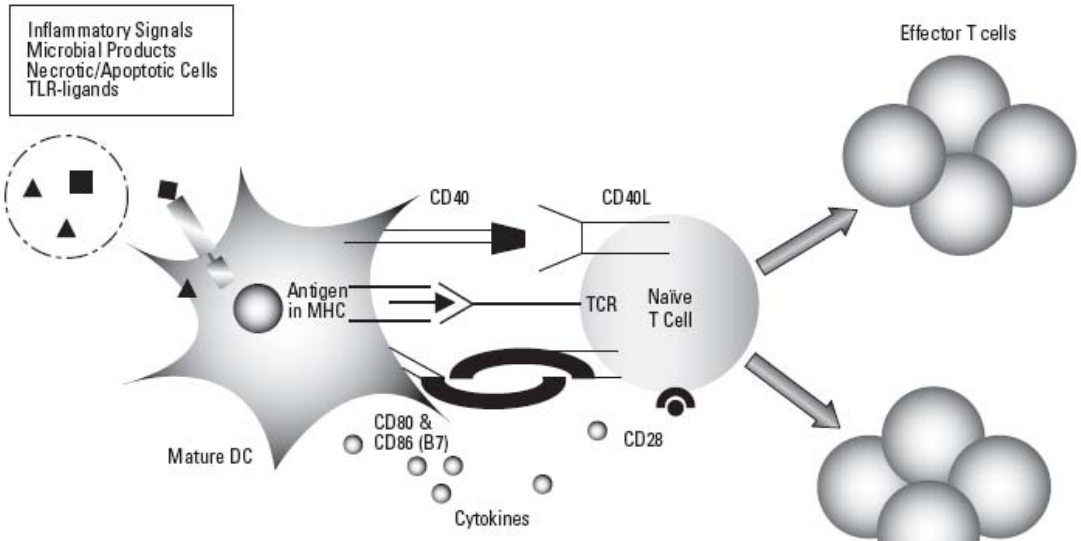


Figure 1: Exogenous and endogenous antigen presentation. (a) Following engulfment, a pathogen or immunogenic protein is contained within a phagosome or endosome. Fusion of the phagosome with the lysosome creates a phagolysosome bringing together the engulfed antigens and degradative enzymes and MHC II molecules. Following proteolytic cleavage, MHC II chaperone protein (CLIP) is displaced by the peptide (9 to 13 amino acids), which binds within the MHC II cleft. The vesicle containing the peptide-MHC II complex (pMHC II) traffics through the cytosol, eventually fusing with the cell membrane and the pMHC II is now displayed on the cell surface. (b) For antigens gaining access to the cytosol of the cell (self antigens, viruses, or cytosolic bacteria) proteins are degraded by cytosolic proteasomes or immune proteasomes. Degraded peptides are guided to TAP (transporter protein associated with antigen processing) and enter the endoplasmic reticulum. Subsequently, the peptides are loaded into MHC I molecules and following intracellular trafficking, are presented on the surface of the cell.

Antigens generated within the cytosol of the cell, including viral antigens, antigen from bacteria that escape into the cytosol, and many cancer antigens are presented by the endogenous pathway. Cytostolic proteins are degraded by proteasomes in the cytosol, chaperone proteins (TAP) translocate the peptide fragments into the endoplasmic reticulum where it is loaded into MHC I molecules that are subsequently transported to the cell surface for presentation to $CD8^+$ T cells as shown in Figure 1b. While all nucleated cells in the body express MHC I molecules, only DCs are able to efficiently stimulate naïve $CD8^+$ cells [30]. Antigen specific $CD8^+$ T cells properly activated by DCs can kill infected cells directly, a powerful component of cell-mediated immunity. What also makes DC excellent activators of adaptive immunity is that DC regularly present antigen from the same source by both MHC I and MHC II pathways by phagocytosing necrotic or apoptotic cells thus allowing for cytosolic antigens access to MHC II loading compartments [30,31]. Thus, DCs are not only involved in immune surveillance, but also act as a bridge between innate and adaptive immunity. Both the effector and regulatory aspects of CMI and humoral immunity are directly affected by the induction or activation of $CD4^+$ T helper cells. These $CD4^+$ T cells can be further classified as Th1, Th17, Th2 or Treg [32,3334]. A Th2-type immune response is characterized by the production of IL-4, IL-5, IL-10, and IL-13 and the secretion of IgG1 and IgE antibody isotypes. Th1-type responses are characterized by the production of the IFN-γ and TNF-β, IgG2a antibodies and are usually associated with cell-mediated immunity including activated macrophages and delayed-type hypersensitivity [35]. Immune responses of the Th1-type are directed more towards intracellular pathogens and are necessary for clearance of many viruses, some bacteria (e.g. *Mycobacterium tuberculosis*) and anti-tumor effects, whereas a Th2-type response is generally associated with the induction of antibodies that effectively neutralize toxins, viruses, and bacterial adhesion [36,37]. Th-17 responses are considered inflammatory in nature and are characterized by production of IL-17 [32]. These responses appear to provide protection during acute inflammatory reactions but have been associated with chronic inflammatory diseases. The role of Th-17 cells in vaccinology or infectious disease has yet to be elucidated.

Induction of the appropriate immune response (humoral vs. CMI vs. regulatory) is essential for vaccine efficacy [37,38]. For example, in the BALB/c model of leishmaniasis, an immune response dominated by IL-4 and IgG1 (i.e., Th2-biased response), in comparison to a protective Th1-biased response (IFN-γ and IgG2a), does not protect nor allow these mice to clear the infection [39-41]. Furthermore, in regions where tuberculosis is endemic, a large portion of the population is infected and possesses a pre-existing immune response to *Mycobacterium* species, usually Th2 dominant [42]. It its hypothesized that the current tuberculosis vaccine (Bacillus Calmette-Guerin or BCG vaccine) is ineffective in preventing disease because the current BCG vaccine is unable to redirect the pre-existing immune response (Treg and/or Th2) in to a protective, Th1 dominant immune response [42,43]. Additionally, the current vaccines used against feline infectious peritonitis viruses enhances humoral immunity which has been shown to exacerbate the disease, whereas a CMI response would be protective [44].

In addition to presentation of antigen to T cells, mature DCs help to shape the adaptive immune response by secretion of cytokines. Activated DCs produce the cytokines tumor necrosis factor alpha (TNF-α), which mediates acute inflammation, and a variety of interleukins, such as IL-1β, IL-6, IL-8, IL-12, and IL-10. The specific combination of cytokines released by activated DCs can influence the ensuing $CD4^+$ T cell response. The bias of the immune response generated after antigen presentation can be characterized by measurement of the cytokine profiles induced upon induction of antigen-specific recall responses (Figure 2). The production of cytokines by DCs is also a critical feature of efficacious immune induction. For example, DC secretion of IL-1β induces secretion of IL-2, which facilitates the maturation and proliferation of naïve antigen-specific T lymphocytes. Conversely, antigen presentation in the absence of effective costimulation (CD80/86, CD40) or cytokine secretion by DCs induces ineffective T cell activation that can result in either tolerance or anergy [30].



Cytokine Signal from DC	T cell Effector Type	Resulting Immune Response
IL-6, IL-23, IL-27	Th 17	IL 17, inflammatory response
IL-12, IL-1β, IL-18	Th 1	IFN-g, activated macrophages, IgG2, IgA
IL-4, IL-13, IL-10	Th 2	IL-4, IL-5, IL-10, anti-inflammatory response, IgG1, IgE
IL-10, TGF-β	Treg	IL-10, suppress activation of T cells and DC

Figure 2: Signals from DCs can influence the differentiation of naïve T cells. Stimulated, mature DCs present not only antigen in the context of MHC but also costimulatory surface molecules necessary for T cell activation. Furthermore, the type and quantity of cytokines secreted by DCs in conjunction with these co-stimulatory molecules can direct the naïve T cell into different effectors phenotypes. IL-12 secretion from the DC initiates a Th1 type response characterized by secretion of IFN-γ. IL-4 secretion from the DC results in a Th2 type response characterized by the secretion of IL-4, IL-5, and IL-10. The cytokines secreted by DCs are induced following ligation of cellular receptors (PRRs or TLRs) and signals from the surrounding tissues (i.e., IL-8). New evidence is emerging regarding the role of DCs in activating Th-17 and Treg cells.

Vaccines

The most potent (i.e., protective) and lasting immune response in a host is induced following a natural infection with the pathogenic organism. However, for many diseases, the clinical outcome for the individual may not favorable because of a lack of treatment for a given disease, untoward morbidity or sequelae, or high mortality. For these reasons, vaccines have been designed to mimic the immune response that would otherwise be

induced by an active infection, thereby avoiding the undesirable effects of a particular disease. To be effective, a vaccine must contain some portion of the disease-causing agent (e.g., bacteria, virus, or toxin) and may include an immune-enhancer or adjuvant. Vaccine regimens generally employ an initial dose or priming dose followed by two to three booster doses. This prime-boost strategy allows for the presentation of high quantities of immunogen in the draining lymph node at several time points. The first dose initiates immune responses that particularly involve DCs and naïve immune cells. Repeated administration of this same immunogen induces activation of not only effector cells (e.g., immunoglobulin-committed B cells and T cells) but also memory immune cells [29]. Upon subsequent exposure to the same immunogen, memory T and B cells provide for a secondary immune response characterized by a greater magnitude (e.g., high antibody titer) and one that occurs at a faster rate than the induction of a primary immune response [29,45]. Regardless of the type of immunogen administered in currently licensed vaccines (e.g. killed organism, subunit), the primary mechanism of protection is mediated by the generation of neutralizing antibodies as opposed to the induction of cell-mediated immunity [46].

Vaccines can be classified into three general categories: modified live, killed/inactivated, or subunit. Each has its own advantages and disadvantages. A list of the current licensed vaccines for use in humans within the United States, is available on multiple websites managed by both the Department of Health and Human Services (DHHS) and the Centers for Disease Control and Prevention (CDC) [12,47-49]. The information provided includes the type of immunogen used, the age at which the vaccine should be administered, and the immunization schedules as recommended/required for the United States as issued by the DHHS.

Live Vaccines

Most successful vaccines currently consist of live, attenuated organisms. Other than natural infection, vaccines containing modified live organisms, relative to other vaccine formulations, induce the most potent and lasting immune response in the host. Modified live vaccines generally require the fewest number of inoculations, require no adjuvants, often confer lifelong immunity, and can be delivered through the same route as

the natural infection would occur [50]. The organism is able to replicate in the host, causing a mild, limited infection that stimulates the host immune response in a very similar fashion to that induced by a natural infection. Furthermore, these vaccines retain many of the natural microbial compounds that enhance immunity by activating the innate immune system.

Safe use of live vaccines requires that the organism first be attenuated, that is, the virulence capacity of the organism must be reduced. This can be achieved through repetitive passages (100s to 1,000 times) in a non-human host or in vitro. Alternatively, attenuated organisms can be developed by inducing genetic changes so that critical virulence attributes have been deleted or inactivated in the target organism. The Sabin oral polio vaccine and Flu-mist are two examples of modified-attenuated, live vaccines that are delivered along the same routes as the natural infection [51]. A closely related but non-pathogenic organism can also be used if the non-pathogen and pathogen share immunoprotective epitopes. For example, Jenner observed that cowpox infection prevented smallpox, and attenuated Ankara strain of vaccinia virus was used to vaccinate against smallpox [4]. Likewise, attenuated *Mycobacterium bovis* used in the BCG vaccine is protective against disease caused by virulent *M. tuberculosis* [50].

The largest drawback to modified live vaccinations is that they are able to replicate in the host and, thus, are capable of persistent infection and reversion to the more virulent form. If the host is immunocompromised, the organism may be able to persist, and an otherwise non-pathogenic strain may be able to induce disease in the absence of a competent immune system. The live organism may also be able to spread and induce disease in other non-vaccinated individuals. Through horizontal gene transfer and natural random mutation, attenuated organism may acquire virulence factors and become able to cause disease. The attenuated strain of poliovirus used in oral vaccines has been shown to circulate throughout a region and occasionally revert to virulence [51]. While modified live vaccines are very effective at inducing both cellular and humoral immunity, they can cause severe reactions, ranging from inflammation at the site of inoculation to systemic disease. Furthermore, many current diagnostic tests cannot distinguish between an individual who is naturally infected and an individual that

received a modified live vaccine [52-54]. Effectiveness of live vaccines also requires that they be properly handled before administration. Keeping attenuated vaccines viable (i.e., proper storage) has been problematic in the worldwide effort to eradicate polio [51].

Killed vaccines

Killed or inactivated vaccines are comprised of the whole organism that has been treated with either heat or chemicals. In this way, the organism is not able to replicate in the host, yet cellular integrity of the pathogen is preserved. Dependent upon in vitro growth conditions, killed vaccines are also potent inducers of the humoral immune response because most of the virulence factors and epitopes are present [50]. Killed vaccines do not carry the same risks as live vaccines; the organism cannot replicate and, therefore, cannot establish persistent infection, spread to other individuals, or revert to a virulent form [50]. These types of vaccines are generally cost effective to produce, possess a longer shelf life and are less sensitive to changes in temperature and handling when compared to modified live vaccines [55]. Some killed vaccines can be administered orally (e.g., typhoid and cholera) more closely mimicking natural infection [35]. Many injectable vaccines that contain killed/inactivated organisms include: polio virus (Salk injectable polio vaccine), whole-cell *Bordetella pertussis*, Hepatitis A virus, *Yersinia pestis* (causative agent of plague), and encephalitis viruses [56].

The use of killed vaccines often requires multiple doses for the induction of protective immunity. The degree of cellular immunity induced following immunization with killed vaccines can be weak. Like modified live vaccines, killed vaccines are highly reactogenic and are associated with a high incidence of side effects. For example, the whole cell killed pertussis vaccine can cause a high fever accompanied by severe pain, redness and swelling at the injection site due to the presence of bacterial lipopolysaccharide (LPS), a cell wall component and potent immunostimulator, and other TLR ligands in the vaccine [57,58]

Subunit vaccines

Subunit vaccines contain only a portion of the organism. Toxoids, inactivated bacterial toxins, were the first subunit vaccine to be employed for human use. Diphtheria and tetanus toxoids are formaldehyde-inactivated forms of the bacterial toxin that induce immune protection against the native toxin (i.e., neutralizing antibody). Other subunit vaccines currently in use include hemaglutin-binding proteins of influenza virus and polysaccharide capsules of bacteria such as the vaccines that include conjugated forms of HiB (*Hemophilus influenza* type B), pneumococcal (*Streptococcus pneumoniae*), and meningococcal (*Neisseria meningitides*) polysaccharides[56,59]. Because of the poor immunogenicity of carbohydrate immunogens, these compounds are generally conjugated to a protein in order to enhance the immunogenicity; this strategy has been specifically used when developing vaccines for infants or the elderly.

Another type of subunit vaccine being developed does not include protein or other structural components of the pathogen but utilizes the DNA of the pathogen. By injecting the DNA sequence encoding for a protective epitope, immunity can be induced against a specific pathogen that bears the target epitope [56]. DNA can be delivered using a viral vector with the epitope encoded on a plasmid or DNA-containing particulates that deliver the DNA to DCs [60]. Host cells then express the epitope, it is presented in the context of MHC I or II molecules, subsequently inducing strong cellular immunity [29]. While many DNA vaccines are still experimental, there are currently several DNA-based human vaccines in phase I, II or III human trials, including vaccines against cytomegalovirus, Dengue virus, human immunodeficiency virus, herpes simplex virus-2, hepatitis B and melanoma (skin cancer) [61].

Subunit vaccines offer several advantages including targeting the immune response to protective epitopes but retaining or deleting epitopes that can be used to differentiate ‘vaccinated’ individuals from naturally exposed/infected individuals [62]. Subunit vaccines may also eliminate many of the side effects and reactivity associated with modified live or killed whole organisms as they lack many of the microbial components that trigger innate immune recognition. The purified protein or other subunit components can be prepared free of LPS, CpG-DNA, or other bacterial TLR ligands that

can induce an overt inflammatory response. Thus, subunit vaccines are very safe, and using new technologies, can be very cost effective to produce.

However, subunit vaccines still have many weaknesses. In general, subunit vaccines lack strong immunogenicity and require multiple doses for protection [63]. Poor immunogenicity also generally requires that subunit vaccines be delivered with an adjuvant or immunoenhancer (e.g., monophosphoryl lipid A). Many of the bacterial components that trigger a more robust immune response also enhance the protective response by inducing affinity maturation of the antibody response, increasing serum antibody titers, and immunoglobulin class switching [64]. While current subunit vaccines can be formulated to induce high titer antibody responses, the induction of protective T cell responses ($CD4^+$ or $CD8^+$ cell-mediated immunity) are generally lacking.

Adjuvants enhance the immunogenicity of vaccine components where a live attenuated vaccine may not be desirable. Increasing numbers of immunocompromised patients, elderly populations and infants represent a special problem to health officials as live-attenuated vaccines are not recommended in these groups. Subunit and recombinant protein vaccines are easier to produce and are generally considered safer than live vaccines, but require adjuvants to be efficacious [65].

Adjuvants

An adjuvant is an agent that stimulates the immune system, increasing the response to a vaccine, while not having any specific antigenic effect. Adjuvants are immunoenhancing materials that perform three major functions, i) provide a "depot" for the antigen, creating an antigenic reservoir for slow release, ii) facilitate targeting of the antigen to immune cells (APCs) and enhance phagocytosis, and iii) modulate and enhance the type of immune response induced by the antigen alone (e.g., isotype switching induce Th1 vs. Th2 bias) [66-69]. Adjuvants may also provide the danger signal the immune system needs in order to respond to the antigen as it would to an active infection [29]. A list of currently licensed adjuvants is listed in Table 1.

Table 1: Vaccine adjuvants currently used in licensed vaccines [a]

Humans, US	Humans, United Kingdom & European Union	Livestock, Worldwide (general categories)[b]
Aluminum hydroxide, Aluminum phosphate, Potassium aluminum sulfate (Alum)	Aluminum hydroxide, Aluminum phosphate, Potassium aluminum sulfate (Alum)	Aluminum hydroxide, Aluminum phosphate, Potassium aluminum sulfate (Alum)
	Calcium Phosphate	Saponin (QS-21)
	MF-59 (Squalene, in Fluad)	Oil Emulsions paraffin, mineral oil, lanolin, squalene, ISA-70, Montanide IMS)
	AS04 (liposome formulation containing MPLA and QS-21) (FENDrix, Cervaix)	Glycerin

[a]Adapted from: [71,259-261]; [b]Many livestock adjuvant-vaccine formulations are proprietary and full composition does not have to be disclosed

The first function, providing a depot for the immunogen, is accomplished by entrapping the antigen in a poorly metabolized, non-dissolving or slowly dissolving substance, or otherwise sequestering the antigen to allow for the slow clearance of the antigen from the body. Some of these types of adjuvants are discussed in more depth in other sections of the review. Aluminum phosphate and aluminum hydroxide, commonly referred to as alum, are the adjuvants most often used in human vaccines and the gel-like matrix of alum creates a slow-release environment for the immunogen. Oil-water emulsions also work by sequestering the antigen and slowly releasing it. The classic water-in-oil emulsion, incomplete Freund's adjuvant, is widely used in livestock

vaccines, even though it has a tendency to induce granulomas [65]. It is not used in vaccine formulations for human use because of this tendency. Other mineral oil emulsions, such as Drakeol, Marcol, ISA 206, and ISA 25 from Seppic Montanide are carriers also used in various livestock vaccines [65]. Recently, MF59, a variation of the biodegradable oil squalene, has proven to be a potent adjuvant with a satisfactory safety record and, thus, is suitable for human use [65,70]. Virosomes, virus-like particles, immunostimulatory complexes (ISCOMs), and liposomes all allow for the slow clearance of antigen by incorporating the antigen into small particles composed of stabilized lipids, phospholipids, or proteins. GlaxoSmithKline's new class of adjuvants (AS02A, ASO1B, AS04 and AS15-SB) combine stable mineral oil liposomes containing a squalene derivative, and immunostimulating monophosphoryl lipid A [61,71]. Furthermore, antigen sequestering can be achieved by incorporating the antigen into microspheres composed of polymeric units of a biodegradable material. As the microsphere degrades, the antigen is released. Thus, many different carrier formulations provide antigen depots once injected.

The second function of adjuvants is to enhance the immune response by targeting the antigen to immune cells, enhancing phagocytosis, and/or activating the APC. This can be accomplished by properties of the antigen, by a property of the carrier, or by inclusion of immunostimulatory molecules. Pertussis toxin binds with high affinity to epithelial cells, enhancing uptake of the vaccine [29]. Other toxins, cholera toxin and *Escherichia coli* heat-labile toxin (LT), bind selectively to M cells of the intestinal tract [35]. These cells translocate the vaccine particle across the epithelial barrier to a region rich in lymphocytes [29,65,72]. While bacterial toxins such as cholera toxin and *E. coli* LT augment a strong humoral immune response, the response to the anti-toxin may overshadow the response to the conjugate antigen [29]. LPS, another bacterial-derived immunostimulant, is derived from the outer membrane of gram-negative bacteria such as *B. pertussis*. These bacterial products directly interact with the innate immune system via LPS receptors CD14 and TLR-4 [72]. Human TLRs, when triggered by LPS, stimulate the activation of NF-κB, a transcriptional activator for the production of pro-inflammatory cytokines [65]. Because humans are very sensitive to endotoxins, LPS is generally too toxic for inclusion

in many human vaccine preparations, and the majority of injectable solutions for medical use are pyrogen-free.

These first two mechanisms of immunity are illustrated in Figure 3. Some adjuvants may interact directly with TLRs on APC (Figure 3b), and can be derived from pathogens that display highly conserved structures (e.g. PAMPs) [73]. As illustrated in Figure 3, an adjuvant can interact with the PAMP directly or release antigen as in the more traditional depot effect. Many adjuvants exhibit a combination of these characteristics.

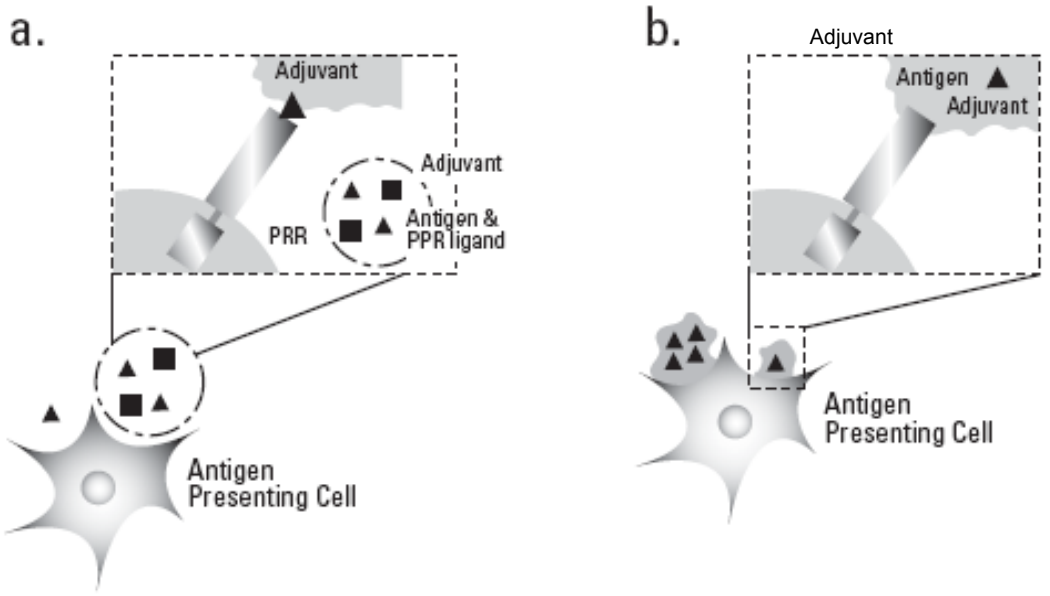


Figure 3: Recognition of antigen and PRR ligands by immature DCs. An adjuvant may act as a depot, releasing both vaccine antigen and stimulatory PRR ligand over time (a) as in many alum or mineral oil formulations containing MDP, MLPA or CpG. Conversely, the adjuvant may be directly recognized by the PRR (such as mannose receptor or TLRs) (b), as may be used in whole cell, killed bacterins vaccines or some polymer adjuvants.

Many biologically derived materials exhibit the third mechanism of adjuvanticity, modulation of the immune response mechanism. Monophosphoryl lipid A (MPLA) is a non-toxic LPS derivative obtained from *Salmonella* and has been shown to enhance IFN-γ production and induction of $CD4^+$ T cell-mediated immunity [35,74]. MPLA has been shown to interact through TLR-4, however it is not fully dependent upon TLR-4 for its

effect [75,76]. Ligation of TLR-4 and activation of TRIF transcription factors is responsible for activating both DCs and intraperitoneal macrophages resulting in T cell stimulation without activation of IL-6, IFN-γ and other inflammatory molecules responsible for the toxic side-effects associated with LPS [76]. A summary of immune modulation by vaccine adjuvants is listed in Table 2.

Cytokines, when included in a vaccine mixture, can enhance the immune response and/or induce immune deviation. In theory, the inclusion of recombinant cytokines can enhance the activation of the APC and also selectively direct the immune response. Delivery of IL-6 or IL-12 along with antigen induces elevated serum antibody titers of both IgG1 and IgG2a isotypes, including increased production of mucosally secreted IgA [35,77]. Inclusion of a plasmid encoding IL-2 in intranasal vaccines shifted the immune response to tetanus toxoid (TT) and cholera toxin (both dominant Th2-type antigens) to a Th1-type immune response [35]. The antibody response to antigen delivered by osmotic pump was greatly enhanced by the inclusion of IL-1β with the antigen [78]. The immune response to intramuscular plasmid DNA vaccination is enhanced by the inclusion of the gene sequence for GM-CSF [29]. Inclusion of exogenous cytokines in a vaccine mixture acts directly on the APC or T cell providing the secondary signal needed to induce immune activation. Many of these properties have led to inclusion of cytokine adjuvants in experimental vaccines that are currently in phase I and phase II clinical trials (Table 3)

Table 2: Antibody isotype bias induced in laboratory animals by administration of immunogens in various adjuvants.

Adjuvant	Example	Basic Characteristics	Dominant Antibody Isotype	Reference
Inorganic Salts	Aluminum Hydroxide, Aluminum Phosphate, and Calcium Phosphate	hydrogel emulsion- creates depot effect, enhance macrophages maturation	IgG1, IgE	36,96,97,99
Oil Emulsions	Mineral Oil (i.e., Freunds incomplete)	basic water-in-oil emulsion with long standing record as research gold standard.	IgG1	36,63,108
	MF59	Blend of muramyl tripeptide, squalene, polyoxyethylene, sorbitan monooelate, and sorbitan trioleate	IgG2a	36,74
	QS-21	purified saponin from *Quillaja saponica*, used to stabilized lipid emulsions	IgG2a	35,110
	Montamide ISA-51 & ISA-720	'ready to use' oil for water-in-oil emulsion	IgG1	62,66,202
Isocoms	ISCOMATRIX	complex of saponins and lipids	IgG1	66,110
Microbial Derived	Monophosphoryl Lipid A (MPLA)	detoxified TLR-4 ligand	IgG1 & IgG2a/c	75,76,107
	Macrophage Activating Protien-2	TLR-2 ligand from *Mycoplasma* spp, and purified derivatives.	IgG2a	123,124

Table 2: Antibody isotype bias induced in laboratory animals by administration of immunogens in various adjuvants.

	Virosomes	stabilized lipid complexes containing viral proteins such as influenza hemaglutinin	IgG2a, IgA	36,103,116
	LT/CT	modified bacterial toxins for mucosal adherence heat-liable enterotoxin and cholera toxin	LT: IgG1, IgG2a, & IgA CT: IgG1	36,141
	CpG	non-methylated bacterial DNA, a TLR-9 ligand	IgG2a	36,64,129
Cytokines as adjuvants	IL-1	pro-inflammatory cytokine	IgG2a, IgA	35,66
	IL-2	lymphoproliverative cytokine	IgG2a	35
	IL-12	pro-inflammatory cytokine	IgG2a, IgA	144
	IL-6	anti-inflammatory cytokine	IgG1, IgA	145
Natural Polymers	Polysaccharides	coating or emulsified with solid antigen	IgG1 or IgG2a depending on route	36
Synthetic polymers	Polyanhydrides	antigen and immunostimulators emulsified into biodegradable particles ranging from 50 μm to 20nm	variable depending on inclusion of immune-stimulants and polymer chemistry	36,148,153
	Polyesters	antigen and immunostimulators emulsified into biodegradable particles ranging from 50 μm to 20nm	variable depending on immune-stimulants and antigen incorporated	36,68,189,197

Table 3: Adjuvants currently being tested in U.S. human clinical trials.*

Adjuvant	Phase	Disease
Aluminum Hydroxide	I	Influenza, SARS
	III	Leishmania
Alhydrogel	II	Anthrax, Plague, Leishmania
Montanide ISA	I/II	Melanoma, solid tumors, Malaria
QS21	I	Cancer: breast, prostate, lung, HIV
	II	Melanoma (skin)
MF59	I/II	Influenza A (H9N2), Bird flu (H5N1), HIV
ISCOMATRIX	II	Melanoma
MLPA, MPL & other TLR-4 ligands	I	HIV, Visceral Leishmaniasis
	II	Allergy (Tree Pollen)
MDP & other TLR-2 ligands	I	HIV
AS15-SB, (liposomes with MPLA and QS21)	III	Lung Cancer
AS02A, AS01B	I/II	Malaria, HIV, Melanoma
CpG (TLR-9 ligand)	I	Malaria, Cancer: breast, melanoma
	II	Allergy: Ragweed
Imiquimod (TLR-7 ligand)	I/II	Influenza, Melanoma
Heat Liable Toxin (LTK63 & LT-R192G)	I	HIV, Tuberculosis, *E. coli* (ETEC)
Diphtheria Toxin	II	Hepatitis B
IMP321 (Th1 activating peptide)	I	Hepatitis B
IL-12	I	HIV, Leishmania, Melanoma
	II	Prostate Cancer
IL-15	I	HIV
IL-2	I	HIV, Melanoma
GM-CSF	I /II	HIV, Cancer: melanoma, lung, ovarian, B cell lymphoma, Hepatitis B
Type I interferon	I	Influenza

Table 3: Adjuvants currently being tested in U.S. human clinical trials.*

Virus carrier: fowlpox, vaccina virus, canarypox	I/II	Cancer: solid tumors, breast, prostate
Bacterial carrier: *Salmonella typhi* CVD	I	HIV
PLG microparticles	I	HIV

* Data from http:clinicaltrials.gov.

Immune modulation can be influenced by other characteristics of the adjuvant/delivery system [79]. As mentioned above, an immune response has been historically categorized as either Th1- or Th2-like. With the discovery of Th-17 cells and the increasing role of antigen derived Treg in controlling disease; the Th1-Th2 paradigm may need expansion. Being said, the Th1-Th2 paradigm provides a model and reference of understanding of pathogenesis of disease. Many different factors can contribute to Th1-Th2 bias of the immune response including route of antigen delivery (intramuscular, subcutaneous, intranasal, oral), antigen dose, duration of antigen presentation, number or frequency of immunizations and inclusion of co-stimulatory molecules (e.g. LPS, exogenous cytokines) with the antigen [80]. Adjuvants can affect all of these factors in different ways, and hence the role of the vaccinologist is to use the correct adjuvant to induce a protective immune response [80]. In the mouse model of leishmaniasis, induction of Th2-biased immune responses by vaccination does not protect the mouse from infection nor does the mouse clear the parasite (i.e., cutaneous lesions develop and persist). On the other hand, induction of Th1-biased immunity was shown to prevent subsequent infection and lesion development illustrating that the Th1-Th2 bias of the immune response is important in the ability to induce active protection [39-41]. Furthermore, in examining the efficacy of BCG vaccination on the clinical outcome of tuberculosis, pre-existing immune responses (usually Th2 dominant) need to be overcome and appropriately redirected in order for vaccines to be efficacious [42,43]. Current vaccines against feline infectious peritonitis viruses enhances humoral immunity which actually

exacerbates the disease, whereas a CMI would be protective [44]. In laboratory animals, Ova-peptide (derived from hen egg ovalbumin, Ova) delivered in alum did not induce a T cell response that could be restimulated in vitro [81]. Delivery of the same peptide within PLGA microspheres induced a significant in vitro proliferative response and production of IFN-γ when lymphocytes were restimulated in vitro with Ova [81]. Cunningham et al., showed that they could alter the Th1-Th2 bias of the immune response to FliC flagellar antigen of *Salmonella* by changing the antigen delivery system [82]. Antigen delivered in native state, on the surface of whole bacteria, induced predominantly IgG2a antibodies (Th1 response) whereas recombinant soluble or polymerized FliC induced primarily IgG1 and Th2 cytokines (IL-4) [82].

Th1-Th2 immune modulation

Induction of the appropriate type of immune response is essential for development of protective immunity. Once naïve T cells have been primed and a Th1 or Th2 type of immune response has been initiated, further immunizations to that antigen using different adjuvants cannot shift the initial immune bias [83,84]. New or novel antigens are not affected by this previous vaccine induced bias [83]. However, it is believed that repeated immunizations that favor a Th2 immune bias create a situation of immunological memory that affects the ability of the immune system as a whole to initiate Th1 immune responses to subsequently encountered immunogens[85].

Table 2 summarizes the dominant antibody isotypes induced by some adjuvants, a reflection of Th1-Th2 biasing of an adjuvant. As illustrated by these examples, the form (e.g. particulate or soluble) of the antigen, delivery system, and route of delivery can all affect the Th1-Th2 bias of a subsequent immune response to a vaccine, and the type of immune response (cell-mediated or humoral) that will be protective varies with the disease in question. Antigen, adjuvants, and delivery systems need to be chosen with care to obtain the most protective response. Current licensed vaccines for the most part are lacking in their ability to induce Th1 type immune responses without also generating undesirable toxic side-effects such as the severe inflammation associated with whole-cell pertussis vaccines [36]. While traditional alum-based vaccines initiate the Th2 response

[65,86], a Th1 response may be more effective for preventing some diseases [87]. Alum is still widely used in veterinary vaccines, but is frequently associated with granulomas in tissues and subsequent carcass losses [88]. Oil-based liposomes are capable of inducing a strong Th1 response, but are also associated with adverse tissue reactivity, granuloma formation, and subsequent carcass loss [89,90].

In the United States, the only adjuvant currently approved for use in humans is alum. However, in England and other European Union countries, MF59 is also used. MF59 is based on a biodegradable plant oil emulsion containing muramyl tripeptide [91]. Highly purified muramyl tripeptide (MTP) is a synthetic component similar to that found in mycobacterial cell walls and MTP retains immunostimulatory properties while eliminating much the toxic effects associated with the whole bacterium [74]. MF59 is used in the H5N1 bird flu vaccine developed by Novartis. MF59 was chosen for dose-sparing effects and is recommended in elderly (65 and older) including those with underlying chronic conditions such as diabetes [92,93].

Vaccine adjuvants straddle a fine line between tissue toxicity and efficacy. Multiple studies in livestock species have shown that greater immunogenicity is achieved when adjuvants causing severe tissue reactivity were used. Greater antibody titers were observed in swine vaccinated with bacterins prepared with a paraffin oil or lecithin (>20%) adjuvant; however, these adjuvants are highly irritating leading to severe diffuse granulomatous tissue at the injection site with multiple foci of necrosis [94]. While adjuvants containing lower amounts (5-10%) lecithin-based oil or aluminum hydroxide ($Al(OH)_3$) induce less tissue irritation, the corresponding antibody titers were also much lower [94]. Vaccine adjuvants for veterinary medicine have many of the same concerns as adjuvants used for human medicine. Tissue irritation, granuloma formation and abscess formation at the injection site are undesirable from an animal welfare viewpoint, but also can be costly to the producer due to carcass losses at time of slaughter [94].

Another consideration for the development of new adjuvants is for the induction of mucosal immunity. With few exceptions (*C. tetani*, rabies virus, and other insect vector borne pathogens), most pathogens enter the host via the mucosal surfaces (e.g., upper respiratory, gastrointestinal, vaginal, or urinary tracts). Induction of mucosal

antibody (i.e., secretory IgA) by appropriate delivery of the antigen to the mucosal associated lymphoid tissue (MALT) is the most effective way to neutralize these pathogens or their secreted toxins [72].

Alum Adjuvants

Salts of aluminum hydroxide or aluminum phosphate, commonly referred to as alum, have long been used in vaccines and have an extensive safety record. Alum was first used as an adjuvant in 1926 [95]. Until recently, it was the only adjuvant approved for use in humans [95,96]. Gels of aluminum phosphate are commercially available for clinical use and generate consistent, predictable results [97]. Alum-based vaccines are prepared by suspending the antigen in a phosphate buffered solution and allowing the antigen to adsorb to the aluminum hydrogel [97]. The amount of antigen that adsorbs onto alum depends upon the forces within the antigen, and between the antigen and the alum, including hydrophobic interactions, van der Waal forces, ionic charges, and hydrogen bonding. The typical quantity of alum in a human vaccine dose is 0.5 mg, the upper allowable limit by the U. S Food and Drug Administration (FDA) and WHO is 1.25 mg per injection [97]. Alum has proven safe for routine use in children, and enhances the production of antibody to protein toxoids and polysaccharide vaccines [97]. Alum has a synergistic effect when combined with other adjuvants and can enhance the adjuvant properties of liposomes, QS-21, MPLA, and CpG [97]. However, alum is not ideal for small peptide vaccines or for use with recombinant proteins due to their inherent low immunogenicity [35,80,98].

Recently, the use of alum in vaccines has come under scrutiny. Alum has been occasionally associated with severe tissue reactions such as erythema, subcutaneous nodules, granulomas, and has been thought to induce hypersensitivity and macrophagic myofasciitis [96,99,100]. It is well established that alum-based vaccines induce IgE and IL-4, which are associated with allergy and type IV immediate hypersensitivity [96]. While alum is effective at inducing strong humoral immunity, alum-based vaccines generally fail to induce cell-mediated immune responses, such as cytotoxic T cells or delayed type hypersensitivity [36]. Alum enhances a strongly biased Th2 immune response in animal

models [99]. Alum-based vaccines have other drawbacks besides the immune bias. Alum, because it is a semi-particulate hydrogel, cannot be lyophilized or frozen [101], thus limiting shelf life and storage conditions. Because the mode of action of alum includes the formation of antigenic deposits at the site of injection, alum is not suitable for oral or intranasal immunization [35,80,102]. Finally, alum proved to be ineffective when used in conjunction with DNA-based vaccines [103].

The mechanism of adjuvanticity for alum has been traditionally thought of as providing an antigenic depot in the tissue. The evidence of the depot effect, or delayed antigen release, of alum adjuvants was established by White in 1967 and Harris in 1935, by inducing immunity in a second animal by implanting granulomatous tissue that had developed as a result of immunizing the donor animal with an alum-based vaccine [97]. Alum particles have been observed at the site of injection up to a year after immunization [97]. Alum-precipitated antigens are somewhat particulate, and therefore, more readily ingested by phagocytes [104]. Macrophages recovered from muscle tissue following injection of an alum-based vaccine and macrophages cultured in vitro in the presence of alum show persistence of crystalline inclusions [99]. Alhydrogel and Adju-phos, commercially available prepared alum gels, produce particles roughly 3 to 4.5 μm in size [97]. Excess alum in a vaccine mixture enhances the adjuvant effect, however alum is slightly cytotoxic to macrophages [97]. Recent studies with cultured macrophages showed that aluminum hydroxide induces a distinct maturation pattern characterized by the expression of surface markers that resemble those found on mature myeloid DCs (HLA-DR^{high}/$CD86^{high}$/$CD83^{+}$/$CD1a^{-}$/$CD14^{-}$) endowing them with the ability to enhance activation of $CD4^{+}$ T cells [99]. Other recent studies have shown that alum may facilitate this DC maturation by inducing the release of uric acid crystals[105]. Uric acid crystals are an endogenous ligand for TLR-2[43,106]. Further evidence for TLR activation was shown by a diminished response to antigens in alum injected into MyD88-deficient mice [105].

Adjuvant Activity of Calcium Phosphate

Calcium phosphate has been used for many years as the adjuvant in childhood DTP (diphtheria-tetanus-pertussis) vaccine formulations in France [96,97]. Furthermore,

calcium phosphate is a normal body constituent and is readily absorbed [96,97]. In contrast to aluminum phosphate, calcium phosphate does not induce IgE production in animals or humans [96,97]. Because of this property, the most common use of calcium phosphate is the delivery of allergens in desensitization therapy for allergic patients [96,97]. In laboratory animals (e.g., mice and guinea pigs), calcium phosphate elicits a lower antibody response than alum-based preparations, however, the opposite is true in humans [96]. Using calcium phosphate-based vaccine, children and pregnant women developed higher neutralizing antibodies than those receiving an aluminum phosphate-based vaccine [97]. The mode of action is thought to be the same as for alum compounds, functioning to create a depot for the immunogen and facilitating the uptake of the particulate antigen by APCs [97].

Freund's Complete Adjuvant and Freund's Incomplete Adjuvant

Freund's complete and incomplete adjuvants (CFA and IFA, respectively) are the standard classical adjuvants to which all other adjuvants are compared [80]. This very potent adjuvant system is comprised of a water-in-mineral oil emulsion with the emulsifier mannide monooleate [107]. Freund's complete adjuvant also contains heat-killed *Mycobacterium tuberculosis* whereas IFA contains only the mineral oil emulsion and emulsifier [29,63,107]. Classically, proteinaceous antigens administered in CFA induce a very strong immune response, including cell-mediated responses, whereas immunogenic proteins administered intraperitoneally in IFA was thought to induce tolerance [83,108]. Advances in both knowledge of the immune system (induction of tolerance and Th2 responses) and methodology in measuring immune responses have shown that administration of antigens in IFA actually induces a Th2 response. This response is characterized by the induction of memory T cells that home on to the spleen, rather than the draining lymph nodes [108]. In addition, the cytokine response produced by these cells is small in quantity (as compared to Th1 cytokines in a lymph node) and may be below the limits of detection [83,109]. The presence of the mycobacterial products in CFA provide a potent danger signal and induces co-stimulatory signals necessary for induction of Th1-type cytokines. Thus, the resultant immune responses induced by CFA and IFA provide the basis for the differential Th1-Th2 skewing of the immune response (i.e., immune

deviation) observed when these two similar adjuvants are employed in a vaccine [108]. Complete Freund's adjuvant is capable of inducing high antibody titers and long lasting T cell responses, but is so reactogenic that its use even in laboratory animals is discouraged [107]. The immune enhancing mechanisms of these adjuvants, the delayed release of antigen, slower antigen clearance, and targeting of the antigen to APCs is due to the mineral oil emulsion [29]. Variations on mineral oil emulsion vaccine adjuvants are marketed by Chiron and Norvartis as Montamide ISA-51 and ISA-720 [92].

MF59 Oil-emulsion Adjuvants

Introduced in Europe in 1997, MF59 is an oil-in-water microemulsion that includes squalene (derived from biodegradable plant oil), Polysorbate 80, and Span 85 (stabilizers) and small amount of muramyl tripeptide, a novel synthetic component derived from mycobacterial cell walls [63,74]. In clinical trials, the muramyl tripeptides proved to be still too toxic and are excluded from current formulations [63,70,74]. MF59 has been shown to stimulate a strong Th2 biased immune response to a large number of antigens and may be more suitable for subunit vaccines than alum [36]. MF59-based vaccines that have incorporated recombinant antigens induce high titer antibody responses and T cell proliferative responses [74]. Combination of MF59 with influenza subunits enhanced the immune response of elderly patients over that obtained using other adjuvants and is being evaluated for use in children [36]. MF59 does not induce Th1-type immunity (e.g., IFN-γ) and, therefore, may not be suitable for vaccines where cell-mediated immunity is needed for protection [74]. The mechanism of adjuvanticity for MF59 appears to be in directing delivery of the immunogen to APCs [74]. Studies with MF59 have shown that macrophages, but not DCs, are the main cell type involved in clearing the oil depot from tissue, and DCs are the key APCs within the T cell zones of the lymph node [36]. It was proposed that following uptake, adjuvant-induced cell death allowed for the transfer of the antigen from the macrophage to the DC for T cell induction (i.e., cross-presentation) [36]. Another observation that arose during the development of MF59 is that there is a difference in emulsion particle size and the resulting immune response in different animal species. Small laboratory animals (mice, guinea pigs and rabbits)

develop high antibody titers following immunization with oil emulsion formulas regardless of particle size. However, non-human primates (baboons, chimpanzees) and goats require stable, small droplet emulsions for optimal antibody induction [70]. The key lesson here is that not all animal species respond equally to an adjuvant and testing in both large and small animals may be necessary to ensure activity of a novel adjuvant. Mineral oil emulsions of various compositions are widely used in veterinary adjuvants, and as their safety record is improved, they are also being developed for human use [61,62,65].

Immunostimulating Complexes (ISCOMs)

ISCOMs were first described in 1984 by Morein et al. [110]. Cholesterol mixed with plant-derived saponins under controlled conditions creates 40 nm cage-like particles referred to as immunostimulating complexes. These synthetic adjuvants are based on the concept of packaging the antigen into micro/nanoparticles or micelles, where the particle size is a crucial determinant of efficient uptake. Many different plant-derived saponins have been investigated for adjuvant activity including saponins derived from *Buplerum chinense, Glycyrrhia uralensis, Quillaja brasilensis* and *Quillaja saponaria* [110-114]. These heterogeneous compounds stabilize the lipid-cholesterol structure while adding immunostimulatory properties. However, these compounds are also generally hemolytic and their tissue-reactive toxic nature has plagued development. While saponins have been used in veterinary vaccines for many years, a balance between potency and adverse reactions will need to be achieved for widespread acceptance in human vaccines [64,107]. A detoxified saponin derivative, QS-21, has exhibited marked decrease in toxicity while maintaining the strong immunoenhancing properties [35,110] This adjuvant has been shown to induce a strong Th1 immune response (CTL, IL-2, IFN-γ, and IgG2a) because of the lipid-cholesterol makeup. Like virosomes, ISCOMs have the ability to fuse with cellular membranes and to deliver the immunogen into the cytosol of the target cell. This results in the endogenous processing and presentation of the immunogenic peptide via MHC I [35,64,110]. This property also makes ISCOMs good vehicles for intracellular delivery of DNA-based vaccines [110]. To increase antigenic loading of ISCOMs, affinity tags or

aliphatic regions can be incorporated into recombinant proteins for higher efficiencies of incorporation into ISCOM membranes; alternatively, chelating agents (e.g., Cu^{2+}) can be used to increase antigen binding [107].

Virosomes and Virus-Like Particles

Virosomes are particles of stabilized membrane lipids and functional viral fusion proteins that can be used to deliver vaccine antigens [103,115]. While theoretically a wide number of virus fusion proteins could be used, the majority of virosomes utilize the hemaglutinin (HA) and neuraminidase (NA) from influenza virus [103]. Virus-like particles are the spontaneous assembly of viral coat proteins lacking in viral genetic material [116]. Virosomes and virus-like particles can be generated by either inserting the viral fusion proteins and antigen into pre-formed small phospholipid vesicles (liposomes) or by separation and reconstitution of viral envelopes with the vaccine antigen [103]. These particles retain the receptor binding capacity and mimic infectivity of native viruses without the risks associated with attenuated viruses and are capable of delivering vaccine antigens directly into the cytosol of the target cell [103]. This allows for induction of both humoral and cell mediated immunity because some of the virosome-delivered antigens have the potential to be presented via MHC II following endosomal processing, and virosomes that escape into the cytosol will allow for antigenic presentation via the MHC I pathway [103]. This type of delivery system has been shown to greatly enhance production of serum IgG and IgA at mucosal surfaces [35]. A synergistic effect is observed when other adjuvants or immunomodulators are included, such as heat-labile toxin of *E. coli* [35]. Virosomes and other virus-like particles are proving efficient for delivery of many types of proteinaceous antigens (i.e., viral coat proteins) or DNA-based antigens intranasally directly to the mucosal surface [35].

Lipopolysaccharide (LPS)

Many antigenic preparations, particularly recombinantly derived antigens, contain residual amounts of bacterial LPS and other TLR ligands that may provide adjuvant activity [117]. LPS is known to stimulate a variety of cells to produce cytokines and

chemokines that control DC movement and maturation [118]. An unusual feature of its adjuvanticity is that LPS can be delivered at a different site and a different time than the antigen and still enhance the immune response to the given antigen. But despite its potency, LPS has been used only as an experimental adjuvant due to its toxicity and pyrogenicity in humans. Chemically modified forms of its active component such as monophosphoryl lipid A (MPLA), have been shown to possess many of the adjuvant properties of LPS but without the associated toxicity.

Monophosphoryl Lipid A

Gram-negative bacterial extracts have strong immuno-potentiating effects, however are too toxic for routine use in human vaccines. Most of the immunostimulatory or toxic effects are derived from the lipid A portion of LPS, which is located in the outer-membrane of gram-negative bacteria [107]. Further analysis showed that by removing a phosphate group, sugar moiety, and the ester-linked fatty acid group the toxicity could be reduced 100 to 1000 fold, while still retaining the immunostimulatory function [107]. MPLA, the resulting molecule, was derived from *Salmonella minnesota* [35]. Similar to LPS, MPLA interacts with TLR-4 on APCs, although immune enhancement is observed in the absence of TLR-4 [75,76]. MPLA initiates signaling through TRIF transcriptional activation rather than NFκB, which induces many pro-inflammatory cytokines associated with the toxic effects of LPS [76]. Equivalent T-cell mediated responses were observed in mice immunized with Ova adjuvanted with LPS or MPLA indicating that the mechanism of TLR-4 signaling (TRIF vs. NFκB), and not the magnitude of the response, was responsible for the reduction in toxicity [76]. Binding of MPLA to TLR-4 initiates the synthesis of IL-1β, IL-12, and IFN-γ, all of which are necessary for DC maturation, migration and initiation of the T cell response [35,119]. In animal studies, MPLA induced a strong systemic Th1 type immune response, including cytotoxic T lymphocytes (CTL) [35]. Furthermore, MPLA was shown to enhance the production of complement fixing antibodies and increased production of secretory IgA [35]. While MPLA enhanced the resulting immune response to a given antigen in comparison to the immune response to the soluble antigen alone, MPLA is more effective when combined with other adjuvants

or delivery systems such as Alum, QS-21 (Quil A) and polymeric microspheres, or other adjuvants that provide a depot effect [35]. Several vaccine formulations using MPLA as an adjuvant are in clinical trials for humans and livestock species [61,65].

TLR2 Ligands

Since the discovery of TLRs as a key sensing and signaling mechanism for APCs, efforts have been made to exploit TLRs as receptors for vaccine adjuvants[120,121]. Many different derivatives of gram positive cell wall components have all been found to trigger immune activation through TLR2. OspA of *Borrelia burgdorferi* was used in the vaccine against Lyme disease[122]. Muramyl-dipeptide (MDP) has been synthesized from several gram positive bacteria including several *Mycobacterium* species, *Corynebacterium granulosum*, and *Bordetella pertussis*. MDP derivatives have been shown to induce dichotomous effects on the immune system. When delivered in soluble delivery systems, MDP enhances humoral immunity; when delivered in liposomes, MDP enhances CMI[116]. Addition of MDP to a vaccine formulation acts synergistically with mineral oil and alum carriers, enhancing the CMI response[107]. Macrophage activating lipopeptide-2 (MALP-2) is another TLR2 targeted ligand showing promise as a vaccine adjuvant. MALP-2 is an agonist of the TLR2-TLR6 heterodimer from *Mycoplasma fermentans* and has been shown to activate APCs via MyD88 signaling and activation of NFκB transcription factor[123]. TLR2 and TLR6 are also present on B cells[123]. Studies in mice lacking either B or T cells showed that MALP-2 activated B cells in a T cell-independent manner but enhanced T cell function via a B-cell dependent mechanism[123]. Pam2Cys is a synthetic compound with structural similarity to MALP-2 and has been shown to enhance the CMI and humoral response in an experimental vaccine for *Listeria monocytogenes* and an intranasal administration of an influenza vaccine in mice[124]. ESAT-6, a protein derived from the cell wall of *M. tuberculosis*, can also be synthetically produced[125,126]. ESAT-6 can act both as a protective antigen against tuberculosis or can non-specifically enhance CMI to co-administered antigens[52,125-127].

CpG Adjuvants

Prokaryotic DNA contains unmethylated CpG dinucleotides within specific nucleic acid motifs that are recognized by the innate immune system of vertebrates [128]. These immunostimulatory motifs are the ligand for TLR-9 which is found primarily in intracellular vesicles of phagocytic cells [128]. Signaling through TLR9 CpG-ligands induce the production of reactive oxygen species and activation of NFκB [129]. These immunostimulatory sequences are species specific and unique sequences have been described for laboratory animals (mice, rats and rabbits), humans, and non-human primates, as well as companion and farm animals [65]. For humans, there have been two types of CpG motifs described, type K (also known as B-type) and type D (or A-type) [128]. The type K CpG motifs primarily stimulate B cell and monocyte proliferation, IgM, IL-10, and IL-6 secretion. Type D CpG motifs primarily activate DCs, a response which is characterized by upregulation of CD80, CD86, MHC II, and TNF-α and IL-8 secretion [128]. Regardless, CpG motifs are capable of stimulating enhanced secretion of immunoglobulins, and may be capable of modulating pre-existing immune responses [64,129]. Addition of CpG to vaccine formulations has been shown to induce both cellular and humoral response to immunogens, inducing a Th1 bias. CpG has been shown to induce demonstrable immune responses to weak immunogens such as malarial antigens, anti-*Haemophilus influenzae* glycoconjugates and melanoma antigens [129]. When both alum and CpG motifs were included in vaccine formulations, the resulting immune response was Th1-biased, with no IgE production or eosinphilia [84]. Furthermore, addition of CpG motifs to intranasal vaccine formulations enhanced the total serum titer to TT and influenza (viral) antigens in mice indicating that they may be useful as immune enhancers for mucosal delivery of antigens [130]. CpG motifs are also used to enhance the response to antigens encapsulated in biodegradable polymeric microspheres described in this review [129,131,132]. CpGs have been included in many experimental vaccines demonstrating enhanced protection against a variety of pathogens including Ebola virus, *Bacillus anthracis*, *Francisella tularensis*, *Listeria monocytogenes*, and *Cryptococcus neoformans* and in models of polymicrobial intra-abdominal sepsis [122,133-136].

Bacterial Toxins

Bacterial toxins have a high degree of immunogenicity and immune enhancing capabilities along with a high degree of cellular receptor specificity. These properties have led researchers to study the potential of bacterial toxins as vaccine adjuvants. Pertussigen, a complex mixture derived from *B. pertussis*, including pertussis toxin, has been used experimentally as an adjuvant [137]. Pertussigen enhances levels of IgE and hypersensitivity reactions to co-delivered antigens and may help adjuvant the response to TT and diphtheria toxoid which are part of the trivalent childhood DPT vaccine [6,137]. Heat-labile enterotoxin (LT) from *E. coli* has also been shown to enhance mucosal immunity to co-administered antigens [35]. LT exhibits adjuvant efficacy for induction of mucosal and parenteral immunity in mice. LT was also used as an oral adjuvant for *Campylobacter* killed whole-cell vaccines. In rhesus monkeys, LT was shown to be safe and provided superior performance over the *Campylobacter* killed whole-cell vaccines alone [138]. Cholera enterotoxin (CT) is another bacterially derived protein that shows high immunogenic potential when delivered to mucosal surfaces [139,140]. LT is highly homologous to CT, but CT stimulates predominantly Th2 responses to conjugated antigens while LT stimulates mixed Th1-Th2 response [141]. However, cholera-like toxin adjuvants delivered by the nasal route have been found to be taken up by the olfactory nerve and the central nervous system, leading to potential unwanted side effects [142] and CT can induce diarrhea in humans. Not much is known about the cell-mediated immunity or delayed hypersensitivity response to CT. The ability of CT to act as a mucosal adjuvant has been confirmed by many investigators with a variety of antigens, and administering CT by a route different from the antigen is not immunoenhancing [139,140].

Cytokines

The cytokine network controlling immunity and T cell development is complex and much research remains to be done to elucidate these pathways [143]. The effect of a few cytokines and their relevance to immune activation has been well studied and these cytokines have been explored as adjuvants to provide potentially less toxic approaches to enhancing vaccine efficacy. For example, granulocyte macrophage-colony stimulating

factor (GM-CSF) has been included in experimental vaccines due to its ability to enhance APC recruitment and activation [116]. In attempts to improve the pneumococcal polysaccharide vaccine against *Streptococcus pneumoniae*, IL-12 was included as a mucosal adjuvant [144]. The inclusion of IL-12 enhanced mucosal and systemic IgG2a and IgA following intranasal vaccination and showed a marked reduction in bacterial nasal carriage and prevention of bacterial systemic invasion [144]. Inflammatory cytokines in the IL-1 family have been shown to enhance the production of serum and mucosal IgG and IgA antibodies and cell-mediated responses to co-delivered Ova and tetanus toxoid [35]. The choice of cytokine included in a vaccine formulation must be chosen with care. In a recent study, polylactide microspheres were investigated as intranasal delivery of recombinant V antigen (rV) of *Yersinia pestis* co-encapsulated with IL-6, IFN-γ, or IL-4 [145]. While all formulations induced mucosal IgG1 and IgA antibodies, only formulations including IL-6 with the rV induced protection from systemic bacterial challenge [145]. The challenge of cytokine delivery is the rapid utilization of cytokines and their pluripotent biological effects. One mechanism to reduce these effects is to deliver a plasmid including the sequence of the cytokine [66,116]. With DNA based vaccine technologies, this has proven very effective for enhancing the response to the DNA-based antigen. Inclusion of the sequence for IL-2 or IL-12 with the sequence with HIV antigen enhanced the production of a strong Th1 immune response [35].

Polymer Vaccines

Biodegradable polymers have been studied for many years because they show promise for the development of single dose vaccines [146,147]. Polymeric compounds have the ability to sustain the release of the vaccine antigen by a controlled mechanism over an extended period of time, thus eliminating the need of subsequent doses of vaccines. Other potential advantages of these materials are that immunomodulatory properties (i.e. adjuvanticity) can also be achieved with the proper tailoring of the polymer chemistry [148]. Studies evaluating the use of controlled-release, single dose polymeric vaccines in both laboratory animals and livestock species (i.e., sheep, mini-pigs, cattle, and horses) have shown promise when encapsulating protein antigens [149-153].

Biodegradable polymers also offer the advantage that MPLA, CpG DNA motifs or other immunoenhancing molecules can be incorporated to create a pathogen-mimicking solid particle [154]. Polymeric vaccine particles have been shown to induce demonstrable immune responses when administered by several routes including, parenteral (e.g. intramuscularly or subcutaneously), intranasal, or orally [35].

These materials also have the added advantage over stable (non-degradable) devices (e.g. pumps) in that after administration, there is no need to remove them, therefore eliminating another surgical procedure. Furthermore, most are manufactured from synthetic parent compounds, eliminating many potential reactive antigenic or allergenic epitopes that can accompany the use of animal or plant derived materials.

The two most widely studied polymer classes for controlled release vaccines are polyesters[155-164] and polyanhydrides [87,165-176]. Other classes of polymeric compounds have been evaluated and shown to successfully deliver antigen to laboratory animals [177-190]. Key findings of research done with these polymeric systems as vaccines carriers are discussed below and some of the chemistries are shown in Table 4.

Table 4: Structure of biodegradable polymers studied for use as vaccine adjuvants

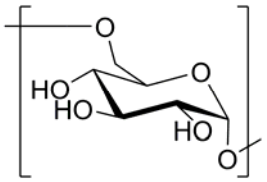
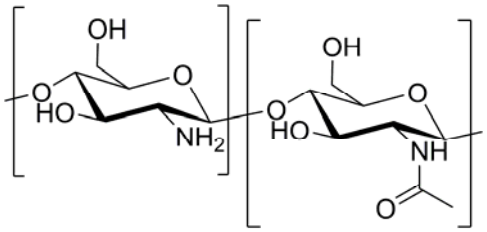
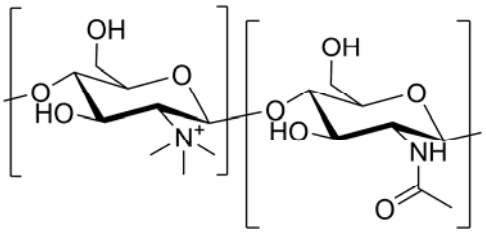
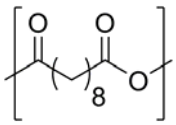
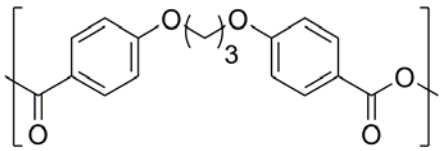
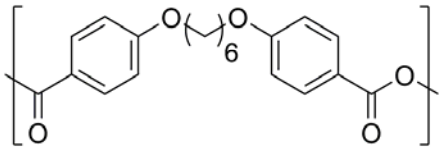
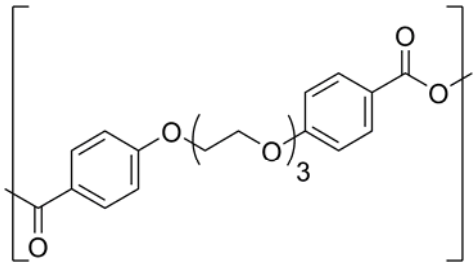
Polymer	Structure	Reference
Polysaccharides		
Dextran		229
Chitosan		230 35
N-trimethyl chitosan		230
Polyanhydrides		
Poly(sebacic acid) SA		148,214,219,226
1,3-bis(*p*-carboxyphenoxy)propane CPP		174,226
1,6-bis(*p*-carboxyphenoxy)hexane CPH		148,214,219
1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane CPTEG		217,221,222

Table 4: Structure of biodegradable polymers studied for use as vaccine adjuvants

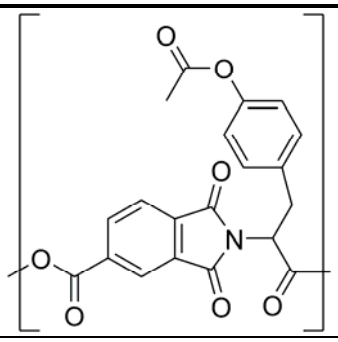
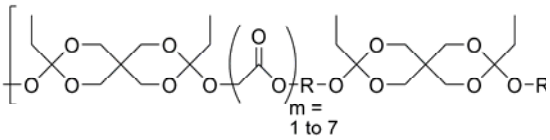
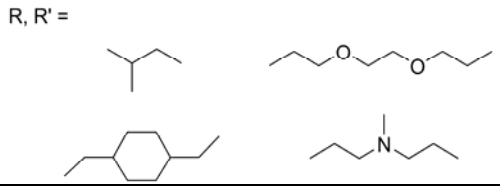
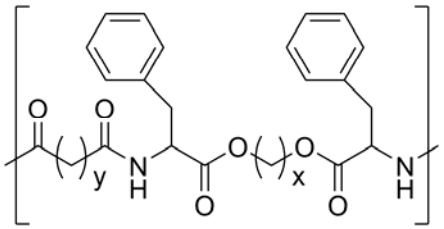
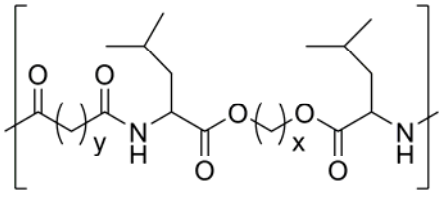
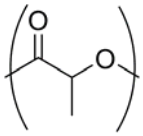
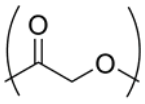
Poly(trimellitylimido-L-tyrosine)		226
Poly(ortho ester)s	 	60
Poly(ester-amide)s Phenylalanine-based PEA		235,236
Leucine-based PEA		235,236
Polyesters Poly(lactic acid) LA		157,159,203
Poly(glycolic acid) GA		157,159,203

Table 4: Structure of biodegradable polymers studied for use as vaccine adjuvants

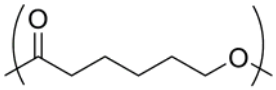
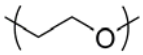
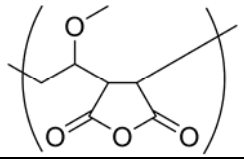
Poly-caprolactone		225
Poloxamers Poly(ethylene glycol) PEG		237,238
Poly(vinyl methyl ether-*alt*-maleic anhydride) PVM/MA		224

Table 5. Advantages and disadvantages of polymers used as vaccine adjuvants.

Polymer	**Antigens**	**Advantages**	**Disadvantages**	**Ref.**
Polysaccharides				
Dextran	*Streptococcus bovis* *Lactobacillus spp* *Mycobacterium* tuberculosis Tetanus toxoid (TT)	Induces strong humoral responses	Not desirable for some diseases (e.g., tuberculosis)	101
Chitosan *N*-trimethyl chitosan	Diphteria toxoid	Enhanced immune response compared to alum Mucoadhesive properties		230 35 230
Polyanhydrides	TT *Salmonella enteriditis* (HE) Plasmid DNA	Biocompatible non-mutagenic, non-cytotoxic degradation products Degradation products have	Processing and storage	214,219,226 13, 90, 217,22

Table 5. Advantages and disadvantages of polymers used as vaccine adjuvants.

		low acidity Enhanced protein stability Immunomodulatory Mucoadhesive properties		1,22
Poly(orthoester)s	Plasmid DNA	Enhanced immune response when compared to naked DNA	Plasmid DNA unsuccessful in human clinical trials	60
Poly(ester-amide)s	Melanoma antigen derived peptides (MART) HIVgp120 MHC II restricted T-cell epitope from influenza A virus hemagglutinin (HA)	Degrades by enzymatic cleavage Enhanced cell-mediated immunity		235
Polyesters	TT Diphtheria toxoid *Yersinia pestis* HIVgp140 *Bordetella pertussis* Measles virus antigen Ovalbumin Type II collagen Malarial antigens Cancer cell antigens *Eschericia coli* Ricin toxoid *Vibrio cholerae* Influenza virus antigens Hepatitis B viral antigens Plasmid DNA	Degradation products are biocompatible and easily metabolizable Antigen-loaded microspheres enhance uptake by APCs Experiments have shown increase in both humoral and cellular immune responses	Acidic microenvironments detrimental to antigens No protective immunity in humans have been reported yet Poor mucoadhesive properties	99,101,101,57,159,161

Table 5. Advantages and disadvantages of polymers used as vaccine adjuvants.

Poly(ethylene glycol) PEG	Ova, Hepatitis B viral antigen, Plasmid DNA	Can target APCs in LNs	237 ,23 8
Poly(vinyl methyl ether-*alt*-maleic anhydride) PVM/MA	*Salmonella enteriditis* (HE)	Th1/Th2 balance Non-specific protection against *Salmonella*	224

Polyesters

Microspheres composed of polyesters have been the most widely studied. Polymers of lactic acid and glycolic acid (e.g. poly(lactide-co-glycolide), PLGA) have been utilized in biomedical applications such as bone pins and dissolvable sutures for many years and recently have proven effective as vaccine delivery vehicles for the induction of protective immunity in laboratory animals [155-159]. The greatest benefits of PLGA in biodegradable materials is that the degradation products, lactic acid and glycolic acid, are naturally occurring metabolites and are readily absorbed by neighboring cells[160,161]. However, as the polyester degrades and the acidic monomers are released, an acidic microenvironment is created. Prolonged exposure to aqueous or acidic environments has been shown to be detrimental to the stability and immunogenicity of proteins, especially the proteins used in recombinant and subunit vaccines, e.g., tetanus toxoid (TT) and diphtheria toxoid [162,163]. Some attempts to minimize this acidity have been recently evaluated by incorporating a basic compound like magnesium carbonate ($MgCO_3$) into PLGA microspheres[164]. However, subsequent analysis indicated that while $MgCO_3$ did not significantly improve peptide stability, it did enhance the antibody production, acting as a potential adjuvant.

Antigen-loaded PLGA microspheres function as an adjuvant by at least two mechanisms: 1) creating a depot for the antigen in vivo, and 2) enhancing phagocytic

uptake of the antigen-loaded particle by APCs[159]. The uptake of PLGA microspheres by macrophages or DCs has been demonstrated following administration by intraperitoneal or intradermal routes, respectively[191]. Other immunostimulatory properties of PLGA were observed in studies showing an enhanced cytokine production and proliferation when cells were incubated in vitro with blank PLGA microspheres[161]. Similarly, oral administration of

PLGA nanoparticles containing type II collagen promoted the induction of tolerogenic immune responses that ameliorated arthritis [192]. The prolonged presence of the nanoparticles in the Peyer's patches and the induction of elevated TGF-β suggested the differential activation of DCs that modulated the subsequent immune response. Vaccine formulations based on PLGA, PLA, or PGA variants have been successful in inducing immune responses in laboratory rodents to a large number of antigens including: *Yersinia pestis* antigens, HIV gp140, *B. pertussis* antigens, measles virus antigen, OVA antigen, TT, diphtheria toxin, type II collagen, malarial antigens, cancer cell antigens, *E. coli* adhesion proteins, *Vibrio cholerae* antigens, influenza virus antigens, hepatitis B viral antigens, and ricin toxoid[35,161,192,193]. These vaccines have been delivered by a variety of routes including intradermally, intravaginally, intranasally, orally, or parenterally into laboratory animals to induce both serum antibodies, mucosal IgA, cell-mediated responses and facilitated the induction of secondary immune responses (e.g., isotype switching) as determined when individuals were analyzed up to a year after single immunization [161,194]. Many groups have reported the successful induction of immunity following use of a single dose vaccine formulation composed of PLGA microspheres of various compositions[157,195-198 199,200]. Furthermore, encapsulation of antigens in PLGA microspheres was shown to enhance antigen presentation via MHC I leading to increased activation of antigen specific cytotoxic T cells [147,193,195]. However, most of these studies were conducted in vitro, and some investigations included MPLA, a known Th1 immune response activator, in the microsphere while others used multiple injection regimens in vivo. There is no consensus opinion, however, as to whether PLGA-based vaccines are more efficacious than current adjuvant systems such as alum. Antibody responses induced in mice and guinea pigs following vaccination with TT-loaded PLGA were

greater than those induced by single injection of soluble TT alone or two doses of alum absorbed TT. Additionally, a stronger anamnestic response (higher titer) was observed when individuals that had received the TT-loaded PLGA microparticles were boosted one year later[194]. On the other hand, Walker et al. observed that encapsulation of TT in PLGA microspheres did not induce serum antibody titers higher than alum-based TT vaccines[199]. Only small amounts of antigenically active TT were released in the first two days from PLGA microspheres, even though protein continued to be released for up to 11 weeks [194]. Collectively, evaluation of PLGA studies does not provide strong correlation between release of antigenic peptides, length of in vitro release of peptides, and immune response to those peptides in vivo.

Some studies have suggested that immunization with PLGA microspheres effects immune deviation. Moore et al. showed the ability of HIV gp120 protein loaded PLGA microspheres to shift the T cell response from a dominant Th2 or mixed Th1/Th2 to a more dominant Th1 immune response as indicated by the presence of IFN-γ producing $CD4^+$ T cells[201]. In other studies, the Th2-biased hepatitis B core antigen has been formulated with the Th1 immune stimulator MPLA in PLGA nanoparticles to develop a stronger Th1 response[68]. More recently, a vaccine formulation prepared against malaria and composed of PLGA microspheres and Montanide ISA 720 was shown to induce an antibody response (IgG isotype class switching) characteristic of Th1 response[202].

Variations in reported efficacy of PLGA microspheres may be due to dose of antigen, method of encapsulation (e.g. spray drying vs. solvent evaporation), route of immunization, and/or the size of the microspheres[193,203]. Following primary immunization with small microspheres (10-20 μm), a greater anamnestic response was generated one year later following a low dose booster than that observed in animals initially receiving larger microspheres (> 60 μm)[194]; however, nanoparticles (200 - 600 nm) were less effective at inducing cell-mediated immune response than microspheres[193]. This may be because microspheres < 10μm in diameter are readily phagocytosed by macrophages and DCs that would enhance antigen processing and presentation[204-209]. On the other hand, the route of immunization with PLGA microparticles influenced the type of immune response generated. The intraperitoneal route induced Th1 cell-mediated

response while the intramuscular route induced a Th2 humoral response[193]. Despite all the extensive research done with PLGA as antigen carriers, some with success in animal models, no formulation has been reported to induce a protective immunity in humans[210].

Polyanhydrides

Polyanhydrides are a class of surface erodible, biocompatible polymers that have been extensively used as carriers for controlled drug delivery[87,165-176]. These biodegradable polymers are currently approved by the FDA for use in a variety of biomedical applications and can also be fabricated into protein-loaded microspheres[211]. Biocompatibility studies have shown that these biomaterials degrade into carboxylic acids, which are non-mutagenic and non-cytotoxic products[212,213]. The surface erosion mechanism leads to a controlled release profile with predictable degradation profiles, which can range from days to months, depending on the co-polymer composition[214,215]. In addition, studies involving polyanhydride delivery systems for vaccines have shown attractive features such as improved adjuvanticity, antigen stabilization, and enhanced immune responses[165,175,176,216].

The main advantage of polyanhydrides over polyesters as antigen carriers is associated with the enhanced protein stability following encapsulation. Studies have shown that polyanhydrides are capable of stabilizing polypeptides and sustaining their release without the inclusion of potentially reactive excipients or stabilizers[217-220]. The hydrophobicity and surface erosion characteristics of polyanhydrides prevent water from penetrating to the interior of the microsphere thus preserving the encapsulated antigen in its native state (i.e., increased stability). Furthermore, the degradation products of polyanhydrides are less acidic than those of polyesters, which may further enhance the stability of encapsulated antigens and reduce tissue reactions to the polymer[217,219]. Despite these beneficial characteristics, the use of polyanhydrides for vaccine delivery has not been extensively evaluated.

Recently, Kipper et al. performed in vivo studies to evaluate the induction of immune responses following immunization with antigen-loaded microspheres based on the anhydride monomers sebacic acid (SA) and 1,6-bis(*p*-carboxyphenoxy)hexane

(CPH)[148]. Microspheres encapsulating TT antigen were injected in C3H/HeOuJ mice. These studies demonstrated that TT maintained its immunogenicity and antigenicity following encapsulation. The type of immune response generated, Th1 vs. Th2, was evaluated by antibody isotypes. It was observed that TT loaded 20:80 CPH:SA microspheres enhanced the immune response after a single dose and indicated a Th2 dominant response. However the 50:50 CPH:SA produced a balanced Th1-Th2 response. Total TT-specific IgG titer remained high regardless of dominant isotype. The preferential enhancement of the Th1 immune response resulting in more balanced immune response (i.e., immune deviation) is a unique and valuable feature of this delivery vehicle that makes it a promising adjuvant candidate for vaccines. Currently, the groups led by Narasimhan and Wannemuehler are corroborating the immunomodulatory properties of the CPH:SA system with other antigens as well as investigating the adjuvant properties of novel amphiphilic polyanhydride chemistries. Copolymers of CPH and 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), which contains ethylene glycol moieties in the polymer backbone, are promising candidates for the development of vaccines as it has been shown to provide a conducive environment for protein stabilization[217,221-223].

Anhydride monomers have been copolymerized with other chemistries and their potential as adjuvants have been evaluated. An immunogenic subcellular extract obtained from *Salmonella enteritidis* cells (HE) has been encapsulated in nanoparticles of the copolymer comprised of methyl vinyl ether and maleic anhydride (PVM/MA), best known as Gantrez® polymer [224]. In this study, 80% of the Gantrez®-HE immunized mice survived even when the nanoparticle formulation was administered 49 days previous the lethal challenge. As early as 10 days after immunization, a Th1 immune response was demonstrable in these mice as determined by the IgG2a antibody titer in the serum. On the other hand, a dominant Th2 immune response was present at 49 days after immunization (IgG1>IgG2a). Since it is known that a Th1/Th2 balance is required to protect against *S. enteretidis* infection, this copolymer is a promising candidate for the development of future vaccines. In this regard, blank nanoparticles of Gantrez® administered subcutaneously four weeks prior to challenge induced a level of protection

similar to that induced by antigen-loaded nanoparticles or the Rv6 commercial available vaccine against *S. entereditis* serovar abortusuis [225]. While the authors did not demonstrate the presence of antigen-specific immunity, this data suggests that the blank nanoparticles were able to induce and sustain sufficient innate immunity to provide non-specific protection against subsequent *Salmonella* infection. In the same study, abortusovis antigen-loaded poly(ε-caprolactone) microparticles did not induce protection.

In another attempt to design suitable carriers specifically intended for vaccine delivery, Hanes et al. synthesized poly(anhydrides-co-imides) with the adjuvant L-tyrosine incorporated in the polymer backbone [226]. In these studies, a predictable and controlled protein release was observed from microspheres of poly[trimellitylimido-L-tyrosine-co-sebacic acid-co-1,3-bis(carboxyphenoxy)propane] and polymeric implants were well tolerated after subcutaneous implantation in rats. More recent studies demonstrating the suitability of polyanhydrides for use in single dose vaccines involved the design of a core-shelled cylindrical device composed of a biodegradable hydrophobic coating and laminated core of polyanhydrides and polyphosphazenes [227]. Polyanhydrides based on SA were used as isolating layers of the cylinder in order to produce a pulsatile drug release, a mechanism which would minimize doses of vaccines. Even though these polyanhydride systems showed promising characteristics for vaccines design, no further in vivo studies evaluating the characteristics of the proposed adjuvant were validated.

A comparative study between polyanhydrides and polyesters has demonstrated the potential capabilities of polyanhydrides for oral vaccination [228]. Microspheres (0.1-10μm) composed of fumaric acid (FA) and SA proved to have strong adhesive interactions with the mucosal gastrointestinal lining of rats, as opposed to poly(lactic acid) (LA), which showed minimal uptake. The adhesive interactions are ideal to prolong the biological activity of the delivered antigen or bioavailability of encapsulated drugs. Not surprisingly, plasmid DNA- and anticoagulant drug dicumarol-encapsulated FA:SA microspheres enhanced gene activity and plasma drug levels, respectively, when compared to the controls. In the same studies, blends of FA and LA were used for insulin delivery and groups that received the formulation were able to regulate glucose levels as opposed to the groups that received insulin only. Even though the biological activity of

insulin was preserved, it was the adhesive characteristic of FA the responsible for the efficient delivery.

Other Polymers

Naturally-derived

Several naturally derived polymeric materials, such as dextran, chitosan, starch, and alginate have been evaluated in laboratory models for use as vaccine adjuvants. In the case of dextran, it has been chemically modified or use in conjunction with other adjuvants in order to improve its immunogenicity. Immunization of cattle with dextran in combination with mineral oil against *Streptococcus bovis* and *Lactobacillus* spp. induced the highest serum IgG responses when compared with other adjuvants (i.e. FCA, Quil A, alum), presumably due to the combined effect of both substances [177]. In studies involving vaccination of cattle against *M. tuberculosis*, diethylaminoethyl (DEAE)-dextran induced high levels of IL-2 and low levels of IFN-γ, indicating a strong humoral response not desirable for this particular disease [178]. Interesting results were obtained when a dietary supplementation of Lactobacillus casei with dextran enhances humoral immune responses, and chickens were able to maintain the growth of the bacteria in their intestines and prevent possible infections [179]. Vaccines that have been evaluated utilizing cross-linked dextran microparticles, containing conjugated TT induced serum antibody to TT for long periods, eliminating the need of additional booster doses [229].

Chitosan, a cationic polysaccharide derived from chitin in the exoskeleton of crustaceans, can also be formulated into microparticles capable of encapsulating antigen [230]. Studies with chitosan showed that the immune bias induced by vaccination with antigen containing chitosan microparticles was more dependent on the route of delivery (e. g., intranasal vs. parenteral) than the nature of this adjuvant [35,180]. An intranasal delivery of N-trimethyl chitosan chloride (TMC) containing diphtheria toxoid enhanced the immune response when compared with the conventional alum adsorbed vaccine [230]. This enhancement of nasal vaccination is likely a result of the mucoadhesive properties of chitosan, which enhance penetration across nasal mucosa [181,182]. More recent studies with chitosan and TMC establish that chemical variables, such as molecular weight in

chitosan and degree of quaternization in TMC influence the magnitude of the immune response after nasal administration [183].

Another natural polymer with potential in vaccines is starch, which also has been assessed in mucosal vaccines. Some advantages of starch include its inert properties, proven safety, and commercial availability [184]. Heritage et al. found that human serum albumin delivered on starch microparticles grafted with polydimethylsiloxane stimulated systemic and mucosal immune responses [185]. Similarly to studies done with chitosan, the route of administration of starch influences the immune response [186]. Among oral, subcutaneous, and intramuscular administrations, the subcutaneous induced stronger humoral responses. However, when comparing oral and intramuscular routes, stronger humoral response was induced after oral primary administration and a stronger cell-mediated response after oral booster doses. Although the adjuvant capabilities of starch were proved with success in mice studies, a human vaccine trial was not successful [231].

Alginate microparticles offer several advantages for vaccine applications, including good biocompatibility, ease of preparation, and antigen protection during fabrication and administration [184,232]. Alginate microparticles have been administered to several animal species (i.e. mice, rabbits, cattle, and chicken) [232]. The enhancement of the immune response induced in the animals after oral administration with antigen-loaded alginate microparticles shows promise for the development of veterinary vaccines. Nevertheless, in vitro studies show that alginate is not the optimum chemistry to activate human-derived DCs, as it decreases the expression of co-stimulatory molecules and antigen presenting complexes when compared to non-treated cells [233]. Other in vitro studies that simulated gastric fluid environment showed that alginate microparticles were not able to stabilize live rotavirus vaccines [234].

Synthetic polymers

Other novel polymer chemistries have been researched to overcome the limitations of available polymers as vaccine carriers. The novel poly(ester-amide) (PEA) copolymers, composed of amino acid residues, diols, and dicarboxylic acids, have been shown to enhance cellular immunity [187]. Polyamide gives PEA its superior mechanical

and thermal properties, while the polyester portion is responsible for its flexibility and hydrolytic susceptibility, allowing PEA to degrade within a reasonable period of time. It is biodegradable, however, in contrast to polyester and polyanhydrides, PEA degrades by enzymatic cleavage within the body [235,236]. Thus, shelf life and handling does not affect its degradation rate and the polymer remains intact until needed for therapy. PEA has been conjugated with several therapeutics peptides, including human melanoma antigen-derived peptides (MART), a synthetic peptide based on the gp120 protein of HIV, and a MHC II-restricted T-cell epitope from the influenza A virus hemaglutinin (HA) protein [187]. In general, the studies evaluating PEA-peptide conjugates demonstrated that cellular immunity, encompassing both MHC I- and MHC II-restricted T-cell responses, was enhanced.

More recently, in vivo studies in mice have shown that poly(ethylene glycol)-stabilized poly(propylene sulfide) nanoparticles target the APCs directly in the lymph nodes [237,238]. In these studies it was found that particles in the size range of 20 to 45 nm enter lymphatic vessels and subsequently target DCs in the lymph nodes. The cross-linked polymer system used here degrades into a water soluble polymer under oxidative conditions.

Polymers in plasmid DNA vaccines

Plasmid DNA vaccines represent a promising alternative against intracellular pathogens. Even though plasmid DNA immunogens have elicited strong cell-mediated responses in small laboratory animals, these have not had success in limited human clinical trials [188]. Ideal adjuvants will improve the magnitude of plasmid DNA expression, must protect DNA from enzymatic degradation, and must facilitate the DNA plasmid uptake into cells. Several polymer chemistries have been evaluated in conjunction with DNA vaccines and a thorough discussion of this topic is beyond the scope of this review. In short, microspheres of polyesters, polycarbonates, polystyrene, and poly(orthoesters) have been used in DNA vaccination and their administration resulted in enhanced immune responses when compared to naked DNA administrations [60,189,190].

The Ideal Vaccine Adjuvant

Vaccines and their adjuvants interact with the patient's immune system in a variety of ways. Thus, there is no single set of characteristics that would describe an ideal vaccine adjuvant for all situations. An adjuvant must be appropriate to the particular delivery route (*e.g.* intramuscular, mucosal, intra-peritoneal, etc.), desired immune response (cell-mediated vs. humoral), pathogen, and stage of a disease. Additionally, biological traits of the patient may also be important including species, race, age, medical history, and genetic makeup. All of these factors may influence the effectiveness of a vaccine adjuvant, and the effects of these factors may be unknown. Nonetheless, there are certain characteristics that a good vaccine adjuvant must possess. These characteristics can be broadly grouped into two categories: biological characteristics and practical or economical characteristics.

Because vaccine adjuvants may enhance the immune response through different modes of action, the particular mechanism of adjuvanticity is of paramount importance. The mechanisms of adjuvant activity have been classified in different ways by different authors [66,119,239]. The broadest classification distinguishes among two types of mechanisms: immune stimulation and targeting antigens to particular cell or tissue types [65]. Adjuvants which act through the later mechanism target vaccines to DC, through interactions with transmembrane TLR proteins or other cell surface receptors [97], or by virtue of their size [240,241]. Polymer microspheres and liposomes < 10 μm in diameter may be readily phagocytosed by macrophages and DCs [97]. This specific targeting can reduce the quantity of antigen required to induce protective immunity. A good immunostimulatory vaccine adjuvant must stimulate the desired immune response without toxicity or inducing excessive inflammation. While some immunostimulatory adjuvants of bacterial origin have potent adjuvanticity (*e.g.* LPS), they can also be extremely toxic (e.g. induction of tumor necrosis factor) [242]. Less toxic adjuvants, such as alum, may also be less potent or ineffective at eliciting cell mediated immunity [97,243,244].

Good immunostimulatory vaccine adjuvants activate DCs to mature into APC and migrate to the draining lymph node, coincident with induction of the cytokine profile

appropriate to the desired immune response mechanism (*i.e.,* IFN-γ, IL-2, and IL-12 for the Th1 response and IL-4, IL-5, and IL-6 for the Th2 response). Like adjuvants that target DCs, some immunostimulatory vaccine adjuvants also interact with TLR proteins. Though these proteins have affinity for a variety of ligands, different subpopulations of DCs express different TLR profiles and, thus, have different degrees of sensitivity to different antigens and adjuvants [31,245,246]. Furthermore, the same TLR may activate different intracellular signaling cascades leading to different activated phenotypes in different DC subpopulations. Regardless of the mechanism of adjuvanticity, vaccine adjuvants must activate this desired adaptive immune response without over stimulating innate immune function.

Economical and practical considerations must also be taken into account when selecting an ideal vaccine adjuvant. Singh and O'Hagan [64,74] list biodegradability, ease of manufacture, and low cost among important characteristics of vaccines. Other practical aspects to be considered include stability over time, ability to provide immunity with a single dose, and suitability for mucosal delivery. Such characteristics would enable more practical and economical strategies to fight infectious disease in remote areas that lack developed public health infrastructure and in communities that do not have access to modern medical care [247]. Finally, while the "depot" effect (long thought to be the primary mechanism of adjuvanticity for alum) is no longer regarded as the essential mechanism behind adjuvant effectiveness [97,248], formulations such as degradable polymer microspheres may provide sustained exposure to antigens, obviating the need for multiple administrations. Practical considerations such as stability and cost may preclude the widespread use of some otherwise potent protein adjuvants such as cytokines [64,244].

Thus, we can summarize the ideal vaccine adjuvant as one which selectively targets the antigen to the desired population of APCs, minimizes the amount of antigen required, induces the desired adaptive immune response, while minimizing the innate immune response, is minimally toxic, low-cost, stable for long-term storage, and provides protective immunity in a single dose via a convenient delivery route.

New Research Tools to Study Disease Prevention

New adjuvants are also needed that can be used to precisely tune the nature or outcome of the immune response to more effectively protect against particular diseases such as cancers and HIV. This may be done by controlling the induction of particular cytokine profiles and by more effectively targeting antigens to specific tissues, cells, or intracellular compartments (e.g. DNA vaccines to the nucleus of a cell). These new adjuvants could also be used as research tools to study the induction or regulation of different immune response mechanisms that are associated with autoimmune diseases, allergies, or tolerance. Sadly though, many of these new adjuvants are still being developed experimentally and much more research is needed to bring them to an application. As shown in Table 1, the number of adjuvants in licensed vaccines are very few. Even the materials being tested in current clinical trials represent relatively few new immunostimulating adjuvants, especially against infectious diseases (Table 2). Furthermore, there may be a need for a considerable shift in thinking about how vaccines are tested for efficacy. Antibody titer is almost universally used as the test for vaccine efficacy but often high antibody titers do not translate into the best protection [249]. Many times a highly immunogenic antigen does not correlate to a protective immune response. This 'deceptive imprinting' is a common evasion mechanism by pathogens and partially responsible for the slow development of HIV vaccines[250]. Also there is the caveat that laboratory mice are not humans (or other livestock species) and what works in a mouse may not translate to other species. Numerous studies have highlighted differences in mouse and human immune systems including differences in complement reactivity[251], induction of Th17 cells [252], or response to a vaccine based on particle size [70].

In 2006, the National Research Council convened a Workshop on Immunomodulation [253], which made several recommendations to improve vaccine design including an improved molecular level understanding of the innate immune system, the need for effective delivery mechanisms, the identification of potential molecular targets to modulate innate immunity without undesirable side effects, and new strategies to target DCs and optimize antigen presentation. A key need that was identified by this panel was that in order to solve these important problems, it is critical for researchers from multiple

disciplines to work together. These fields may include biochemistry, immunology, materials science, cell biology, computational biology/materials science, pathology, oncology, microbiology, and combinatorial science. It is important to combine expertise from antigen biochemistry, cell biology, and immunology to understand the mechanism of immunogenicity and how the preservation of various epitopes contributes to immunogenicity. As these antigens are combined with adjuvants, it is important for materials scientists to work closely with immunologists to understand how protein antigens can be stabilized during encapsulation and delivery and how adjuvants interact with APCs. As these adjuvanted systems enter the body, they encounter plasma proteins that may adsorb on to the surface of the adjuvant. How this affects the release of the antigen and how this influences APC activation or antigen processing is of great significance to the initiation of the desired immune response. Finally, the use of the appropriate animal models to study these phenomena is critical and immunohistochemical methods are needed to study how these adjuvants affect the local tissue response.

In this regard, the authors, who belong to chemical engineering and veterinary microbiology departments have worked towards providing a highly cross-disciplinary research environment for students and postdoctoral researchers in their respective groups. The chemical engineering graduate students have the opportunity to take courses on immunology and molecular biology techniques, participate in journal clubs, and several of them have completed an immunobiology certificate program on their way to a Ph.D. Likewise, the microbiology students have the opportunity to take courses on polymeric biomaterials and nanotechnology. The two research groups have joint meetings every week and the students present the research results in a variety of formats, including tag-team talks and “storyboarding”. Such an approach has immensely benefited students from both disciplinary groups and has prepared them to address diverse research problems with new and innovative perspectives. Similar examples of cross-disciplinary research groups exist and are much needed as scientists embark on new therapies for diseases such as cancer, HIV, and respiratory infections.

Over the last 200 years, the use of vaccines has proven to be one of the most successful medical interventions in the reduction of disease caused by infectious agents [1]. However, many challenges still remain with regard to fully realizing the health benefits of active immunization programs. Some of these obstacles include the implementation of improved adjuvants, development of single dose vaccines, methods to overcome the poor immunogenicity of recombinant and subunit immunogens, and the ability to rapidly and rationally develop vaccines against emerging pathogens. In this regard, the mechanisms underpinning the effective modulation of cellular and molecular events associated with adjuvant enhancement of immune responses are still largely unknown. There is growing interest in the development of vaccine delivery systems based on micro- and nano-scale devices composed of biodegradable polymers, because they have the potential to act as effective adjuvants by encompassing all three of the classical adjuvant properties: providing an antigenic depot with a tailored and pulsatile release of the antigen over time, directing particulate antigens to the APCs and modulating the activation of innate immunity by altering polymer chemistry [95]. However, the mechanism of adjuvanticity and the ability of adjuvant chemistry to selectively modulate the immune response are still largely unknown. In order to address these challenges, it is important to perform fundamental and systematic studies of the role of polymer chemistry in regulating activation of APCs (e.g., DCs), antigen uptake, processing, and presentation, migration to the draining lymph node, and modulation of the immune response.

The mechanisms by which adjuvants enhance and/or redirect the immune response (e.g., formation of high titer antibodies, $CD4^+$ helper T lymphocytes and/or $CD8^+$ T lymphocytes) in order to establish long term immunologic memory are poorly understood. Upon antigen stimulation, T cells differentiate into two distinct populations described as Th1 and Th2 type immune responses [254]. Furthermore, Th1- and Th2-related cytokines (IFN-γ or IL-4/IL-13 respectively) can impact both the quality and magnitude of humoral and cell-mediated immunity. Humoral immunity, characterized by the activation of B cells that differentiate into antibody secreting plasma cells, is effective at neutralizing toxins, viruses, complement fixation, and opsonization of extracellular pathogens whereas the cell-mediated immunity (i.e., activation of cytotoxic T cells and

macrophages) are crucial for protection against intracellular pathogens [255]. The balance of humoral and cell-mediated immune responses has been shown to be important in the favorable outcome of many disease states. In this regard, vigorous and inappropriate cell-mediated immune responses have been implicated in the induction of autoimmune diseases (multiple sclerosis and Crohn's disease) while robust humoral immune responses are associated with allergic reactions [120]. In order to control the induction of appropriate immune responses and reduce the risk of autoimmunity or allergic responses, there is an urgent need to develop new, well-characterized adjuvants that allow for tailored immune activation and deviation. In spite of these implications of immune deviation, the mechanisms by which adjuvants influence whether Th1 or Th2 cells dominate an immune response are not well understood. Additionally, it is also important to consider the use of adjuvants to induce regulatory T cell responses and to avoid the aberrant induction of Th17 cells that have been associated with chronic inflammatory diseases.

Both in vitro and in vivo studies with the adjuvants discussed above indicate that adjuvant chemistry and particle size may play an important role in regulating the cellular and molecular mechanisms responsible for modulating host immune responses. Additionally, in order to understand intra-cellular trafficking at a molecular level, it is important to study the use of reporter molecules (e.g., quantum dots (QDs)) embedded within the adjuvant. Encapsulating QDs within adjuvants will provide adequate stability even in acidic conditions within cells and may be used to effectively track the transport of the nanospheres in intracellular compartments. The luminescence properties of QDs are expected to persist so long as the integrity of their nanocrystal structure is maintained, providing superior performance compared to conventional fluorescent dyes such as FITC, whose fluorescence is sensitive to pH [256,257]. QDs can also dramatically enhance *in vivo* imaging of APC migration by using red and near-infrared emitting QDs as an alternative to Cy5, Cy5.5, or other traditional organic dyes. QDs have substantially larger absorption cross-sections than even the best commercial dyes developed specifically for such imaging applications [258]. This improves the effective brightness of the fluorescence emission signal considerably. Additionally, QDs have unrivaled photostability that allows continuous long-term excitation without a substantial loss in fluorescence [256].

Finally, in order to understand the cellular and molecular mechanisms that establish immunologic memory, it is very important to correctly choose appropriate in vitro/in vivo models that will promote the induction of cell-mediated as well as humoral (i.e., antibody) immune responses. Because there are likely to be subtle immunogenetic differences between mouse strains (and eventually individual human subjects), the use of combinatorial approaches evaluating cell-adjuvant interactions may provide a robust and versatile approach to the development of vaccines that will effectively stimulate immunity for different conditions and/or applications. These approaches may be used to rapidly screen a large number of adjuvant chemistries for their ability to differentially activate APCs, which will aid in the rational use of cocktails of micro- or nano-particles in vaccine formulations. These formulations will possess the ability to stimulate the appropriate immune response depending upon the disease. The availability of transgenic models (e.g., OTI and OTII transgenic mice) provides for the capability to critically evaluate the activation of $CD4^+$ and $CD8^+$ pathways while other molecular biology tools enable researchers to evaluate the effect of new adjuvants on antigen processing and presentation both in vitro and in vivo.

In summary, an integrated and cross-disciplinary approach is needed that combines the development of novel adjuvants with: i) molecular level studies that will elucidate the mechanisms of chemistry-mediated cellular activation by adjuvants; ii) cellular level studies that will elucidate the uptake mechanisms of antigen-loaded adjuvants by immune cells and the activation and migration of these cells; and iii) in vivo studies that highlight the underlying mechanisms governing immune modulation. Such an integrated approach is essential to solve the important challenge of rationally designing vaccine delivery systems that will effectively stimulate the immune system. It can provide new insights into the mechanisms of adjuvanticity and on the complex relationships between adjuvant chemistry, molecular mechanisms of APC activation, antigen uptake, processing/presentation by APCs, migration to the draining lymph node, and modulation of the immune response. To carry out such an approach, it is important to assemble highly cross-disciplinary teams of researchers with expertise in the areas of

molecular and cellular immunology, intra-cellular trafficking, biomaterials chemistry, toxicology, nanotechnology, and pathology.

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CHAPTER 3

Activation of Dendritic Cells and Early T cell Activation Using Polyanhydride Microspheres

Jennifer H. Wilson Welder[1], Maria P. Torres[2], Senja Lopac[2], Bradley VanDeWoestyne, Balaji Narasimhan[2]*, Michael J. Wannemuehler[1]

[1]Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011

[2]Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

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*Corresponding Author, nbalaji@iastate.edu

Abstract

Biodegradable polymers have been widely studied for various drug delivery applications including vaccines. Work from this laboratory previously showed that polyanhydride based materials enhanced protein stability, a quality necessary for vaccine antigen delivery. The present studies were designed to evaluate the adjuvant activity of polyanhydride microspheres prepared in the absence of additional stabilizers, excipients, or immune modulators. The microspheres were composed of varying ratios of either CPH:SA or CPTEG:CPH and added to cultured bone marrow-derived dendritic cells (DCs) . Upon stimulation with polyanhydride microspheres, DCs increased surface marker expression of MHC class II, T cell costimulatory molecules CD86 and CD40, and the C-type lectin CD209 in a dose and chemistry dependent manner. It was demonstrated that microspheres also induced secretion of IL-12p40 and IL-6, and the amount secreted was dependent on polymer chemistry. Using ovalbumin (Ova)-specific OT I and OT II

transgenic (Tg) T cells, DC stimulated with polyanhydride microspheres and Ova induced antigen-specific proliferation of both $CD4^+$ OT II and $CD8^+$ OT I T cells. In vivo, microspheres co-delivered with Ova in a Tg T cell transfer model (DO11.10) enhanced antigen-specific $CD4^+$ T cell expansion. These cells produced cytokines consistent with activated T cells upon incubation with Ova. Taken together, this work demonstrated that polyanhydride microspheres act as classical immunomodulators by activating antigen presenting cells and enhancing antigen-specific T cell responses, both in vitro and in vivo.

1 Introduction

The World Health Organization (WHO) estimated that in 2002, 2.1 million deaths worldwide were due to diseases that could have been prevented by routine vaccination [1]. In an effort to minimize these casualties, the WHO and the United Nations Children's Fund (UNICEF) together with other partners developed a Global Immunization Vision and Strategy (GIVS) four years ago. One of the main strategic areas of GIVS is the introduction of new and efficacious vaccines and delivery technologies to combat diseases for which no treatment currently exists [2]. The rapid development of protein based biopharmaceuticals suggests that many future vaccines will involve the delivery of peptide or protein subunits. Currently, such vaccines lack a suitable carrier, thus, there is an urgent need to develop better adjuvants for delivery of efficacious vaccines that will benefit public health [3].

An adjuvant is a substance which when incorporated into a vaccine will enhance the immune response to the antigen. Classically, adjuvants fulfilled one of three roles, 1) act as a depot, preventing rapid clearance of the antigen, 2) direct the antigen to antigen presenting cells (APC) for phagocytosis, processing, and presentation, and 3) induce co-stimulatory signals on APCs necessary for activation of naïve T cells [4, 5].

When designing novel vaccine adjuvants, it is essential to have a detailed understanding of the complex interplay between the cells of the innate and adaptive immune systems. As the first line of defense, cells of the innate immune system are involved in the recognition of foreign invaders by means of pathogen-associated-

molecular patterns (PAMPs) [6]. Many of these cells are also APCs that express antigenic peptides derived from pathogens on their surface in the context of major histocompatabilty molecules (MHC) types I and II. Upon encounter with PAMPS, APCs become activated undergoing phenotypic changes that allow them to become an effective link between pathogen detection and induction of adaptive immunity. Dendritic cells (DCs) residing within the skin, most organs, and the lamina propria of mucosal tissues are uniquely situated for pathogen detection as well as the most efficient APC for activating naïve helper T cells, killer T cells, and B cells [6, 7]. Residing in an immature state, DCs sample their surroundings for possible pathogens through pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) and C-type lectins [8, 9]. After ligation of the PRR, DCs engulf the pathogen and migrate to lymph nodes (LNs) where they undergo maturation. Dendritic cell maturation includes increased surface expression of co-stimulatory molecules (i.e., CD80, CD86, CD40) and antigen presenting complexes (MHC I and MHC II) and secretion of T cell activating cytokines such as interlukins (IL-1, IL-6, IL-10 and IL-12) and tumor necrosis factor alpha (TNFα)[10]. The specific pattern of DC maturation (surface marker expression and cytokine secretion) can polarize the ensuing adaptive immune response.

While the Th1-Th2 paradigm has dominated immunology for the last 15 years, current understanding has expanded this to include Th17 and CD4+ mediated regulatory responses (Treg) [11]. Th1-type immune responses are characterized as cell-mediated immune responses, associated with the production of IL-12p70 by APCs and interferon-γ (IFNγ) from T cells [12]. Th1-type immune responses are often necessary for clearance of intracellular viral and bacterial pathogens. Excessive Th1 immune responses have been implicated in sarcoidosis, tuberculosis, and collagen-induced arthritis [13-15]. Th2-type immune responses are associated with the induction of IgE, eosinophil activation, release of IL-4, IL-5, IL-10, and IL-13 from T cells [16] and promote allergic reactions. In addition, Th2-type responses are necessary for elimination of parasites and some extracellular bacteria [11]. Th2 dominant immune responses may be responsible for increases in asthma, atopic dermatitis, and certain cancers (basal cell carcinomas and gastric cancers) [17-21]. Recently, IL-17 secreting CD4+ T cells or Th17 cells have been

described and are associated with pro-inflammatory responses needed for the clearance of extracellular pathogens [22]. The dysregulation or over expression of Th17 responses have been linked to inflammatory autoimmune diseases such as inflammatory bowel disease, multiple sclerosis, and rheumatoid arthritis [22, 23].

Currently, the common adjuvants employed in human vaccines are aluminum-based (e.g., alum) and often require multiple doses (i.e., booster shots) to achieve protective immunity that is primarily an antibody-mediated immune response (i.e., poor induction of cell-mediated immunity) [24]. The use of aluminum salts is often associated with the induction of adverse reactions at the site of injection. While polyanhydride-derived polymers have been shown to be tissue compatible [25-28], biodegradable polymers have also shown promise as vaccine adjuvants [29]. The use of polymer adjuvants provides multiple advantages over other adjuvants including the controlled release of antigen which enhances the induction of an immune response, improves patient compliance by administering in a single injection, and modulates the ensuing immune response in respect to Th1-Th2 bias [29, 30]. The controlled release of antigens following parenteral administration of biodegradable polymeric microspheres has been extensively studied [3, 31-33]. Microspheres greater than 10 μm provide an antigenic depot at the site of injection, while smaller microspheres can be efficiently phagocytosed by APCs and carried to lymph nodes [3].

Polyanhydrides are a class of biodegradable polymers that have shown promise as carriers for controlled drug delivery and have been approved by the FDA for use in humans [25, 27, 34-44]. Biocompatibility studies have shown the safety of these biomaterials as these degrade into non-mutagenic and non-cytotoxic products [45, 46]. Another advantage of these polymers is their degradation by a surface erosion mechanism resulting in controlled release of the antigen with predictable degradation profiles that can vary from days to months depending on the polymer chemistry. The polyanhydride chemistries used in this study are based on the aliphatic sebacic acid (SA), the aromatic 1,6-bis(p-carboxyphenoxy)hexane (CPH), and the amphiphilic 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (Figure 1). The biocompatibility of CPH:SA and CPTEG:CPH copolymer libraries has been recently studied using high

throughput cell-based screening methods and were found to induce no discernible cytotoxic effects at concentrations as high as 2.8 mg/mL which are much higher than that expected to be used for in vivo applications [47].

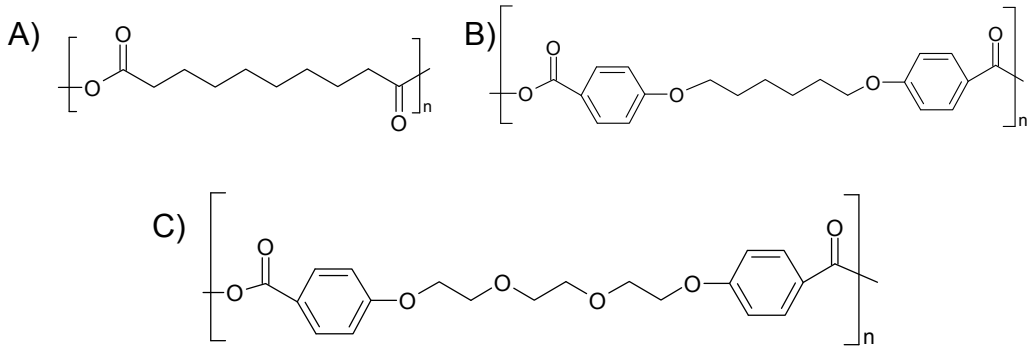

Figure 1: Chemical structures of (A) poly(SA), (B) poly(CPH), and (C) poly CPTEG. n represents degree of prolimerization.

Previous work has shown that polyanhydrides are suitable vaccine carriers with enhanced adjuvanticity and possess immunomodulatory capabilities associated with polymer chemistry [29]. These studies showed that a single dose of CPH:SA microspheres was able to enhance and modulate the antigen-specific immune response, depending on the composition of the polymer. Specifically, tetanus toxoid (TT)-loaded 20:80 CPH:SA microspheres induced a IgG1 dominant response while the 50:50 CPH:SA formulation induced a balanced IgG1/IgG2a antibody response. Although no in vivo experiments have been published with the CPTEG:CPH system, this system has shown excellent characteristics for protein stabilization and release [47, 48].

The purpose of the current study was to mechanistically evaluate the adjuvanticity and immunomodulatory capabilities of CPH:SA and CPTEG:CPH microspheres including the inherent stimulatory capacity of these polymers. Because DCs are the most potent APCs involved in the induction of an immune response, the in vitro evaluation of murine DC activation after incubation with polyanhydride microspheres was evaluated. The surface expression of the MHC II, the co-stimulatory molecules CD86 and CD40, and the C-type lectin DC specific ICAM-3 grabbing non-integrin DC-SIGN/CIRE

(CD209) was evaluated using flow cytometry. In addition, the ability of polyanhydride microspheres to induce the secretion of immune activating cytokines was measured. In order to extend the in vitro observations to in vivo applications, two murine models were used to evaluate the induction of antigen-specific immune responses following immunization with ovalbumin (Ova) in the presence of polyanhydride microspheres. The results of the in vitro and in vivo studies demonstrated that the copolymer composition has significant impact on the activation of DCs and the induction of an antigen-specific immune response.

2 Materials and Methods

2.1 Materials

The chemicals needed for the synthesis of CPH and CPTEG monomers include: 4-*p*-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, and tri-ethylene glycol, and sebacic acid (99%) were purchased from Sigma Aldrich (St Louis, MO); 4-*p*-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetic anhydride, methylene chloride, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ).

2.2 Polymer synthesis and characterization

CPH:SA and CPTEG:CPH copolymers were synthesized by melt polycondensation as described previously [44]. The purity and degree of polymerization of the polymers was analyzed using ^{1}H NMR spectra obtained from a Varian VXR-300 MHz NMR spectrometer.

2.3 Microsphere fabrication by cryogenic atomization

Prior to fabricating microspheres, all glassware and equipment was soaked in 70% ethanol to prevent microbial contamination. The procedure used to fabricate microspheres was modified from previously reported studies [49, 50]. Briefly, polymer dissolved in methylene chloride was pumped through an 8700-1200 MS ultrasonic

atomizing nozzle (Sono Tek Corporation, Milton, NY) into 200 mL of ultra cold ethanol overlaid with ~100 mL of liquid nitrogen. This procedure was performed at 4ºC for 50:50 CPH:SA and CPTEG:CPH microspheres in order to maintain the temperature below the glass transition temperature of the polymers during microsphere preparation. After atomization, the polymerized microspheres were stored at -80°C for three days to allow the methylene chloride to be extracted into the ethanol. The microspheres were then collected by filtration and dried under vacuum overnight. The microsphere morphology was characterized by scanning electron microscopy (SEM). The particle size distribution was obtained from SEM images (150-250x) using a soft imaging system software (analySIS®, Soft Imaging System Corp, Lakewood, CO). An average of 800 particles per image was analyzed.

2.4 Endotoxin assay

To ensure that the activation observed was due to the polymers and not endotoxin contamination, endotoxin levels of polyanhydride microspheres were tested with *Limulus* Amebocyte Lysate (LAL) QCL-1000 test kit (Cambrex, Walkersville, MD). Solutions of CPTEG:CPH and CPH:SA microspheres (5 mg/mL) fabricated as described above were prepared using endotoxin-free, sterile water and incubated overnight at 37ºC while shaking. After centrifuging the suspension of microspheres, the LAL test was performed using the supernatant according to manufacturer's procedure. All the polyanhydride microspheres exhibited an endotoxin content of less than 0.1 EU/mL, which is five times lower than the maximum level permitted by the U.S. Food and Drug Administration (FDA) for new drugs tested by the LAL test [51].

Ovalbumin to be used in antigen specific studies was tested for endotoxin levels. Two mg/mL solutions of Ova were prepared in endotoxin free water and LAL performed according to manufacturer's instructions. Ovalbumin as purchased contains high levels of endotoxin. To remove contaminating endotoxin, AffinityPak Dextoxi-Gel endotoxin removing gel columns (Thermo Scientific, Rockford, IL) were used according to manufacturer's instructions. Resulting ovalbumin contained less than 10 EU/mg which

equates to roughly 1 ng endotoxin equivalent per mg protein. Endotoxin-free Ova was lyophilized and stored at -20ºC until needed.

2.5 Mice

C3H/HeNHsd (C3H) and C57BL/6 (BL6) mice were purchased from Harlan Sprague Dawley. Male and female C57BL/6 Tg(TcraTrab)1100Mjb/J (OT I), C57BL/6 Tg(TcraTrab)425Cbn/J (OT II) and DO11.10 TCR transgenic mice were purchased from Jackson Laboratory (Bar Harbor, Maine). BALB/c mice, at least 6 weeks of age, were obtained from the breeding colony maintained by the Hybridoma Facility at Iowa State University (Ames, IA). All of the mice were housed under specific pathogen-free conditions where all bedding, caging, water, and feed were sterilized prior to use. Animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

2.6 Culture and stimulation of DCs

Bone marrow cells were isolated from the femurs and tibia of C3H, BALB/c, or BL6 mice and cultured to derive DCs using minor modifications of a previously developed method [52]. Briefly, after euthanizing mice and excising the tibia and the femur, bone cavities were flushed using a 1 cc syringe fitted with a 25 gauge needle and containing cell culture medium; each bone was flushed three times. After washing the cells by centrifugation, the cell pellet were suspended in complete culture media (cRPMI) (RPMI 1640 supplemented with 2% essential amino acids (Mediatech, Herndon, VA), 25 mM HEPES buffer (Mediatech), 100 units/mL penicillin, 0.1 mg/mL streptomycin (Mediatech), 0.05 mg/mL gentamicin (Sigma), 1% l-glutamine (Mediatech), 5 x 10^5 M 2-mercaptoethanol (Sigma), 10% heat inactivated fetal bovine serum (FBS) (Valley Biomedical, Winchester, VA)) with 10 ng/mL granulocyte macrophage colony stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ). Then cells were placed in bacteriological petri dishes (4 x 10^5 cells/mL) and incubated at 37ºC under 5% CO_2 atmosphere. On day 3, 10 mL of fresh media was added to each plate. After day 6, veiled projections were visible in the cell cultures, which are the characteristic dendrites of DCs.

Flow cytometric evaluation of the population of non-adherent and loosely adherent cells indicated that 75 to 90% of the DCs expressed CD11c. On day 6, DCs were removed from the plates, washed, resuspended in fresh media, and transferred to 24-well plates (2.5×10^6 cells/well). On day 9, DCs were incubated with the different stimulation treatments. Non-stimulated (background, Bkgd) DCs and LPS (200 ng) stimulated DCs were used as negative and positive controls, respectively. After suspending polyanhydride microspheres in complete culture medium, microspheres were sonicated briefly to create an evenly dispersed suspension. Microspheres were added to the DC cultures at concentrations of 60, 125, or 250 μg/mL. These concentrations corresponded to ratios of 1:3, 1:6, and 1:12 microsphere to DC, respectively. The stimulated cultures were incubated for 48 h (37°C, 5% CO_2). Cultures were routinely observed with an inverted microscope.

2.7 Surface marker staining

The surface expression of cell surface markers, co-stimulatory molecules, and antigen presenting complexes was analyzed with flow cytometry. After 48 h of incubation with stimulation treatments, DCs were harvested and placed in polystyrene tubes (BD FALCON™, Franklin Lakes, NJ). After centrifuging (1200 rpm, 7min), DCs were resuspended in Fc blocking solution containing unlabeled CD36/16 FcγR (10 μg/mL)(eBioscience, San Diego, CA), homologous mouse serum (0.5%), and unlabeled rat IgG (50μg/mL) (Sigma). After blocking DCs for 1 h on ice, cells were stained for the presence of specific cell surface markers using Alexa Fluor® 700 anti-mouse CD11c (clone N418)(eBioscience), FITC conjugated anti-mouse/rat MHC Class II ($I\text{-}E^k$) (clone 14-4-4S)(eBioscience), PE/Cy7 anti-mouse CD86 (clone GL-1) (Pharmingen, Becton Dickinson, Franklin Lakes, NJ), allophycocyanin (APC) anti-mouse CD40 (clone 1C10)(eBioscience), and PE conjugated anti-mouse CIRE (CD209) (clone 5H10)(eBioscience) antibodies. Respective isotype-specific control antibodies (Alexa Fluor® 700 conjugated Armenian hamster IgG (clone eBio299Arm), FITC IgG2a κ (clone eBM2a), PE/Cy7 conjugated rat IgG2b (clone KLH/G2b-1-2), APC rat IgG2a κ (clone eBR2a), and PE-conjugated rat IgG2a (clone eBR2a)) (all from eBioscience),

single color, and unstained controls were also included in the analytical panel. Propidium iodide (PI) was used to establish the live/dead cell gate. Flow cytometric analysis was performed using a FACScanto^{TM} (Becton-Dickinson) and results were analyzed using FloJo (Tree Star, Inc., San Carlos, CA).

2.8 OT I and OT II stimulation

DCs were cultured as described above. On day 10 of culture, non-adherent and DCs were collected and incubated with 250 μg/mL polymer microspheres, and 100 μg/mL Ova for 8-12 h. Lymph nodes were removed from either OT I or OT II mice and single cell suspensions were prepared. Cells were washed by centrifugation and resuspended in complete culture medium. To assess in vitro immune stimulation, 2.5 x 10^5 lymphocytes from OT I or OT II mice were combined with 0.5 x 10^5 DCs in 96-well round bottom culture plates. Plates were incubated at 37°C in humidified 5% CO_2. On day 3, 0.5 μCi of methyl-[^{3}H]-thymidine (specific activity 6.7 Ci mmole^{-1}, Amersham Life Science, Arlington Heights, IL) was added to each well and incubated for additional 18 h. Well contents were harvested onto glass fiber filters and the incorporated radioactivity was measured using liquid scintillation counter. The assays were performed in triplicate and data are presented as mean counts per minute of triplicate wells.

2.9 T cell isolation and immunizations

T cells were purified from lymph nodes and spleens of DO11.10 mice using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Auburn, CA). Purity of isolated cells (89.3% $CD4^+$) was confirmed by flow cytometry. Approximately, 1x10^6 DO11.10 T cells were injected intravenously into the tail vein of BALB/c recipients. Recipient BALB/c were then immunized subcutaneously at the nape of the neck with saline alone, Ova (100 μg), Ova + LPS (25 μg), Ova + PLGA microspheres (0.5 mg), Ova + 20:80 CPTEG:CPH microspheres (0.5 mg), or Ova + 20:80 CPH:SA microspheres (0.5 mg) in a volume of 100 μL. Serum from each animal was collected from the saphenous vein prior to cell transfer and immunizations.

2.10 Cell surface marker staining of lymphocytes

Five days following cellular transfer and immunization, draining lymph nodes were removed and single cell suspensions prepared as described above. Approximately 2.5 x 10^6 lymph nodes cells were placed into four mL snap-cap tubes and incubated with the Fc blocking solution for 30 minutes on ice. Cells were then stained with anti-CD4 PE/Cy7(eBioscience), Biotin-labeled anti-KJ1.26 (Caltag Laboratories, Burlingame, CA), anti-CD62L-PE (eBioscience), and anti-CD44-AF700 (eBioscience). Tubes of cells pooled from all animals were incubated with the corresponding isotype antibody controls as described above. Cells were incubated on ice for 1 h, 1 mL of FACS buffer was added, and the tubes were centrifuged. After respending the cells, streptavidin-FITC (0.5 μg/mL) was added and the cells were incubated on ice for an additional 30 minutes. Cells were washed twice with FACS buffer and then fixed with 0.25 mL BD Stabilizing Fixative (BD Bioscience, San Jose, CA). Flow cytometry was performed using FACScanto™ (Becton-Dickinson) and results were analyzed using FloJo (Tree Star, Inc., San Carlos, CA).

2.11 Culture and in vitro restimulation of lymphocytes

Five days following cell transfer/immunization, lymph nodes draining the immunization site (axillary and brachial) were excised and single cell suspensions prepared. Separate wells containing LN cells were incubated with either anti-mouse CD3 plus anti-mouse CD28 monoclonal antibodies (1.5 μg/mL and 0.5 μg per mL, respectively, eBioscience) or Ova (100 μg/mL). Non-stimulated cells were incubated in cRPMI alone (i.e., no stimulation). Cells were incubated at 37°C in 5% CO_2 in air and culture supernatants were collected at 72 h and frozen for later cytokine analysis.

2.12 Cytokine assay

Cytokines (TNF-α, IL-4, IL-6, IL-10, and IL-12p40) were assayed from cell free supernatants collected from DCs cultured for 48 h in the presence of microspheres. Supernatants were collected and stored at -20°C until analysis. Cytokines were assayed using Luminex® Multiplex assay (Austin, TX).

Cytokines IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, and IFN-γ were assayed from cell free supernatants collected from lymphocytes collected from BALB/c mice that received Tg DO11.10 T cells. Supernatants were collected at 72 h after in vitro re-stimulation with antigen and stored at -20°C until assayed using Luminex Multiplex assay. Multiple experiments were analyzed individually.

2.13 Enzyme-linked immunosorbent assay (ELISA)

Serum samples collected from mice 21 days post-transfer and immunization were tested for Ova-specific antibodies. Costar brand high binding ELISA plates (EIA/RIA high binding, catalog # 3590) were coated overnight with 5 µg/mL Ova. Plates were washed with phosphate buffered saline (PBS, pH 7.4) containing 0.5% Tween 20 (PBST) and blocked for two hours with PBST+ 2% gelatin (Difco, catalog # 214340). Plates were washed and individual serum samples were serially diluted in PBST plus 2 % gelatin and incubated overnight at 4°C. On the third day, plates were washed again with PBST and alkaline phosphatase-conjugated goat anti-mouse IgG1 or IgG2a (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:1000 dilution) was added. After incubating for two hours, plates were washed and p-nitrophenyl phosphate (Sigma 104) substrate (1 mg/mL) in carbonate buffer (pH 9.3) was added to each well. Changes in optical density (OD) were spectrophotometrically measured at 405 nm.

2.14 Statistical analysis

Multivariate analysis, linear regression analysis and non-linear regression analysis was performed on DC activation data using the statistical analysis software JMP® 7 (Cary, NC). Linear and non-linear regression models were fit to describe the effect of dose response on marker expression. One-way analysis of variance (ANOVA) was performed on individual surface markers, with Tukey pair-wise comparison post-tests with GraphPad Prisim 4.0 for Macintosh (GraphPad Software, La Jolla, CA). Where appropriate, ANOVA and Tukey post-tests were performed on T cell activation data.

3 Results

3.1 Microsphere fabrication

After microsphere fabrication by cryogenic atomization, the surface morphology was analyzed with SEM. The electron photomicrographs show that poly(CPTEG) and poly(SA) microspheres had a smoother surface than the other compositions which is attributed to processing conditions and differences in polymer properties (e.g., glass-transition temperature)(data not shown). When the size distribution was analyzed, the majority of the polyanhydride particles, regardless of chemistry, were below 10 μm in diameter. Most of the microspheres were in a size range that is reported to be readily phagocytosed by DCs [53].

3.2 Induction of cell surface marker expression by microspheres

After stimulation for 48 h with polymer microspheres, non-adherent and semi-adherent DCs were harvested and stained for the surface markers CD11c, MHC II, CD40, CD86 and CD209 (CIRE). Multivariate analysis was performed to identify experimental days where microsphere treated DC were different from non-stimulated DC. Linear correlation analysis showed this correlation between surface marker expression and concentration or dose of microspheres incubated with DCs (Table 1). While the slope of the linear correlation line was positive and in most cases was significant ($P < 0.1$), the R2 values were low. Based on these analyses, it was determined that dose accounts for roughly 10 to 20 percent of the variability observed between treatment groups. Linear correlation analysis for the effect of polymer composition (i.e., % of SA or % CPTEG in CPH:SA or CPTEG:CPH) showed a positive correlation with significant values for some polymer composition-surface marker combinations, namely, CD86 and CD209 (Table 2). Again, the R2 values when they were significant, were low. Thus, less than 10% of the differences between treatment groups were attributable to composition (% SA or % CPTEG). Majority of the differences between treatment groups was attributable to the individual day that the experiments were performed (the sum variation of DC batch variation, staining intensity, antibody age variation, machine settings, etc.).

As shown in Figure 2A, the percentage of cells expressing MHC II does not differ between non-stimulated, LPS- or polymer-stimulated DCs ($P > 0.1$). As shown in Figure 2B, there is a significant difference between LPS stimulated group and non-stimulated (Bkgd) DC ($P \leq 0.01$) but no differences between polymer groups at 250 μg/ml dose. Linear fit of dose is shown in Table 1 and linear fit of composition is summarized in Table 2.

CD86 is part of the B7 family of co-stimulatory molecules and binds to CD28 on T cells. CD86 is up-regulated following activation of DC by microbial stimuli [54]. Incubation with increasing concentrations of polyanhydride microspheres increased both the number of DCs expressing CD86 (% CD86) and CD86 MFI (Figure 3A-B, Table 1). There is a significant trend toward increasing CD86 MFI with increasing CPH content in the CPH:SA copolymers (i.e., 50:50 > 20:80 > pSA) and for % CD86 for CPTEG:CPH copolymers (Table 2).

Costimulatory molecule CD40 binds with CD40L on T cells as well as CD154 expressed on a variety of cell types [55]. As shown in Figure 4A, stimulation of DCs with LPS induces increased expression of CD40 as indicated by increases in both percentage of positive cells and MFI. Polyanhydride microspheres also induced increased expression of CD40 in a dose dependent manner (Table 1). The effect of dose is more significant for 50:50 CPH:SA and 20:80 CPH:SA than pSA, although polymer composition was not significant (Table 2, Figure 4B). For CPTEG:CPH microspheres, there was an interesting observation for 10:90 CPTEG:CPH and pCPTEG in that there was greater stimulation of CD40 expression by microspheres of copolymers that have the highest concentration of CPH (i.e., more hydrophobic) and the highest concentration of CPTEG (i.e., more hydrophilic).

CD209 plays a role in pathogen uptake, DC migration, and the initial interactions with T cells [56]. The data shown in Figure 5A demonstrates that only a low percentage of cells express CD209. Expression of CD209 was very dependent on chemistry and dose (Table 1 and 2). The greatest surface of CD209 was observed with the bulk-eroding hydrophilic or aliphatic polymers (pCPTEG, PLGA and pSA) as compared to background or CPH:SA containing copolymers.

Similar experiments were performed with DCs derived from C3H, BALB/c, and BL6 mice with respect to induction of cell surface marker expression. In addition, there were no observable differences in the robustness or magnitude of responses between the different mouse strains in response to polymer stimulation.

Table 1: Statistical summary of linear model fit to DC activation data by polymer composition to assess the effect of dose on surface marker expression.

Polymer	**Surface Marker**	**R^2**	**P value**
50:50 CPH:SA	*% MHC II+*	*0.13*	*0.074*[a]
	MFI MHC II	**0.34**	**0.002**
	% CD86+	0.07	0.114
	MFI CD86+	**0.13**	**0.033**
	% CD40 +	**0.16**	**0.022**
	MFI CD40	*0.10*	*0.073*
	% CD209+	0.09	0.246
	MFI CD209	0.01	0.734
20:80CPH:SA	% MHC II+	0.27	0.371
	MFI MHC II	0.28	0.362
	% CD86+	0.05	0.604
	MFI CD86+	0.03	0.686
	% CD40 +	0.09	0.518
	MFI CD40	0.02	0.769
	% CD209+	Na[b]	Na
	MFI CD209	Na	Na
pSA	% MHC II+	0.28	0.116
	MFI MHC II	0.28	0.114
	% CD86+	0.03	0.597
	MFI CD86	0.10	0.355
	% CD40 +	0.13	0.299
	MFI CD40	0.19	0.202
	% CD209+	0.13	0.635
	MFI CD209	0.15	0.619
10:90 CPTEG:CPH	% MHC II+	0.00	0.864
	MFI MHC II	0.04	0.254
	% CD86+	0.04	0.199
	MFI CD86	*0.08*	*0.071*
	% CD40 +	**0.09**	**0.035**
	MFI CD40	0.06	0.144
	% CD209+	0.00	0.948
	MFI CD209	0.00	0.857
20:80 CPTEG:CPH	% MHC II+	0.04	0.162
	MFI MHC II	0.03	0.228

Table 1: Statistical summary of linear model fit to DC activation data by polymer composition to assess the effect of dose on surface marker expression.

50:50 CPTEG:CPH	**% CD86+**	**0.11**	**0.026**
	MFI CD86	0.00	0.929
	% CD40 +	0.00	0.932
	MFI CD40	**0.13**	**0.008**
	% CD209+	0.04	0.390
	MFI CD209	0.01	0.763
	% MHC II+	0.02	0.424
	MFI MHC II	0.00	0.989
pCPTEG	**% CD86+**	**0.30**	**<0.001**
	MFI CD86	0.04	0.133
	% CD40 +	**0.16**	**0.004**
	MFI CD40	0.04	0.165
	% CD209+	0.03	0.418
	MFI CD209	0.02	0.530
	% MHC II+	0.06	0.271
	MFI MHC II	0.01	0.683
	% CD86+	**0.18**	**0.041**
	MFI CD86	0.03	0.396
	% CD40 +	**0.25**	**0.019**
	MFI CD40	0.09	0.181
	% CD209+	**0.59**	**0.043**
	MFI CD209	0.11	0.476

[a]Significant values $P \leq 0.05$ are shown **bold**, $P \leq 0.1$ *italics*. [b]Na, no analysis available.

Table 2: Statistical summary of linear correlation analysis for surface marker expression as a function of polymer composition (% SA in CPH:SA or % CPTEG in CPTEG:CPH).

Polymer	Surface Marker	R^2	P value
CPH:SA	% MHC II+	0.010	0.2677
	MFI MHC II	**0.034**	**0.0365**
	% CD86+	**0.096**	**0.0002**
	MFI CD86	**0.073**	**0.0014**
	% CD40 +	0.014	0.1657
	MFI CD40	0.007	0.3333
	% CD209+	**0.092**	**0.0234**
	MFI CD209	0.029	0.2126
CPTEG:CPH	% MHC II+	0.012	0.1873
	MFI MHC II	0.000	0.8682
	% CD86+	**0.034**	**0.0188**
	MFI CD86	0.001	0.7604
	% CD40 +	**0.036**	**0.0142**
	MFI CD40	<0.0001	0.9725
	% CD209+	**0.235**	**<0.0001**
	MFI CD209	0.005	0.5812

Significant values $P \leq 0.05$ are shown **bold**.

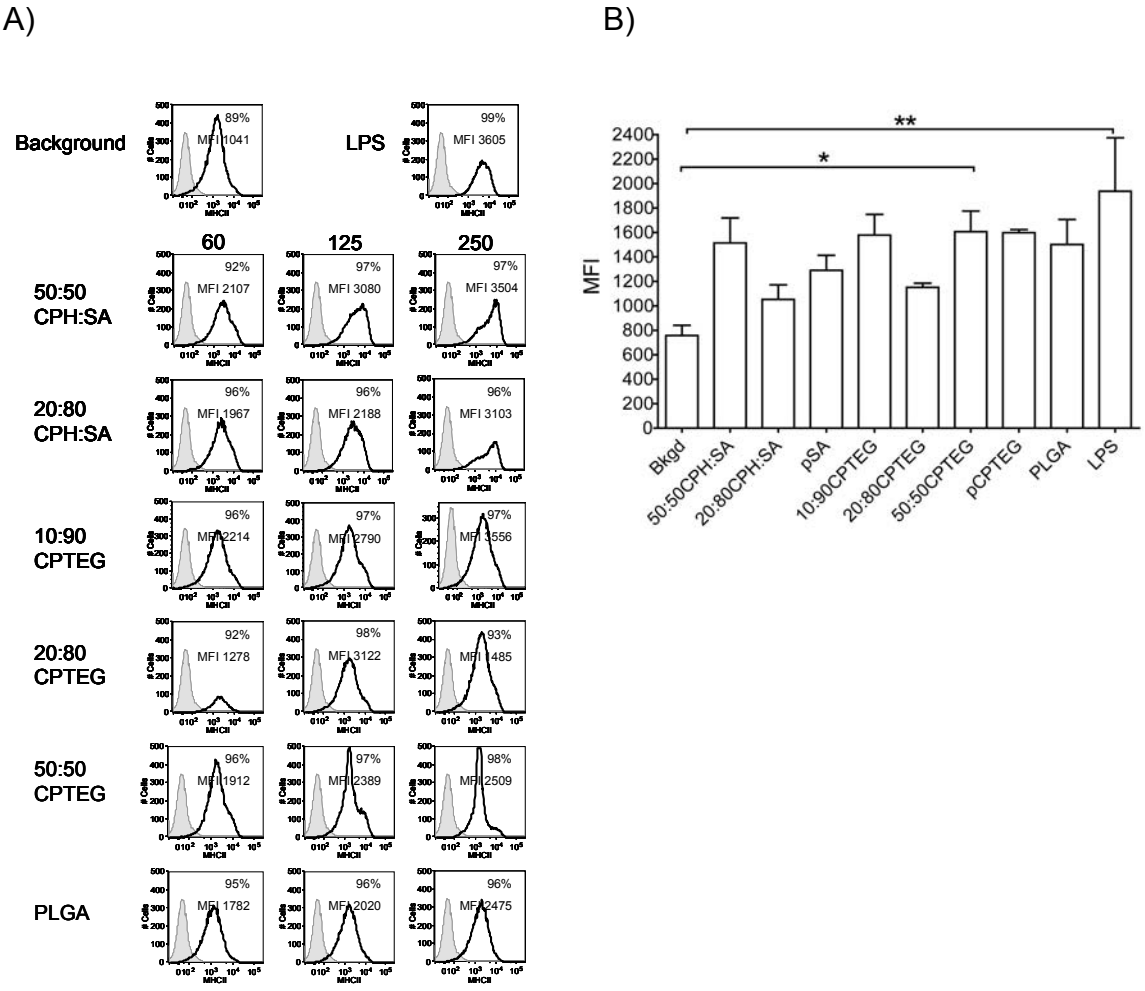

Figure 2: Induction of MHC II surface expression by polyer microspheres. A) MHC II Histograms of DCs incubated with different concentrations and compositions of microspheres. DC was gated on live cells by PI exclusion and forward scatter-side scatter. DC was then sorted on CD11c expression. DCs expressing MHC II (open histograms) are superimposed over isotype controls (solid gray histograms). Concentration of polyanhydride microspheres was 60, 125, and 250 µg/mL corresponding to 1:3, 1:6, and 1:12 microsphere:DC ratios, respectively. One representative experiment out of three independent experiments shown.
B) Mean MFI of MHC II of three independent experiments (error bars = SEM) at 250 µg/ml concentration. Linked bars are significantly different from each other, * indicates $P \leq 0.05$; **, $P \leq 0.01$.

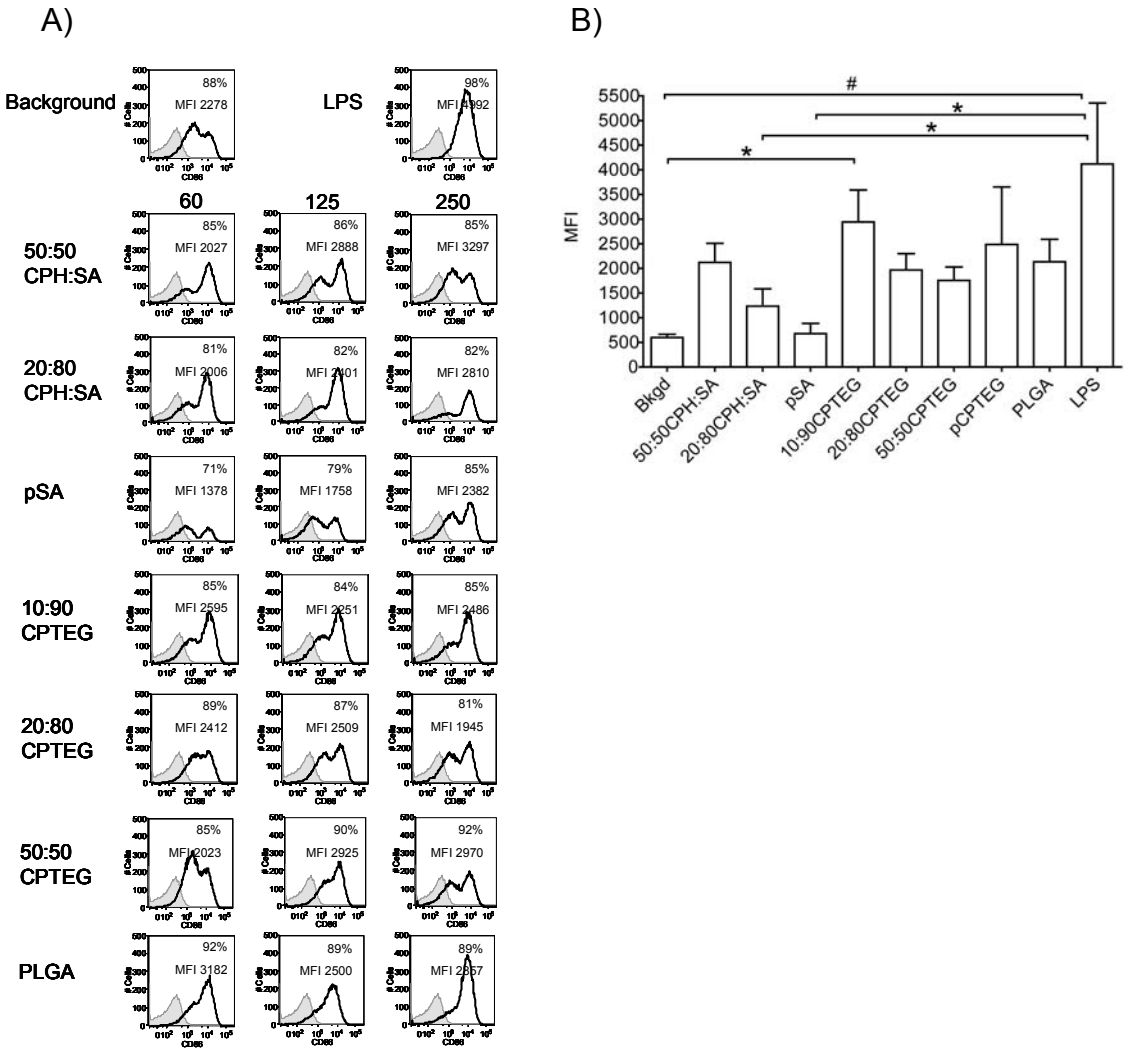

Figure 3: Expression of costimulatory molecule CD86 on the surface of DCs activated with polymer microsopheres. A) CD86 Histograms of DCs incubated with different concentrations and compositions of microspheres. DC was gated on live cells by PI exclusion and forward scatter-side scatter. DC was then sorted on CD11c expression. DCs expressing CD86 (open histograms) are superimposed over isotype controls (solid gray histograms). Concentration of polyanhydride microspheres was 60, 125, and 250 µg/mL corresponding to 1:3, 1:6, and 1:12 microsphere:DC ratios, respectively. One representative experiment out of three independent experiments shown.
B) Mean MFI of CD86 of three independat experiments (error bars = SEM) at 250 µg/ml concentration. Linked bars are significantly different from each other, * indicates $P \leq 0.05$; **, $P \leq 0.01$; #, $P \leq 0.001$.

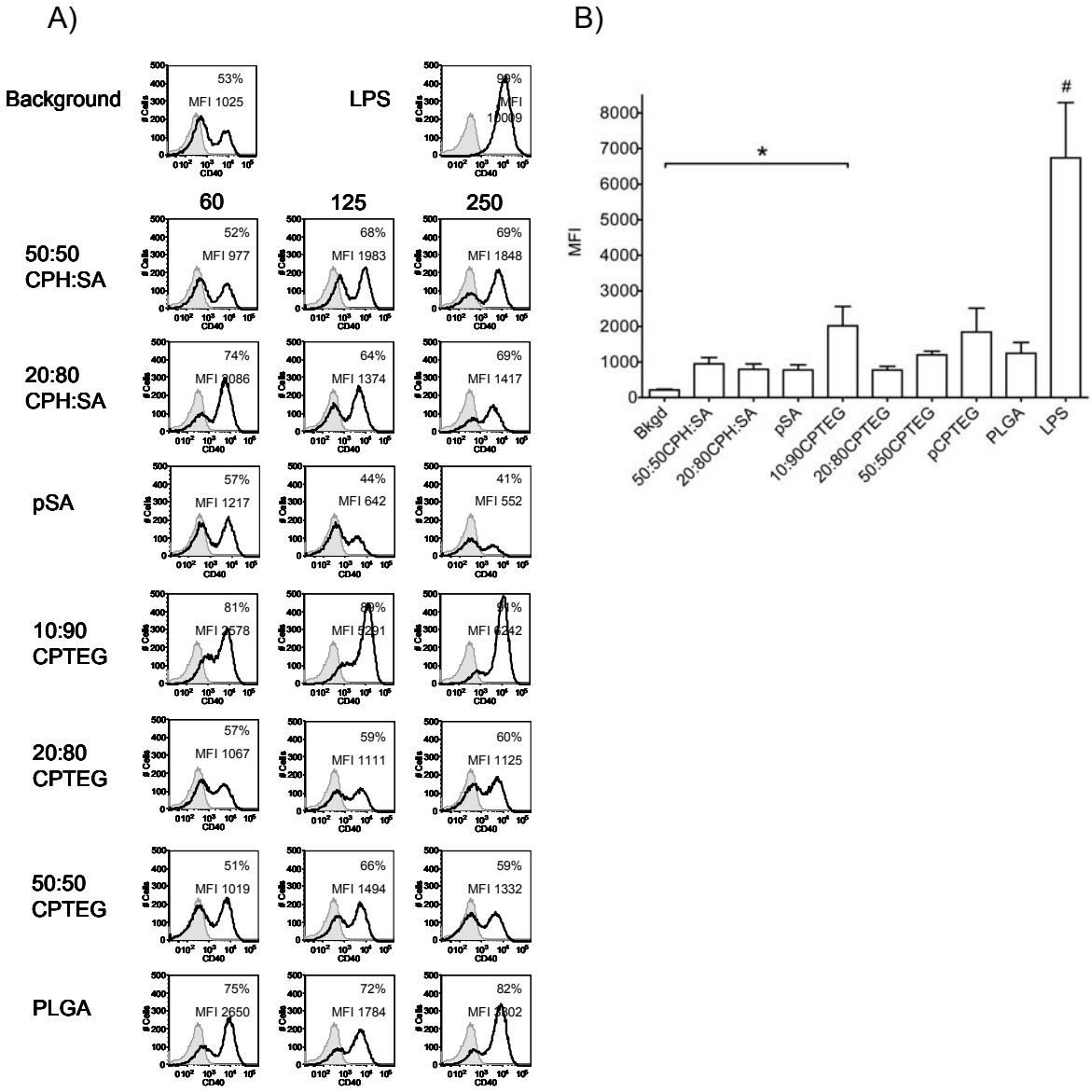

Figure 4: Induction of CD40 expression by DCs stimulated with polymer microspheres. A) Histogram of flow cytometric analysis performed as described in the Materials and Methods. DCs expressing CD40 (open histograms) are superimposed over isotype controls (solid gray histograms). Concentrations of polyanhydride microspheres added to the DCs cultures were 60, 125, or 250 μg/mL corresponding to ratios of 1:3, 1:6, and 1:12 microspheres:DC, respectively. One representative experiment out of three independent experiments shown.
B). Mean MFI of CD40 of three independat experiments (error bars = SEM) at 250 μg/ml concentration. Linked bars are significantly different from each other, * indicates $P \leq 0.05$; bar marked with # is significant from all others, $P \leq 0.001$.

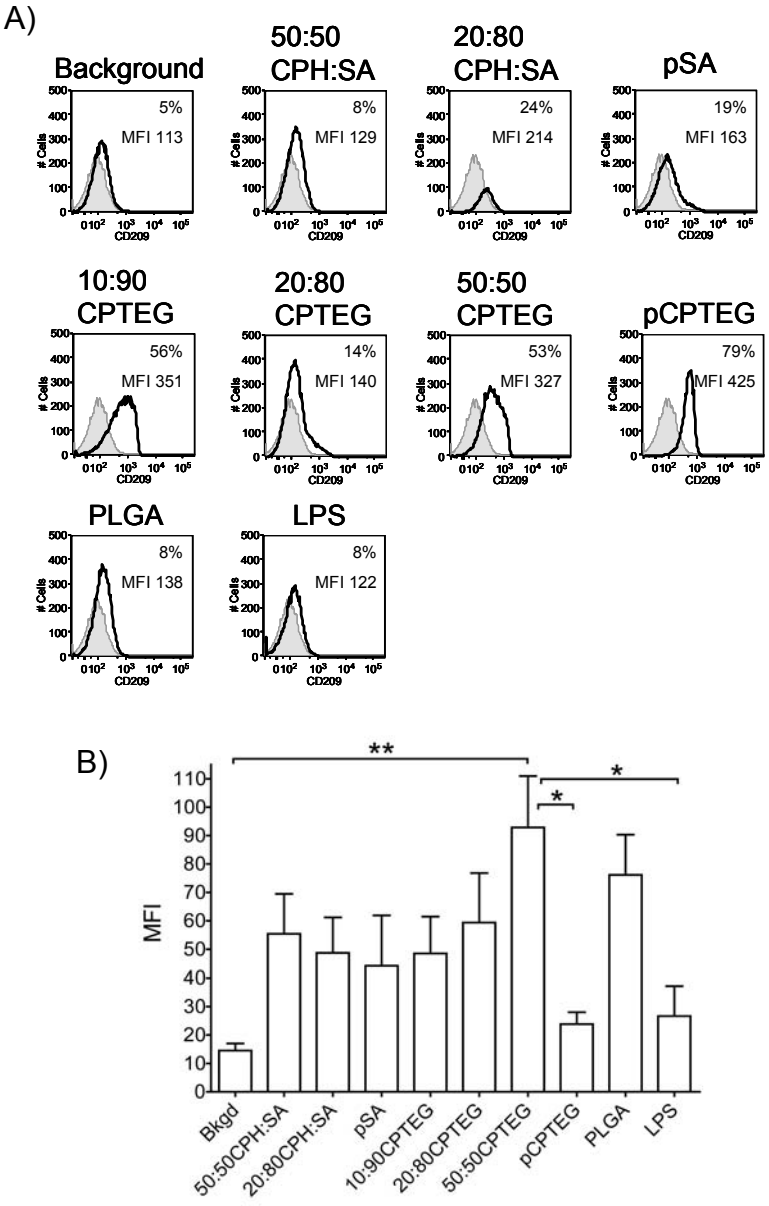

Figure 5: Induction of CD209 expression by DCs stimulated with polymer microspheres. A) Histogram of DCs that were stimulated with 250 µg/mL of microspheres. Flow cytometric analysis was performed as described in the Materials and Methods. Results depicted are representative of one experiment out of three.
B) Mean MFI of three independat experiments (error bars = SEM) at 250 µg/mL concentration. Bars identified with the same letter represent significant differences bewteen those groups ($P < 0.05$). Bkgd = non-stimulated DCs. Linked bars are significantly different from each other, * indicates $P \leq 0.05$; **, $P \leq 0.01$.

3.3 Induction of cytokine secretion by microsphere stimulation

The measurement of the cytokines secreted by stimulated DCs support the flow cytometry findings that polyanhydride microspheres are able to activate DCs in a dose dependent manner (Figure 6A & B). The cytokines that were secreted by DCs stimulated with the microspheres were IL-6 and IL-12p40. The cytokines TNFα, IL-10 and IL-4 were also measured but their levels were below the limits of detection but were detected when DCs were stimulated with lipopolysaccharide, lipotecholic acid, or monophosphoryl-lipid A (data not shown) [57]. The dose response curves for each cytokine were different. Greater amounts of IL-12p40 were produced at doses of 60 or 125 μg/mL in comparison to that induced by 250 μg/mL (Figure 6A). In contrast, production of IL-6 exhibited a positive linear dose response with the greatest amounts induced with the highest dose of polymer tested (250 μg/mL)(Figure 6B). While for most polymers, there was a significant effect of composition of CPH:SA effecting cytokine production, the R^2 values are low indicating that monomer composition is only a small factor in the observed differences between groups (Table 3).

Table 3: Statistical summary of linear fit model to CPH:SA and CPTEG:CPH for the effect of copolymer composition on cytokine release.

Polymer	Cytokine	R^2	P value
CPH:SA	IL-6	0.0298	0.1312
	IL-12p40	0.0742	0.0159
CPTEG:CPH	IL-6	0.0279	0.1180
	IL-12p40	0.0745	0.0096

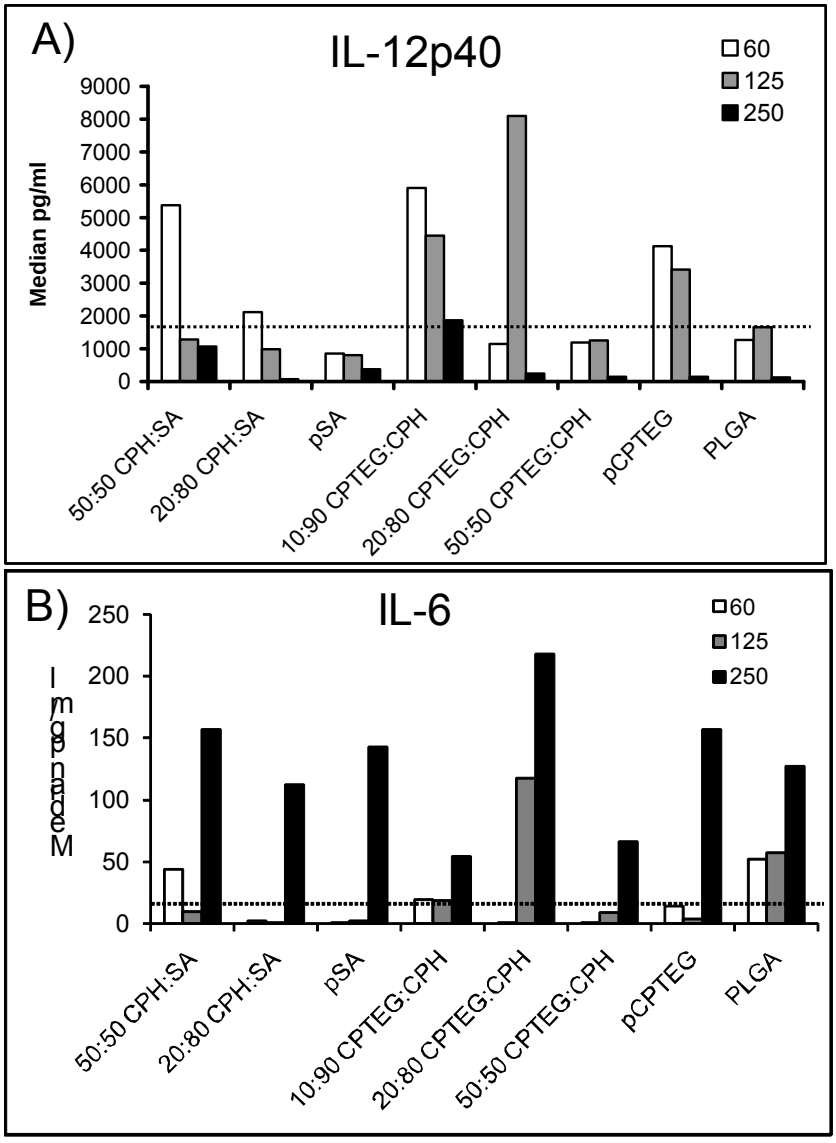

Figure. 6: Cytokines secreted by DCs after incubation with increasing concentrations of polymer microspheres. Panel A:IL-12p40, panel B: IL-6. White bars 60 µg/mL, grey 125 µg/mL, and black 250 µg/mL. Data represented in median concentration of cytokines from four independent experiments. Dashed line represents the amount of cytokine secreted by non-stimulated cells in each experiment (1,522 pg/mL for IL-12p40 and 18 pg/mL for IL-6). LPS was used as a positive stimulant (> 50,000 pg/mL for IL-12p40, and 5,012 pg/mL for IL-6).

3.4 In vitro proliferation of lymphocytes by activated DCs

In order to address the outcome of DC activation by polyanhydride microspheres, lymphocytes were obtained from OT I and OT II mice. These mice express a clonotypic TCR such that in the OT I, approximately 90% of their $CD8^+$ T cells respond to residues 257-264 of Ova and, in the OT II mouse, 90% of their $CD4^+$ T cells respond to peptide 323-339 of Ova. Unfractionated lymph node cells were mixed with DC that had been incubated with microspheres and ovalbumin for 8 to 12 h. Activation of the DCs with LPS served as a positive control and non-stimulated cultures refer to DCs incubated with Ova alone (i.e., no microspheres) (Figure 7). Figure 7 shows the proliferative response of these lymphocytes in the presence of Ova. As observed in other analyses, DCs incubated with pSA induced responses lower than non-stimulated cells. This suggests that the degradation of pSA and the acidic pH generated had detrimental effects on DC and DC-driven T cell activation [47]. A robust proliferative response was observed when $CD8^+$ OT I lymphocytes were incubated with DCs that had been stimulated with PLGA or CPTEG containing microspheres. In contrast, a modest 2-fold increase in lymphocyte proliferation was observed when OT II $CD4^+$ T cells were incubated with DCs stimulated with 50:50 CPH:SA while there was no discernable enhanced response induced by PLGA or CPTEG. Dendritic cells stimulated with LPS induced the greatest proliferative for the $CD4^+$ OT II lymphocytes.

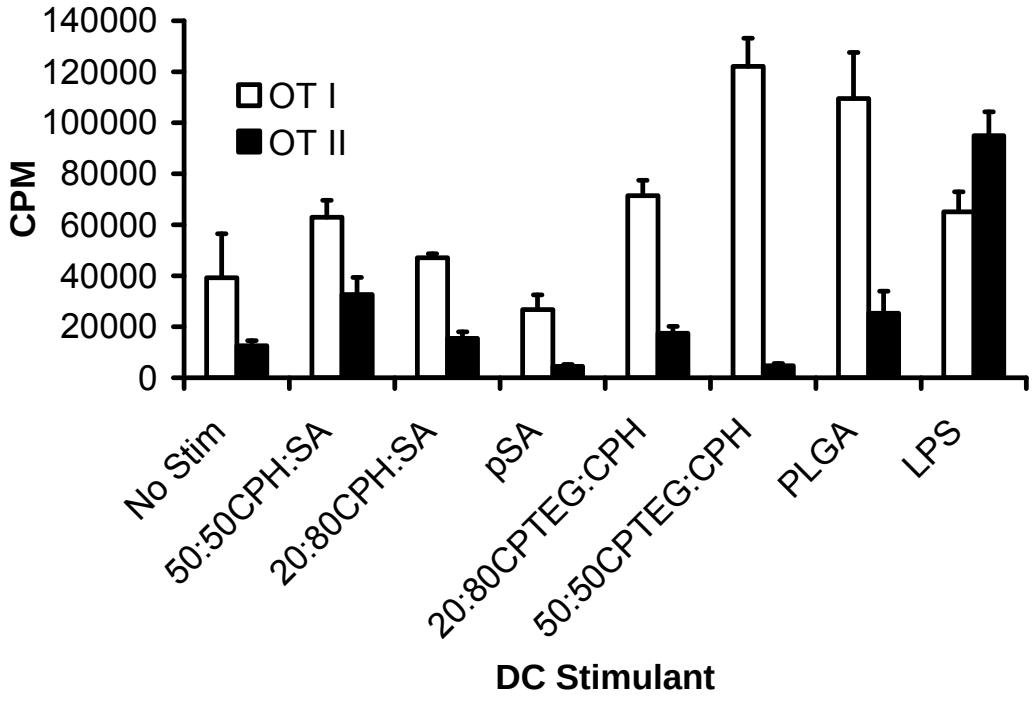

Figure 7: Proliferation of ovalbumin-specific transgenic T cells co-cultured with microsphere stimulated DCs. DCs were stimulated with 100 µg/mL ovalbumin and 250 µg/mL of the indicated microsphere compositions for 8-12 h then lymphocytes recovered from OT I (CD8) and OT II (CD4) mice were added to the stimulated DCs. Cells were incubated for 96 h at which time ^{3}H-thymidine was added and the cells were harvested 18 h later. No Stim wells received Ova and DCs but no microspheres or other stimulant. One representative experiment of three independent experiments is shown. Error bars = SEM of replicate wells.

3.5 Induction of immune response in vivo with polymer adjuvants

In order to evaluate the effects of microsphere as adjuvants, an in vivo model of early events in T cell activation and adaptive immune induction was used. $CD4^+$ T cells from DO11.10 mice were infused into BALB/c recipients. At the same time, the recipients were immunized with 100 μg Ova along with 500 μg of 20:80 CPH:SA, 20:80 CPTEG:CPH, or PLGA microspheres as an adjuvant. 5 days later, mice were euthanized and draining lymph nodes evaluated for evidence of immune activation. Saline treated mice received T cells but no Ova.

$CD4^+$ T cells from DO11.10 mice expressing the Ova Tg TCR can be identified with the monoclonal antibody KJ1.26. Data depicted in Figure 9 is the mean percentage of $CD4^+$ cells positive for KJ1.26 (n = 3-4 mice/group). The percentage of $CD4^+$ lymphocytes positive for $KJ1.26^+$ increased in all mice receiving Ova. A greater increase was seen in animals also receiving an adjuvant along with the Ova.

In order to evaluate the activation state of antigen specific T cell population in the draining lymph node, cells were gated on CD4 and KJ1.26 and evaluated for CD44 and CD62L expression. As T cells move from naïve to effector/memory populations, they become positive for CD44, an integrin receptor, and lose surface expression of CD62L, a homing marker. Nearly all $CD4^+KJ1.26^+$cells are $CD44^{hi}$, but fewer cells (as a percentage) have matured into the memory phenotype ($CD62L^{lo}$) (Figure 9 and Figure 10).

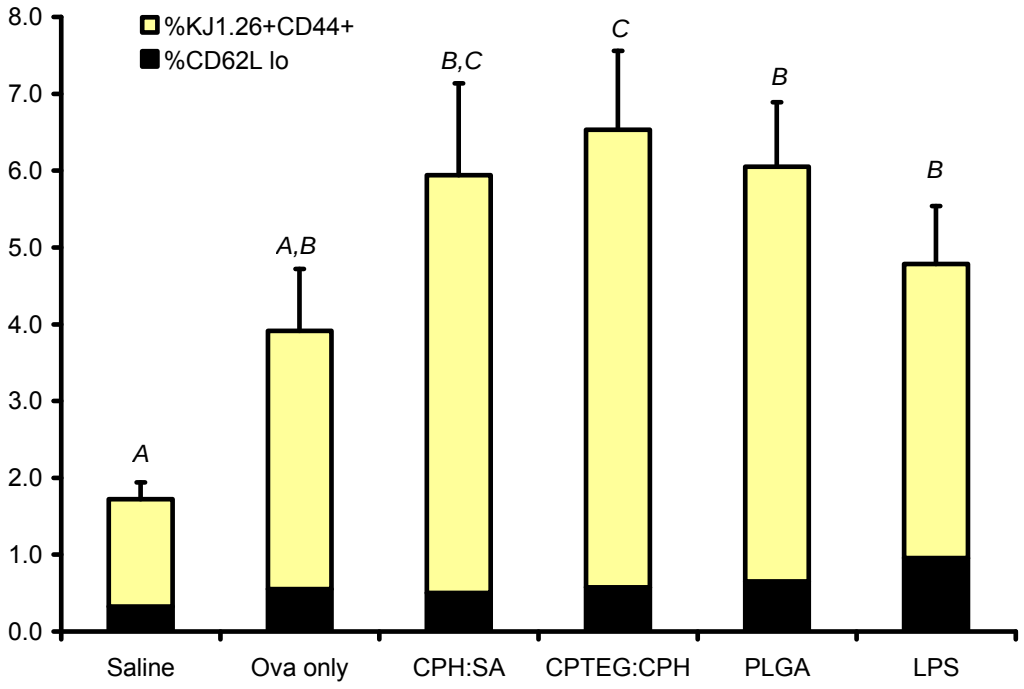

Figure 8: Percentage KJ1.26^{+} T cells in the lymph nodes of BALB/c recipient mice 5 days post-immunization. On day zero, BALB/c mice received 1 x 10^{6} CD4^{+} T cells from DO11.10 and were immunized with one of the following treatments: saline, ovalbumin (Ova) (100 µg) alone, Ova plus 20:80 CPH:SA microspheres (0.5 mg), Ova plus 20:80 CPTEG:CPH (0.5 mg) microspheres, Ova plus PLGA microspheres (0.5 mg), or Ova plus LPS (25 µg). Flow cytometric analysis was performed as described in Materials and Methods. Total bar represents total percentage of KJ1.26^{+} cells which were > 90% CD44^{+}. The lower (black) portion depicts percentage of KJ1.26^{+} cells that were CD44^{+} and CD62L^{lo}. Histograms identified with the same letter are not significantly different from each other but are different from bars marked with a different letter ($P < 0.05$, n=3-5). The data presented is representative of three independent experiments. Error bar is SEM of KJ1.26^{+} cells.

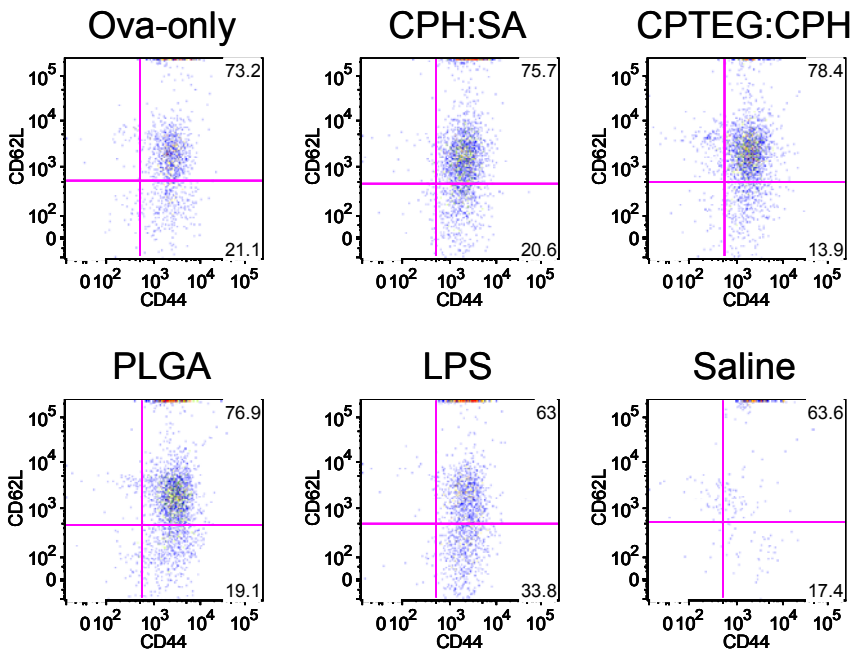

Figure 9: Dot plot of KJ1.26^{+} CD4^{+} T cells recovered from the draining lymph nodes of mice immunized with 100 µg ovalbumin and microsphere adjuvants. Cells were gated on both CD4^{+} and KJ1.26^{+}. Percentages of the right two quadrants are given in the upper and lower right corners. A representative example of each treatment group is shown. n = 3 to 5 mice per group.

To address the induction of the immune response induced by polymer adjuvants, a portion of the lymphocytes recovered from mice described above were stimulated ex vivo with 100 µg ovalbumin and cultured in vitro for three days. Supernatants from these cultures was collected and assayed for the presence of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, and IFN-γ. Results are depicted in Figure 11. IL-4, IL-5, IL-6, and IL-12p40 were below assay detection limits. Very little IL-2, IL-10, and IFN-γ was detected from cells from mice receiving LPS as an adjuvant, whereas cells recovered from mice receiving 20:80 CPH:SA, 20:80 CPTEG:CPH, or PLGA induced Ova-specific T cell recall responses as measured by the production of IL-2, IL-10 and/or IFN-γ.

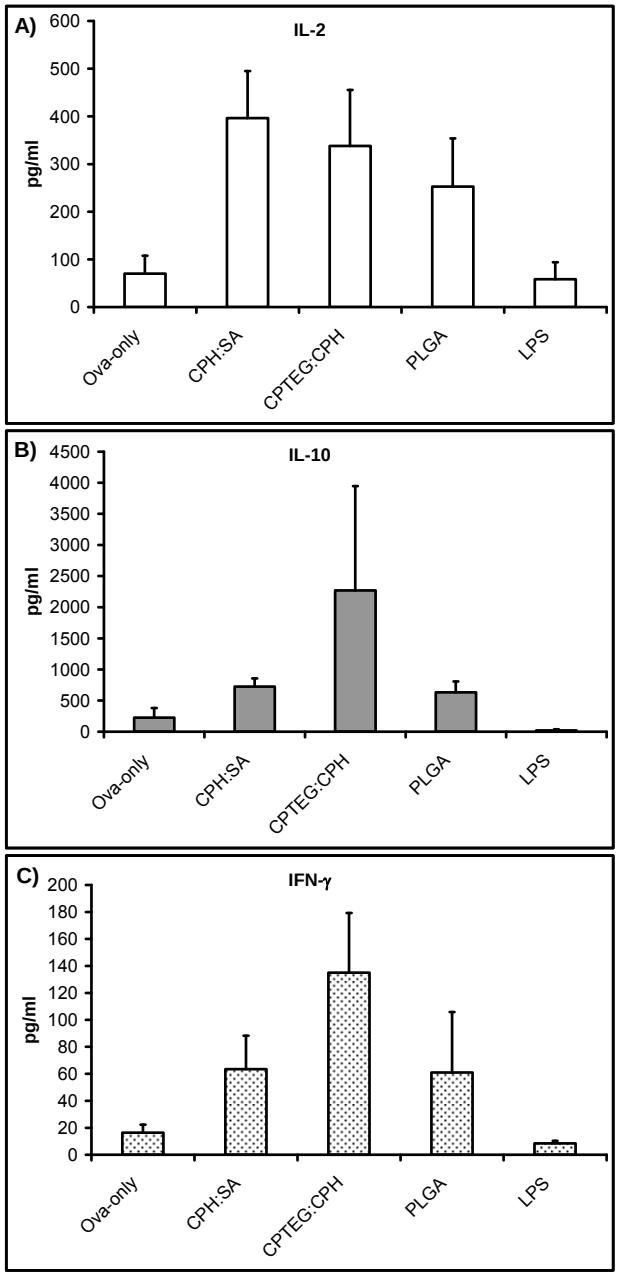

Figure 10: Antigen-specific cytokine responses of lymphocytes recovered from mice vaccinated with ovalbumin in the presence of microspheres. Draining lymph nodes from mice receiving $1x10^6$ DO11.10 $CD4^+$ T cells (Ova responsive), 100 µg ovalbumin and 20:80 CPH:SA microspheres (0.5 mg), 20:80 CPTEG:CPH microspheres (0.5 mg), PLGA (0.5 mg) or LPS (25 µg) were excised 5 days after transfer. Lymphocytes were cultured with 100 µg Ova for 3 days and culture supernatants were analyzed for cytokines. Data represents one experiment of three, 4-6 animals per group, error bars = SEM.

3.6 Antibody production in adjuvanted mice

To further address adjuvant activity of the polyanhydride microspheres, a separate group of mice were immunized as above, receiving Tg DO11.10 T cells and 100 μg ovalbumin in the combination with 20:80 CPH:SA, 20:80 CPTEG:CPH, PLGA, LPS, or saline (i.e., Ova alone). Serum was collected 21 days after transfer/immunization. IgG1 and IgG2a serum antibody titers were evaluated by ELISA (Figure 12A and B). When IgG2a titers were evaluated, overall the magnitude of the IgG2a titers was much lower than IgG1 titers. The only two groups showing any appreciable IgG2a anti-Ova responses was serum collected from mice treated with LPS or CPH:SA.

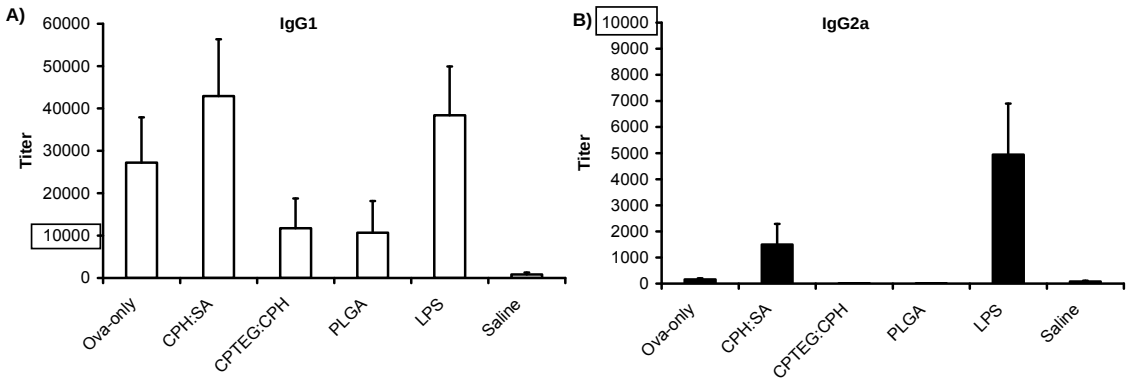

Figure 12: Ovalbumin-specific serum antibody response of mice 21 days after receiving 1 x 10^6 DO11.10 $CD4^+$ T cells. Mice were immunized with 100 μg ovalbumin and the response was adjuvanted with 20:80 CPH:SA microspheres (0.5 mg), 20:80 CPTEG:CPH microspheres (0.5 mg), PLGA (0.5 mg), LPS (25 μg), or saline. Data presented is mean titer of individual mice from three experiments (10-13 mice per group) error bars = SEM. A) IgG1 anti-Ova antibody response. B) IgG2a anti-Ova antibody response. Boxes on y-axis highlight the same magnitude on each panel.

4 Discussion

There is pressing need for novel adjuvants to improve vaccine efficacy and direct the immune response against a particular disease. The rational design of new adjuvants involves developing an in-depth understanding of the complex function of the communication between the innate and adaptive immune systems. These studies demonstrated that polyanhydride microspheres possess characteristics/properties that can be manipulated making them potential promising candidates as vaccine adjuvants. One

important attribute of these biodegradable polymers is that even small amounts of microspheres were capable of significantly activating murine DCs. The ratios (1:3-1:10) of polyanhydride microspheres to DCs used in these studies were significantly lower compared to previous studies employing polyester microspheres [53, 58, 59]. In previously published studies, the polyester microspheres were used at ratios that were more than 50 times the ratio used in the current study [58]. In addition, MPLA was often added to the polyester microspheres when stimulating immune cells [53]. In this study, a head-to-head comparison of two polyanhydrides and polyester chemistries was made with fairly comparable DC activation abilities but marked differences when DC-T cell or in vivo interactions were measured. The ability of a low dose of the polyanhydride microspheres to activate APCs is a very important characteristic to take into consideration when designing vaccines, as the ability to induce enhanced immune responses with a minimum of adjuvant would be a desirable outcome (i.e., cost effectiveness). Similar DC activation experiments were performed using DCs derived from C3H/HeNHsd (C3H), BALB/c, or C57BL/6 (BL6) mice with respect to induction of cell surface marker expression and there were no observable differences in the robustness or magnitude of responses of DCs derived from the different mouse strains. This is important to note as the OT I/OT II and DO11.10 mouse models are in different genetic backgrounds.

Overall, the results indicate that there was differential activation as shown by enhanced surface expression of MHC II and the co-stimulatory molecules CD86, CD40, and CD209 by CPH:SA and CPTEG:CPH containing microspheres, suggesting that adjuvant chemistry plays a major role in the activation of DCs. CPTEG containing compounds are more hydrophilic than SA containing compound, with 50:50CPH:SA being the most hydrophobic of all compositions. Hydrophobicity relates not only to protein interactions but also erosion kinetics. It is very interesting to note that while CPTEG:CPH copolymers induced the greatest stimulation of surface marker expression, the CPH:SA (namely, 50:50 CPH:SA) enhanced a greater proliferative response of $CD4^+$ T cells responses both in vitro and in vivo. These studies clearly show that in terms of cytokine secretion and surface marker expression, CPTEG containing compounds are

slightly superior to CPH:SA or PLGA microspheres at activating DCs. Furthermore, CPTEG containing microspheres were the only formulation to promote the expression of CD209 suggesting different mechanisms of particle recognition and uptake. This being said, there are other properties (erosion type and protein interactions) that may be useful in CPH:SA containing microspheres. While CPTEG:CPH copolymers enhanced surface marker expression (Figures 2-6), CPH:SA copolymers enhanced $CD4^+$ responses (Figures 7 & 12).

It has been recently shown that polyanhydride chemistry affects protein release and stability [48]. In these previous studies, the amphiphilic, high CPTEG-containing microspheres provided for greater protein stability. This observation is in accordance with the fact that carriers containing both hydrophobic and hydrophilic entities may provide a gentler environment for proteins [60, 61]. The amphiphilic nature of these materials also change their erosion properties from surface eroding (e.g. 50:50 CPH:SA) to bulk eroding, like PLGA.

Since it is known that a small number of DCs are sufficient to induce strong immune responses [62], all the polyanhydride chemistries studied here still have potential as adjuvants. A small amount of ligand, originating from pathogens or damaged cells trigger activation in innate immune cells through TLR, C-type lectin receptors and other pathogen recognition receptors [63]. Matzinger proposed that hydrophobic molecules may trigger these pattern (or pathogen) pattern recognition receptors [63]. The polyanhydrides included in these studies are relatively hydrophobic, especially when compared to sugars and lipids, it is likely that the hydrophobic nature of these microspheres activated the DCs by triggering a pattern recognition receptor. As CPH:SA and CPTEG:CPH induced differing levels of surface marker expression and cytokine secretion, it is likely that the two chemistries may trigger intracellular signaling by different receptors and/or different pathways which result in the different responses observed. An optimal vaccine formulation might consist of a cocktail of microspheres of different compositions, with different release profiles that optimizes antigen stability over longer periods of time and at the same time enhances the activation and maturation of DCs leading to the development of the preferred immune response (Th1, Th2 or Th17).

The specific cytokine profile, in conjunction with surface marker expression, can influence the bias and magnitude of an immune response. The secretion of IL-12p40 and the absence of IL-4 suggest that polyanhydride chemistries preferentially induce a Th1-type response. As the mere activation of DCs is not an absolute indicator of an effective immune response, the further evaluation of the activation of other immune effector cells (e.g., T and B cells) is required.

In summary, this study showed that these biodegradable polymers have promising characteristics for the development of vaccine adjuvants. The three major functions of an adjuvant include: (1) providing a "depot" or reservoir for the antigen for a slow release; (2) enhancing antigen phagocytosis by APCs; and (3) modulating and enhancing the immune response against the particular antigen alone. Previous work has evaluated the depot potential through protein stabilization and release of antigen after short-term storage (48). The studies presented here show that the polyanhydride microspheres activate APCs by modulating cell surface marker expression, cytokine secretion, and enhancing the immune response to a co-delivered antigen.

5 Conclusions

Despite the advantages of enhancing and possibly modulating immunogenicity by using polymeric microspheres as vaccine carriers, no vaccines based on polymeric carriers have been approved for human use to date. The ability of polyanhydride microspheres to activate murine DCs in vitro and contribute to immune activation in vivo, in conjunction with their biocompatibility and potential for enhanced protein stability, demonstrates that these are promising candidates for the development of vaccines. In addition, the studies described here clearly point out the role of polymer chemistry in APC activation and the disconnect between experimental models employing a single cell type and the complex interaction within the mammalian host. Regardless, these studies lay the foundation for further investigation into the molecular and cellular mechanisms responsible for this effect.

6 Author's Contributions

JWW, MT and MJW designed the experiments. JWW, SL and MT performed the DC activation studies. MT and SL made and analyzed the microspheres. JWW performed the T cell proliferation and all in vivo studies. JWW performed all data analysis. MT and JWW contributed equally to the writing of the manuscript. BV performed the statistical analysis of the DC activation study and advised on statistics for the remainder. All authors are responsible for critical revisions and intellectually contributed. NB was major professor to MT and SL, MJW was major professor for JWW.

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CHAPTER 4

Immune Response to Ovalbumin Encapsulated in Polyanhydride Microspheres

[1]Jennifer H. Wilson Welder, [2]Brenda Carrillo, [2]Maria P. Torres, [2]Senja Lopac, [2]*Balaji Narasimhan, [1]Michael J. Wannemuehler

[1]Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011
[2]Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

To be submitted to Journal of Controlled Release

Correspondence: Balaji Narasimhan, Department of Chemical and Biological Engineering, Iowa State University, 2035 Sweeney Hall, Ames, IA, 50011. Telephone: (515) 294-8019. Fax: (515) 294-2689. Email: nbalaji@iastate.edu

Abstract

There is a need to develop adjuvants that can stabilize multi-epitope vaccine antigens. Biodegradable polyanhydrides based on the aliphatic sebacic acid (SA), the aromatic 1,6-bis(*p*-carboxyphenoxy)hexane (CPH), and the amphiphilic 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) have exhibited characteristics that make for promising protein-based vaccine adjuvants. The current study evaluates the immune response to ovalbumin (Ova) encapsulated into 20:80 CPH:SA and 50:50 CPH:SA microspheres fabricated by two different methods (S/O/O and cryogenic atomization) using three different mouse strains (BALB/c, C3H/HeN, and C57BL/6). Protein stability was effected by choice of polymer chemistry and fabrication method In addition, the magnitude of antigen-specific immune responsiveness to the encapsulated Ova was mouse strain dependent. While only modest immune responses were observed in BALB/c and C3H/HeN mice given a single dose of polymer encapsulated Ova, a greater response

was seen after the mice were given a small antigenic challenge or boost five days prior to evaluation of antibody responses and lymphocyte proliferation. The immunological response of BALB/c mice was more susceptible to protein instability induced by microsphere fabrication method (S/O/O vs. cryogenic atomization). Microspheres comprised of 50:50 CPTEG:CPH enhanced the stability of encapsulated Ova and enhanced the immune response to encapsulated Ova; however, immunization with this copolymer formulation was only tested in C57BL/6 mice. It is hypothesized that degradation of Ova observed in other studies with CPH:SA co-polymers results in lost epitopes of a weak immunogen. Protein stability must be considered when designing vaccine adjuvant and delivery systems for which biodegradable polyanhydrides have been proposed, especially in the context of designing multi-epitope vaccines that would need to be efficacious in a non-homogenous MHC haplotype population.

1 Introduction

According to the NIH, infectious disease remains the second leading cause of death worldwide [1]. Vaccination is the most cost effective means for the prevention of disease. Many new vaccines under development consist of rationally designed recombinant proteins that are often relatively poor immunogens. Thus, adjuvants are employed in order to enhance the immune response (e.g., antibody titer, T cell memory) to these recombinant antigens. Currently, licensed adjuvants (e.g., alum or MPLA) induce effective humoral immunity but are poor inducers of cell-mediated immunity (CMI). Thus, there is a need to develop adjuvants that will enhance both. Recent studies demonstrate that biodegradable microspheres based on novel polyanhydrides have the properties and characteristics that make them suitable carriers for vaccine delivery [2-7]. These characteristics include an amphiphilic environment for protein stabilization, enhanced adjuvant effect (controlled by polymer chemistry), immunomodulatory capabilities (Th1/Th2 balance), and lower monomer solubility in water (leading to micro-environments with a more suitable pH) [2-7]. Previous immunization studies in mice evaluated the immune response against tetanus toxoid (TT) released from polyanhydride microspheres [5]. Upon release from the microspheres, the TT maintained its

immunogenicity and antigenicity. In addition, the microspheres provided adjuvant-like activity and a prolonged exposure to TT that was sufficient to induce high titer anti-TT antibody responses following a single administration [3, 5]. Additionally, a shift in the induction of antigen-specific IgG1-IgG2a antibody isotypes was observed in a polymer chemistry-dependent manner [5].

The ability of a substance to induce antigen-specific T cells of the desired phenotype and to maintain a sufficient immune response to provide protective immunity is crucial to the rational design of vaccines. The purpose of this study was to evaluate the in vivo induction of an immune response to ovalbumin (Ova) encapsulated in novel polyanhydride microspheres.

2 Materials and Methods

2.1 Materials and polymer synthesis

The polyanhydride chemistries used in this study are based on the aliphatic sebacic acid (SA), the aromatic 1,6-bis(*p*-carboxyphenoxy)hexane (CPH), and the amphiphilic 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) (Figure 1). The chemicals needed for the synthesis of CPH and CPTEG monomers include: 4-*p*-hydroxybenzoic acid, 1,6-dibromohexane, 1-methyl-2-pyrrolidinone, and tri-ethylene glycol, and sebacic acid (99%) were purchased from Sigma Aldrich (St Louis, MO); 4-*p*-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); potassium carbonate, dimethyl formamide, toluene, sulfuric acid, acetic acid, acetonitrile, acetic anhydride, methylene chloride, and petroleum ether were purchased from Fisher Scientific (Fairlawn, NJ). CPH:SA and CPTEG:CPH copolymers were synthesized by melt polycondensation as described previously [8]. The purity and degree of polymerization of the polymers was analyzed using ^{1}H NMR spectra obtained from a Varian VXR-300 MHz NMR spectrometer.

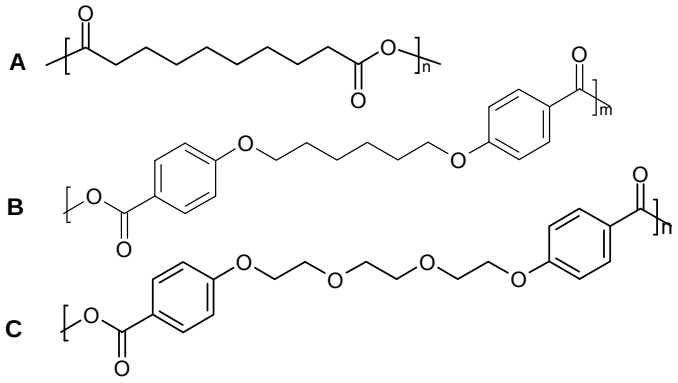

Figure 1: Chemical structures of A) aliphatic poly-sebacic acid (SA), B) aromatic poly-1,6-bis(*p*-carboxyphenoxy)hexane (CPH), and C) amphiphilic poly1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG). m and n represents degree of polimerization. Microspheres were made from copolymers of CPH and SA or CPTEG and CPH in molar ratios of 20% CPH and 80% SA (20:80) or 50% CPH and 50% SA (50:50) or 50% CPH and 50% CPTEG (50:50 CPTEG).

2.2 Endotoxin removal from Ova

Ovalbumin to be used in antigen specific studies was tested for endotoxin levels. Two mg/mL solutions of Ova were prepared in endotoxin free water and assayed with Limulus Amebocyte Lysate (LAL) QCL-1000 test kit (Cambrex, Walkersville, MD). LAL was performed according to manufacturer's instructions. Ovalbumin, as purchased, contained endotoxin in amounts > 100,000 EU/mg protein. To remove contaminating endotoxin, AffinityPak Dextoxi-Gel endotoxin removing gel columns (Thermo Scientific, Rockford, IL) were used according to manufacturer's instructions. Resulting Ova contained less than 10 EU/mg which equates to roughly 1 ng endotoxin equivalent per mg protein. Endotoxin-free Ova was lyophilized and stored at -20ºC until needed.

2.3 Microsphere fabrication

2.3.1 Solid-oil-oil emulsion

Ovalbumin loaded microspheres were fabricated using a modified solid/oil/oil (s/o/o) method [3, 9, 10]. Briefly, lyophilized Ova (2-3 mg) were suspended in a solution of 100 mg of 20:80 or 50:50 CPH:SA copolymer dissolved in 2 mL of methylene

chloride to produce the first emulsion. The suspension was obtained by homogenizing the solution at 20,000 rpm for 3 minutes using a Tissue-Tearor™. The second emulsion was produced by adding 3 mL of Dow Corning oil 550 saturated with methylene chloride. The mixture was poured into 200 mL of ice cold heptane and stirred for 2 h at 300 rpm. Microspheres were collected by filtration and dried under vacuum. Blank microspheres were prepared by the same method, omitting the Ova in the first emulsion.

2.3.2 Cryogenic atomization

Cryogenic atomization (CA) method has also been shown to provide for high encapsulation efficiencies of the desired immunogen [6, 7]. As above, microspheres of 20:80 and 50:50 CPH:SA and 50:50 CPTEG:CPH compositions were prepared using procedures as previously reported [3, 11]. Following suspension of the lyophilized Ova (2-3 mg) in the polymer/methylene chloride solution, solution was stirred at 10,000 rpm for 1 min using a Tissue-Tearor™. The solution was pumped with a syringe pump through an 8700-1200 MS ultrasonic atomizing nozzle (Sono Tek Corporation, Milton, NY) into 200 mL of frozen ethanol overlaid with ~100 mL of liquid nitrogen. After atomization, the resulting polymer/protein solution was stored at -80°C for three days to allow the methylene chloride to be extracted and the resulting microspheres were collected by filtration and dried under vacuum. Microsphere morphology was characterized by scanning electron microscopy and particle size distribution will be obtained from SEM images (250-500x) using analySIS® software (Soft Imaging System Corp, Lakewood, CO).

2.4 Mice

Female C3H/HeNHsd (C3H) and C57BL/6 (BL6) mice were purchased from Harlan Sprague Dawley. Male and female BALB/c mice, at least 6 weeks of age, were obtained from the breeding colony maintained by the Hybridoma Facility at Iowa State University (Ames, IA). All of the mice were housed under specific pathogen-free conditions where all bedding, caging, water, and feed were sterilized prior to use.

Animal procedures were conducted with the approval of the Iowa State University Institutional Animal Care and Use Committee.

2.5 Immunizations and blood collection

Mice were immunized in the right hind leg (IM) with 0.5 mg of microspheres (20:80 CPH:SA Ova-loaded, 50:50 CPH:SA Ova-loaded, 20:80 CPH:SA-blank, 50:50 CPH:SA-blank or 50:50 CPTEG:CPH Ova-loaded) suspended in pyrogen-free saline. Microspheres were sonicated briefly to disperse clumps prior to immunization using a 23 gauge (or smaller) needle. A total volume of 100 μL was administered into the right quadriceps. Mice immunized with Ova alone received 25 μg Ova in 100 μL pyrogen-free saline administered with 26 gauge needles into the right quadriceps. Control animals received 100 μL saline alone. Blood samples were collected from the left saphenous vein prior to immunization and every two weeks thereafter. Serum was collected by centrifugation and stored at -20°C until assayed for Ova-specific antibody by ELISA.

At twelve weeks post immunization (PI), some animals received a booster dose consisting of 25 μg Ova in 100 μL saline IM. This boost dose was given 5 days prior to euthanization.

2.6 Culture and in vitro restimulation of lymphocytes

Twelve weeks following immunization, lymph nodes draining the immunization site (right popliteal and inguinal) were excised and single cell suspensions prepared. Cells were counted and resuspended at 2.5 x 10^6 cells/mL in complete culture medium (RPMI 1640 supplemented with 2% essential amino acids (Mediatech, Herndon, VA), 1% non-essential amino acids (Mediatech), 100 mM sodium pyruvate (Mediatech), 25 mM HEPES buffer (Mediatech), 100 units/mL penicillin, 0.1 mg/mL streptomycin (Mediatech), 0.05 mg/mL gentamicin (Sigma), 100 mM l-glutamine (Mediatech), 5 x 10^5 M 2-mercaptoethanol (Sigma), 2.5% heat inactivated fetal bovine serum (FBS) (Valley Biomedical, Winchester, VA)). Separate well containing lymphocytes were incubated with either concanavalin A (ConA) (Sigma) (1 μg/mL), Ova (50 μg/mL) or medium alone. Cells were incubated at 37°C in 5% CO_2 in air. Culture supernatants were

collected at 72 h and frozen for later cytokine analysis. Medium was replaced with fresh complete culture medium. 0.5 μCi of methyl-[^{3}H]-thymidine (specific activity 6.7 Ci mmole^{-1}, Amersham Life Science, Arlington Heights, IL) was added to each well and incubated for additional 18 h. Well contents were harvested onto glass fiber filters and the incorporated radioactivity was measured using liquid scintillation counter. The assays were performed in triplicate and data are presented as mean counts per minute of triplicate wells.

2.7 Cytokine assay

Cytokines IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-17a and IFN-γ were assayed from cell free supernatants collected from lymphocytes cultured as described above. Supernatants were collected at 72 h after in vitro re-stimulation with antigen and stored at -20°C until assayed using Luminex® Multiplex assay (Austin, TX).

2.8 Enzyme-linked immunosorbent assay (ELISA)

Serum samples collected from mice prior to immunization and every two weeks following immunization were tested for Ova-specific antibodies. Costar brand high binding ELISA plates (Catalog # 3590, EIA/RIA high binding) were coated overnight with 5 μg/mL Ova. Plates were washed with phosphate buffered saline (PBS, pH 7.4) containing 0.5% Tween 20 (PBST) and blocked for two hours with PBST+ 2% gelatin (Difco, catalog # 214340). Plates were washed and individual serum samples were serially diluted in PBST and incubated overnight at 4°C. On the third day, plates were washed again with PBST and alkaline phosphatase-conjugated goat anti-mouse IgG (H&L), IgG1, IgG2a or IgG2c (Jackson ImmunoResearch Laboratories, West Grove, PA for reagents to detect serum antibody from BL6 and BALB/c mice, or Southern Biotechnology Associates, Birmingham, AL to detect serum antibody from C3H mice) (1:1000 dilution) was added. After incubating for two hours, plates were washed and p-nitrophenyl phosphate (Sigma 104) substrate (1 mg/mL) in carbonate buffer (pH 9.3) was added to each well. Changes in optical density (OD) were spectrophotometrically measured at 405 nm.

2.9 Statistical analysis

One-way analysis of variance (ANOVA) was performed on experimental data sets, with Tukey pair-wise comparison post-tests using GraphPad Prisim 4.0 for Macintosh (GraphPad Software, La Jolla, CA).

3 Results

3.1 Twelve week immunization in BALB/c and C3H with Ova-loaded microspheres prepared by S/O/O.

In order to evaluate the ability of Ova-loaded microspheres to induce an antigen-specific immune response, mice received a single immunization dose as described in Materials and Methods. At twelve weeks PI, half of the animals received a 25 μg antigenic challenge (i.e., booster dose). Figures 2, 3, and 4 depict data from BALB/c mice, Figures 5, 6, and 7 depict data from C3H mice, all receiving 50:50 CPH:SA or 20:80 CPH:SA Ova-loaded microspheres fabricated using the S/O/O method. Figure 2 and 5 show the total antibody specific for Ova as measured over time. Figure 3 and 6 depict the isotype specific antibodies to Ova at 12 weeks. Figure 4 and 7 depict the antigen specific proliferation of lymphocytes recovered from these mice at 12 weeks PI. The ConA-induced proliferative simulation index was 10 to 30 times that of background or non-simulated responses (data not shown). After 3 days re-stimulation with Ova, cell free supernatants were collected from cells recovered from the C3H mice and analyzed for antigen-specific cytokines released upon restimulation . Cytokines IL-4 and IL-6 were below detectable limits. IFN-γ and IL-12p40 were secreted by cells stimulated with ConA , but there was no demonstrable secretion of Ova-induced T cell cytokines by cells recovered from the mice immunized with the Ova-loaded microspheres (Data not shown).

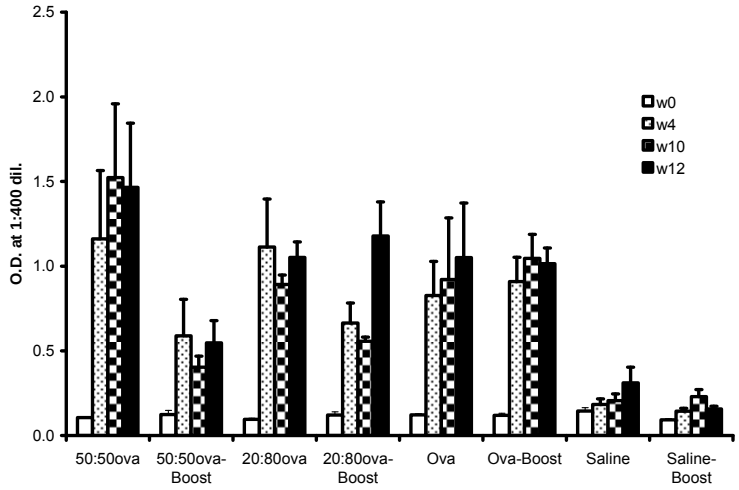

Figure 2: Ovalbumin-specific serum antibody (IgG, H&L) response of BALB/c mice receiving Ova-loaded CPH:SA microspheres fabricated by S/O/O method, with and without Ova boost (25 µg Ova) given 5 days prior to necropsy. Mice were immunized with the noted Ova-loaded CPH:SA microsphere formulations, 25 µg soluble Ova, or saline alone. Mice received a single immunization at week 0 (w0) and samples collected at intervals over 12 weeks (w12). ELISA was performed as described in Materials and Methods. Data is presented as the mean $\pm$ SEM titer of serum samples from five mice per group. Error bars represent the standard error of the mean.

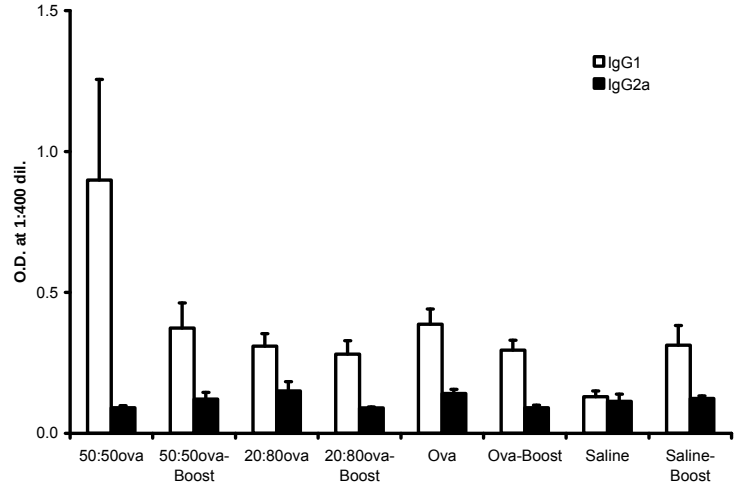

Figure 3: Isotype specific serum antibody to ovalbumin from BALB/c mice receiving CPH:SA Ova-loaded microspheres fabricated by S/O/O method, with and without boost (25 µg Ova) 5 days prior to necropsy. ELISA performed as described in Materials and Methods. Data presented is mean optical density (O.D.) at 1:400 dilution of sera collected at 12 weeks after a single immunization, error bars = SEM (n = 5)

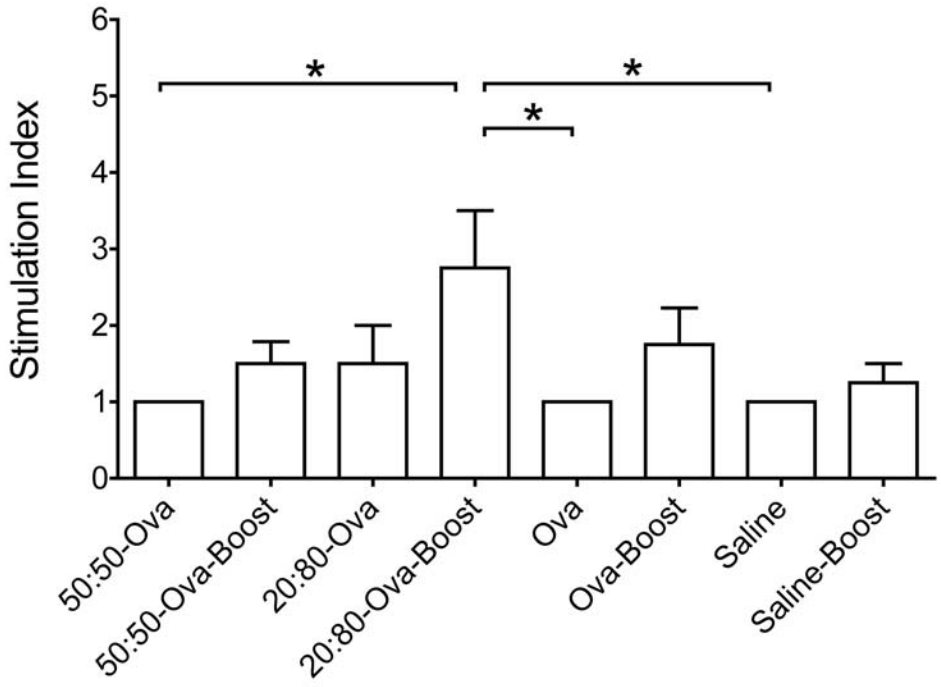

Figure 4: Induction of antigen-specific lymphocyte proliferation in cells recovered from BALB/c mice receiving Ova-loaded CPH:SA microspheres fabricated using S/O/O. At 12 weeks post-immunization, the proliferative response of lymphocytes recovered from the draining lymph was evaluated. Specifically, the treatment groups consisted of mice that had been immunized with Ova-loaded microspheres, soluble ovalbumin (Ova), or saline. Half of the mice in each group received 25 μg Ova 5 days prior to necropsy (Boost). Single cell suspensions of lymphocytes were stimulated with 50 μg/ml Ova in vitro for 72 h at which time ^{3}H-thymidine was added and the cultures were incubated for another 18h. Stimulation index was calculated from counts per minute of Ova-stimulated cells divided by the counts per minute of unstimulated cells. Data is presented as the mean stimulation index $\pm$ SEM (n=5). Linked bars with indicate statically significant difference between groups, * indicates $P \leq 0.05$.

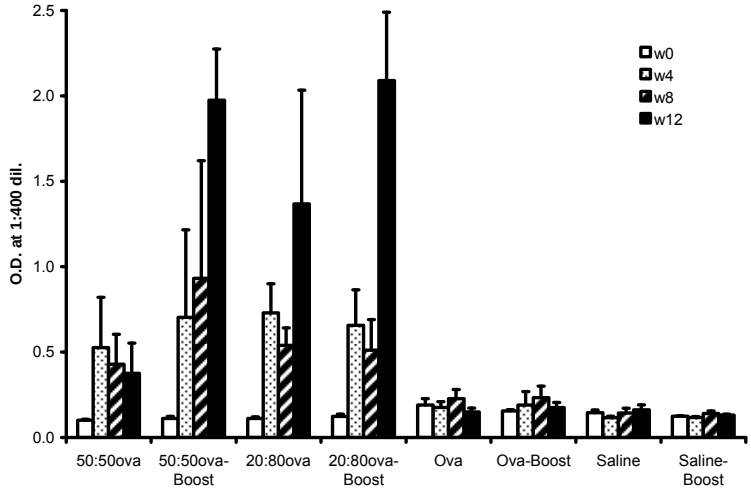

Figure 5: Ovalbumin-specific serum antibody (IgG, H&L) response of C3H mice receiving Ova-loaded CPH:SA microspheres fabricated by S/O/O method, with and without boost (25 µg Ova) 5 days prior to necropsy. Mice were immunized with Ova-loaded CPH:SA microspheres, 25 µg soluble Ova, or saline alone. Mice received a single immunization at week 0 (w0) and samples collected at intervals over 12 weeks (w12). ELISA performed as described in Materials and Methods. Data is presented as the mean $\pm$ SEM titer of serum samples from five mice per group. Error bars represent the standard error of the mean.

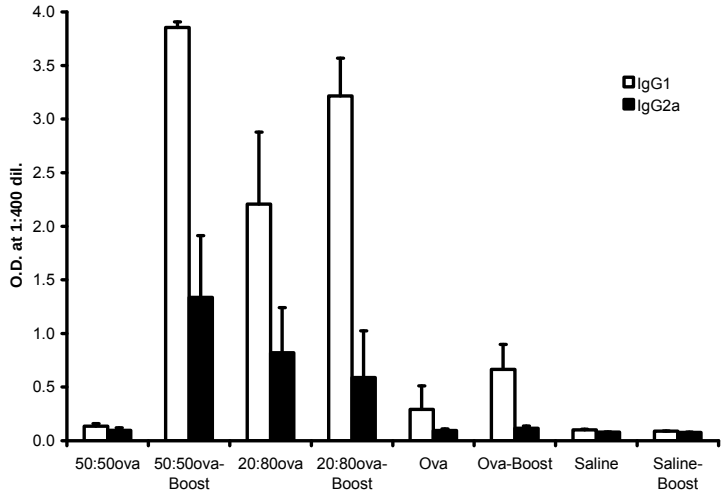

Figure 6: Isotype specific serum antibody to ovalbumin from C3H mice receiving Ova-loaded CPH:SA microspheres (0.5 mg microspheres, 25 µg Ova) fabricated by S/O/O method, with and without boost (25 µg Ova) five days prior to necropsy. ELISA performed as described in Materials and Methods. Data is presented as the mean $\pm$ SEM optical density (O.D.) at 1:400 dilution of sera collected at 12 weeks following a single immunization.(n = 5)

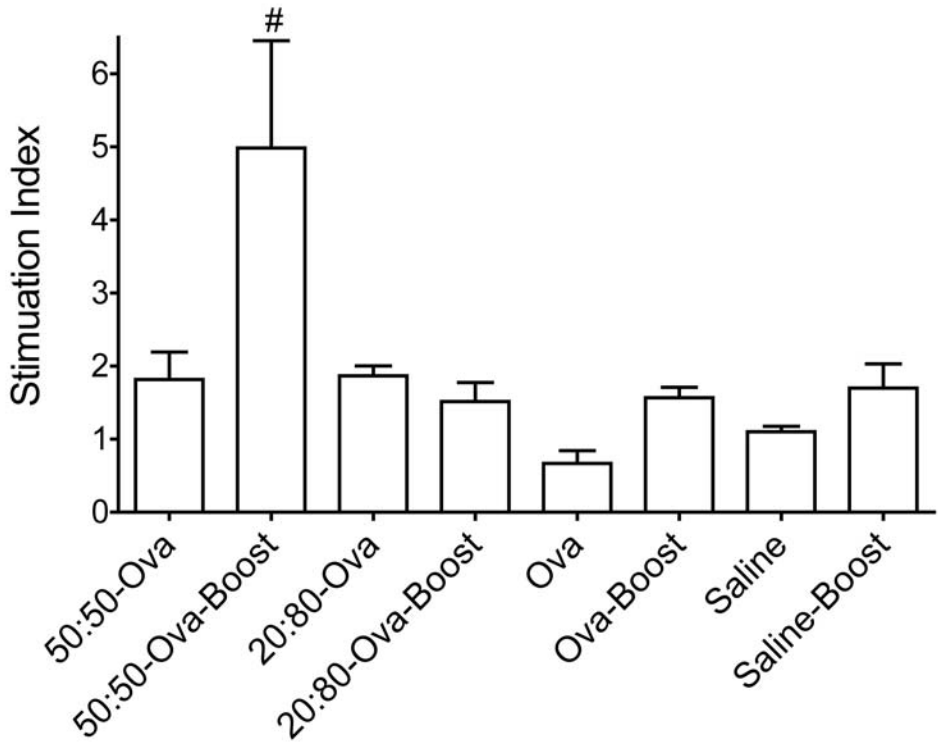

Figure 7: Induction of antigen-specific lymphocyte proliferation from cells recovered from C3H mice receiving Ova-loaded CPH:SA microspheres fabricated using S/O/O. At 12 weeks post-immunization, the proliferative response of lymphocytes recovered from the draining lymph was evaluated. Specifically, the treatment groups consisted of mice that had been immunized with Ova-loaded microspheres, soluble ovalbumin (Ova), or saline. Half of the mice in each group received 25 μg Ova 5 days prior to necropsy (Boost). Single cell suspensions of lymphocytes were stimulated with 50 μg/ml Ova in vitro for 72 h at which time ^{3}H-thymidine was added and the cultures were incubated for another 18h. Stimulation index was calculated from counts per minute of Ova-stimulated cells divided by the counts per minute of unstimulated cells. Data is presented as the mean stimulation index $\pm$ SEM (n=5). # indicates group mean is statically significant from other groups, $P \leq 0.001$.

In addition to antigen-loaded microspheres, for each strain (BALB/c and C3H) there were groups of mice that received blank (no-antigen) microspheres of each polymer formulation and these mice served as adjuvant controls. Consistently, no detectable antigen-specific immune response (antibody or proliferation) was observed in these control treatment groups (data not shown).

3.2 Immunization in BALB/c, C3H/HeN, and C57BL/6 with Ova-loaded microspheres prepared by cryogenic atomization.

In order to evaluate the effect of fabrication method on the induction of the immune response, mice were immunized with Ova-loaded microspheres that had been prepared by the CA method as described in Materials and Methods. At twelve weeks PI, half of the animals received a 25 μg antigenic boost. Figures 8, 9, and 10 depict data from BALB/c mice, Figures 11, 12, and 13 depict data from C3H mice, and Figure 14 depicts data from BL6 mice, all receiving 50:50 CPH:SA or 20:80 CPH:SA microspheres loaded with Ova. In the studies using BL6 mice, induction of Ova-specific antibody responses by 50:50 CPTEG:CPH microspheres loaded with Ova were also evaluated. Figure 8 and 11 show the total Ova-specific antibody response as measured over time. Figure 9, 12, and 14 depict the isotype specific antibodies to Ova at 12 weeks PI. Figure 10 and 13 depict the antigen-specific proliferation of lymphocytes recovered from these mice, 12 weeks PI. The ConA-induced proliferative simulation index was 5 to 20 times background or non-simulated response (data not shown). After 3 days of in vitro re-stimulation with Ova, cell free supernatants were collected from cells recovered from spleen and lymph node of the BL6 mice and analyzed for cytokine release. Cytokines IL-4, IL-6, and IL-17a were below detectable limits. IL-2, IL-12p40, and IFN-γ were secreted when cells were stimulated with ConA. IL-5 and IL-10 were secreted from lymph node cells restimulated with Ova; however, cytokine secretion was greater from cells recovered from the spleen of the immunized mice. There were no detectable differences in the induction of IL-12p40 or IFN-γ secretion when comparing the responses between Ova-immunized and sham immunized animals (data not shown).

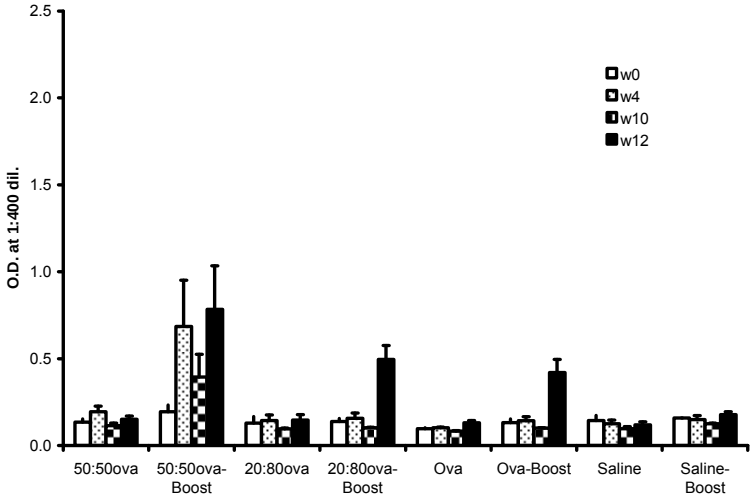

Figure 8: Ovalbumin-specific serum antibody (IgG, H&L) response of BALB/c mice receiving Ova-loaded CPH:SA microspheres fabricated by CA, with and without boost (25 µg Ova) 5 days prior to necropsy. Mice were immunized with Ova-loaded CPH:SA microspheres, 25 µg soluble Ova, or saline alone. Mice received a single immunization at week 0 (w0) and samples collected at intervals over 12 weeks (w12). ELISA performed as described in Materials and Methods. Data is presented as the mean $\pm$ SEM titer of serum samples from 3-4 mice per group. Error bars represent the standard error of the mean.

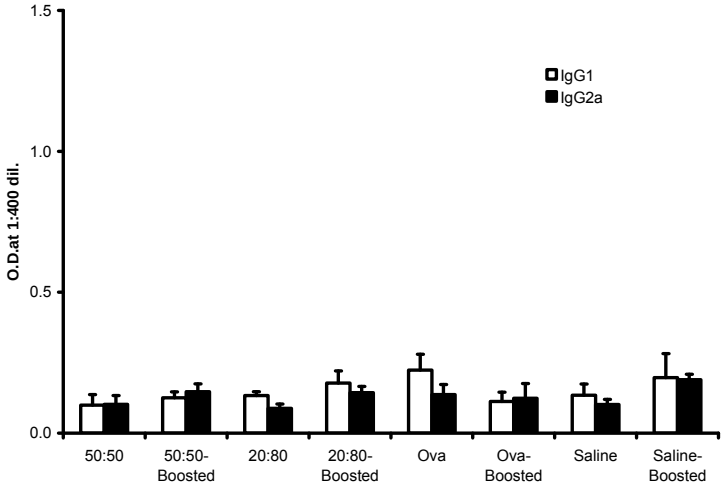

Figure 9: Isotype specific serum antibody to ovalbumin from BALB/c mice receiving Ova-loaded CPH:SA microspheres fabricated by CA, with and without boost (25 µg Ova) 5 days prior to necropsy. ELISA performed as described in Materials and Methods. Data presented is mean $\pm$ SEM of the optical density (O.D.) at 1:400 dilution of sera (n = 3-4).

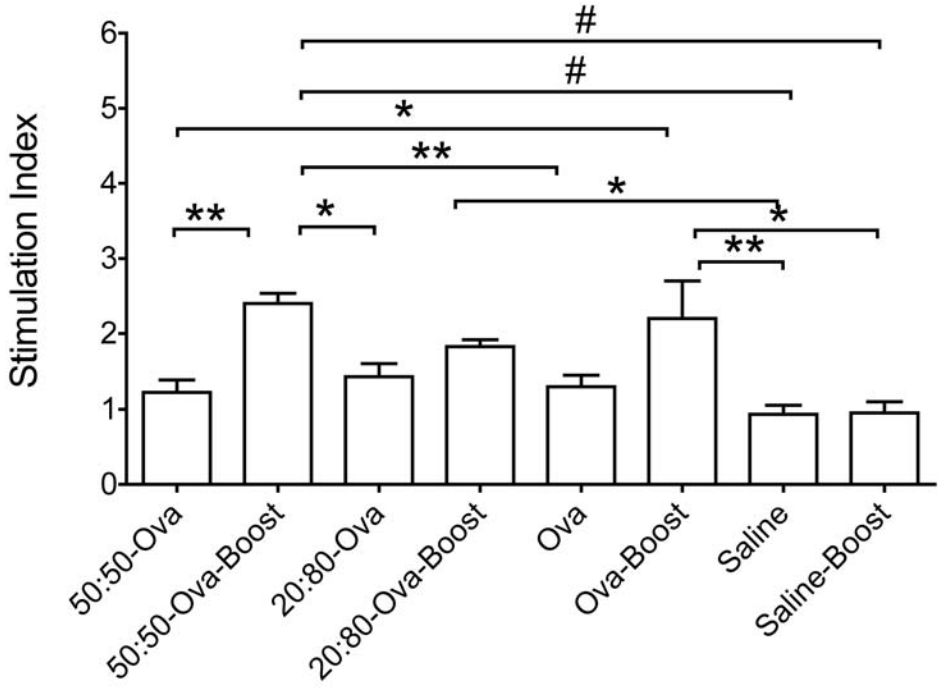

Figure 10: Induction of antigen-specific lymphocyte proliferation in cells recovered from BALB/c mice receiving Ova-loaded CPH:SA microspheres fabricated using CA. At 12 weeks post-immunization, the proliferative response of lymphocytes recovered from the draining lymph was evaluated. Specifically, the treatment groups consisted of mice that had been immunized with Ova-loaded microspheres, soluble ovalbumin (Ova), or saline. Half of the mice in each group received 25 μg Ova 5 days prior to necropsy (Boost). Single cell suspensions of lymphocytes were stimulated with 50 μg/ml Ova in vitro for 72 h at which time ^{3}H-thymidine was added and the cultures were incubated for another 18 h. Stimulation index was calculated from counts per minute of Ova-stimulated cells divided by the counts per minute of unstimulated cells. Data is presented as the mean stimulation index $\pm$ SEM (n = 3-4). Linked bars indicate statistically significant difference in the group means, * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and # indicates $P \leq 0.001$.

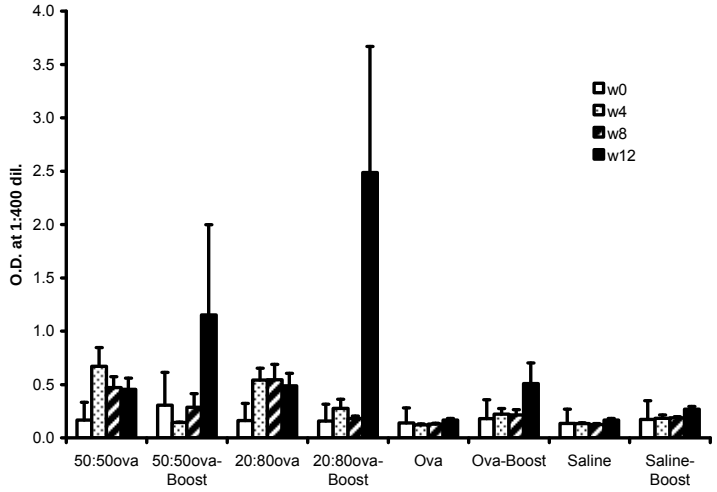

Figure 11: Ovalbumin-specific serum antibody (IgG, H&L) response of C3H mice receiving Ova-loaded CPH:SA microspheres fabricated by CA, with and without boost (25 µg Ova) 5 days prior to necropsy. Mice were immunized with Ova-loaded CPH:SA microspheres, 25 µg soluble Ova, or saline alone. Mice received a single immunization at week 0 (w0) and samples collected at intervals over 12 weeks (w12). ELISA performed as described in Materials and Methods. Data is presented as the mean ± SEM titer of serum samples from 3-4 mice per group. Error bars represent the standard error of the mean.

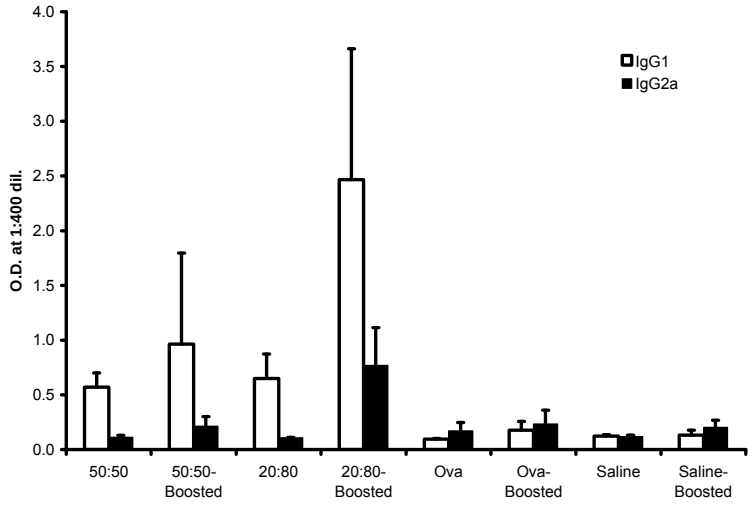

Figure 12: Isotype specific serum antibody to ovalbumin from C3H mice receiving Ova-loaded CPH:SA microspheres fabricated by CA, with and without boost (25 µg Ova) 5 days prior to necropsy. ELISA performed as described in Materials and Methods. Data presented is mean ± SEM of the optical density (O.D.) at 1:400 dilution of sera, (n = 3-4).

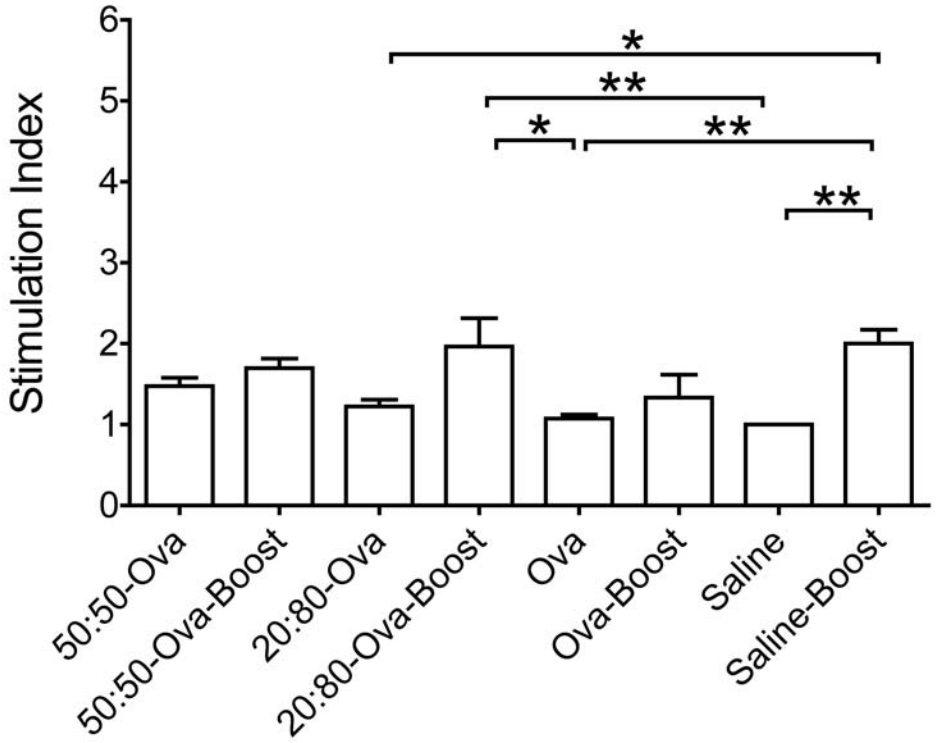

Figure 13: Induction of antigen-specific lymphocyte proliferation in C3H mice receiving Ova-loaded CPH:SA microspheres fabricated using CA. At 12 weeks post-immunization, the proliferative response of lymphocytes recovered from the draining lymph was evaluated. Specifically, the treatment groups consisted of mice that had been immunized with Ova-loaded microspheres, soluble ovalbumin (Ova), or saline. Half of the mice in each group received 25 µg Ova 5 days prior to necropsy (Boost). Single cell suspensions of lymphocytes were stimulated with 50 µg/ml Ova in vitro for 72 h at which time ^{3}H-thymidine was added and the cultures were incubated for another 18 h. Stimulation index was calculated from counts per minute of Ova-stimulated cells divided by the counts per minute of unstimulated cells. Data is presented as the mean stimulation index $\pm$ SEM (n = 3-4). Linked bars indicate statistically significant difference in the group means, * indicates $P \leq 0.05$, and ** indicates $P \leq 0.01$.

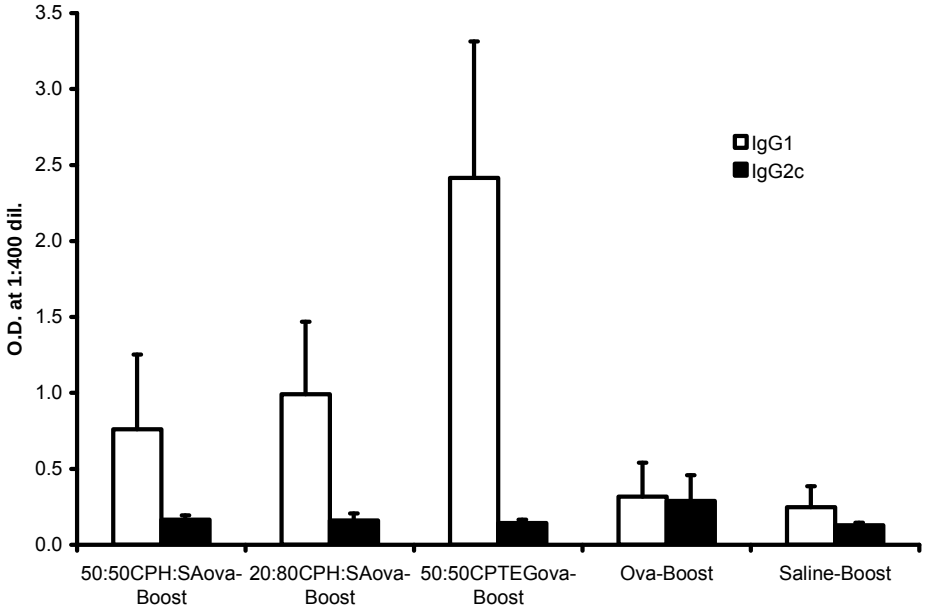

Figure 14: Isotype specific serum antibody to ovalbumin from C57BL/6 mice receiving Ova-loaded microspheres fabricated by CA method, with boost (25 μg Ova) 5 days prior to necropsy. ELISA performed as described in Materials and Methods. Data presented is mean ± SEM of the optical density (O.D.) at 1:400 dilution of sera, (n= 5).

4 Discussion

Based upon prior published reports, biodegradable polymers have demonstrated great promise as adjuvants in single dose vaccine regimen used in laboratory animal studies [11-15]. Vaccine formulations based on PLGA, PLA, or PGA have been successful in inducing immune responses to a large number of antigens including: Yersinia pestis antigens, HIV gp140, Bordetella pertussis antigens, measles virus antigen, OVA, TT, diphtheria toxin, type II collagen, malarial antigens, cancer cell antigens, Escherichia coli adhesion proteins, Vibrio cholerae antigens, influenza virus antigens, hepatitis B viral antigens, and ricin toxoid [13, 16-18]. Several of these studies incorporated mono-phosphoryl lipid A (MPLA), a known adjuvant, into the polymer delivery devise along with Ova complicating the ability to determine whether or not the polymer itself provide any immune enhancing activity [14, 15, 18-20]. Other studies included excipients and stabilizers to enhance immunogenicity of the encapsulated protein [12, 21, 22]. In the current study, no additional immune-enhancers or stabilizers

were included during the fabrication of the microspheres. Therefore, any stability and immunomodulation capabilities were the direct result of the polymers themselves.

In previous studies using TT as the immunogen, a shift from a dominant IgG1 antibody response to a more balanced IgG1-IgG2a anti-TT antibody response was observed and this was attributed to polymer chemistry because different responses were observed between 20:80 and 50:50 CPH:SA microspheres [5]. The current studies were undertaken to confirm and extend the previous observations using a different model antigen and several strains of mice.

Evaluation of the immune response induced by Ova-loaded microspheres was performed using three different strains of mice. The three strains (BALB/c, C3H/HeN, and C57BL/6) were chosen for differences in their genetic immune biases. BALB/c mice are generally considered to be genetically biased towards Th2-type immune responses and are used as prototypic allergy models [23-25]. C57BL/6 mice are considered biased toward Th1-type immune responses and are used as models for typical delayed-type hypersensitivity (DTH) responses and inflammatory autoimmune diseases [26, 27]. C3H mice are considered unbiased toward induction of Th1- or Th2-type immune responses. Furthermore, each of these mice strains express a different MHC haplotype: BALB/c express H-2^d, C3H express H-2^k and C57BL/6 express H-2^b. These H-2 haplotypes can restrict antigen presentation causing some of the differences observed in peptide derived-antigen responsiveness between strains [28]. Complex antigens generally contain multiple epitopes that interact with a wider variety of MHC haplotypes [29]. It is possible that slight differences in antigen presentation are magnified by antigen dose and general protein stability. Others have shown that different mouse strains (BALB/c and NIH) respond with maximal IgG1 responses with different doses of antigen (high or low dose) [30].

In the SO/O fabrication process, lyophilized protein is mixed with polymer dissolved in methylene chloride. This is then emulsified in silicon oil where polymer-protein droplets are formed into microspheres as the methylene chloride is dissolved into the silicon oil where the polymer is not. The resulting microspheres are harvested by filtration and washed with heptane to remove any residual silicon oil and methylene

chloride. In contrast, cryogenic atomization (CA) starts with the same polymer-protein-methylene chloride solution, but the solution is pumped through an atomizing nozzle into an ice-cold ethanol bath. The bath is stirred for three days at zero degrees to evaporate off methylene chloride. The resulting microspheres are harvested by filtration. It was thought that fabrication methods that used fewer solvents and fewer emulsification steps would be beneficial to protein stability. Lopac, et. al., showed that there was no difference in profile of protein release kinetics when Ova was encapsulated in microspheres made by either fabrication method [6].However, as shown by the differences between mice receiving Ova-loaded CPH:SA microspheres fabricated by S/O/O vs. CA, there could be differences in the stability of Ova encapsulated using the S/O/O vs. CA fabrication methods. The enhanced response to Ova-loaded 50:50 CPH:SA (Figures 5 and 7) are also contrary to what one would predict given the results of earlier stability studies from this laboratory group. Determan et. al., showed that incubation of bovine serum albumin (BSA) in saturated solutions of the CPH monomer there was an associated increase in protein aggregation, protein cleavage, and changes in the tertiary protein structure (β-sheet vs. α-helix) [2, 3]. While there are many structural differences between BSA and Ova [23] which make Ova more immunogenic, it was perhaps a false assumption on the part of the authors that these two proteins would behave similarly when encapsulated. Administration of aggregated or chemically denatured Ova has been shown to induce less robust immune responses when compare to native Ova [23, 24, 26]. Lopac et al., showed that there was marked degradation of Ova when encapsulated and released from microspheres containing high amounts of SA (poly-SA and 20:80 CPH:SA) [6]. In those previous studies, CPTEG containing microspheres had a more stabilizing effect on Ova as shown by SDS-PAGE gel. This is also reflected in the current study by the greater response seen in BL6 mice immunized with 50:50 CPTEG:CPH Ova-loaded microspheres (Figure 14).

In general, the immune responses to Ova tended to induce a dominant Th2-type immune response based upon the higher IgG1 versus IgG2a antibody detected in the serum samples of immunized mice (Figures. 3, 6, 9, 12 and 14). When care is not taken to remove contaminating endotoxin-like material from commercial Ova a more Th1-type

immune response was observed (data not shown). While little secreted cytokine was measured from Ova-stimulated cells recovered from immunized mice, the presence of IL-5 and IL-10 are consistent with higher IgG1 antibody response (i.e., Th2 phenotype). Furthermore, the Ova-specific induction of IL-10 secretion from splenocytes recovered from mice receiving Ova-loaded microspheres is consistent with other observations in our laboratory in that CPH:SA microspheres induce demonstrable B cell expansion in vivo and antigen-specific B cell proliferation in vitro (data not shown). One limitation of ^{3}H-thymidine incorporation as a measure of proliferation is that the phenotype (i.e., T cell or B cell) of the proliferating cell is unknown. To address this shortcoming, lymphocytes recovered from the draining lymph nodes of mice receiving Ova-loaded microspheres were stained with CFSE and stimulated in vitro with Ova; the results of this limited study demonstrated that the predominant proliferating cell type was $CD19^{+}$ (B cells) (data not shown).

The induction of modest antigen-specific proliferation, low cytokine secretion, and low to modest levels of Ova-specific antibody, all indicated that the immunization regimen used in these studies failed to induce a robust immune response to Ova. It should be noted that although there is statistical differences in proliferative responses between some groups, a stimulation index less than 2 may not be biologically relevant. Additionally O.D. measurements of less than 0.5 border on non-responsive antibody responses. As the groups immunized with Ova alone failed to induce a robust immune response, one could conclude that in the absence of an adequate adjuvant administered along with the 25 μg dose of Ova resulted in insufficient immune activation. In this regard, 25 μg was the amount of Ova loaded into a 0.5 mg quantity of microspheres using a target encapsulation of 5% antigen in the total mass of the polymer. Previous studies have shown that increasing the loading percentage reduces encapsulation efficiency and may introduce destabilizing factors into the polymer matrix of the microspheres. Enhanced immune responses to Ova have been previously demonstrated when mice were immunized with 100 μg of Ova in the presence of blank microspheres affirming their adjuvant like potential (Chapter 3 and data not shown). Others have observed that a dose of 25 μg of Ova given in Alum-based adjuvants was sufficient to induce an immune

response in the context of allergic hypersensitivity (Edward Rose, University of Virginia, personal communication). Therefore, it is unlikely that insufficient Ova delivered in the context of microspheres was the reason for the lack of immune responses. This 25 μg dose of Ova encapsulated into the microspheres appeared to be sufficient to prime the mice, as observed by many groups responding with increased antibody and Ova-specific proliferative responses five days following administration of an in vivo antigenic boost (Figures 4, 5, 6, 7, 8, 11, and 12). However, if this was the case, every group receiving the antigenic boost would have responded with an anamnestic immune response, but this was not the case. There was no single explanation (e.g., fabrication method, polymer chemistry, dose, or strain) for the variability in the immune responses observed in this study. However, these results suggest that a combination of low (insufficient) dose, Ova instability in CPH:SA polymer microspheres, and differences MHC restricted recognition of Ova epitopes between the different mouse strains influenced the magnitude of the resultant immune response.

5 Conclusion

Polymer chemistry affects not only erosion kinetics but also protein stability. For complex antigens, antigen stability is vitally important to ensure maintenance of full antigenic repertoire and recognition by a wide variety of MHC haplotypes. Co-polymers containing CPH:SA, especially high levels of SA may not be suitable for single dose immunization due to rapid release of antigen and destabilization of the protein during encapsulation and/or release. The effects of chemistry dependent instability are compounded by microsphere fabrication methods. These studies add to the understanding of the complex interplay between polymer chemistry, antigen stability, and immune responsiveness. Induction of efficacious or protective immune responses (antibody, cell-mediated) will require careful selection of dose, polymer adjuvant and fabrication method.

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CHAPTER 5

Evaluation of Storage on Immunogenicity of Tetanus Toxoid encapsulated into Polyanhydride Microspheres

A Brief Communication to be submitted to BMC Research Notes

Jennifer H. Wilson-Welder[1], Matt J. Kipper[2], Balaji Narasimhan[3], Michael J Wannemuehler[1*]

[1]*Department of Veterinary Microbiology and Preventive Medicine, 2180 Veterinary Medicine, Iowa State University, Ames IA 50011-1250, USA*

[2]*Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, CO 80523-1370, USA*

[3]*Department of Chemical and Biological Engineering, 2035 Sweeny Hall, Iowa State University, Ames IA 50011-2230*

**Correspondence to:* Michael J. Wannemuehler, mjwannem@iastate.edu

Abstract

As vaccine stockpiles are critical components of medical preparedness, the ability to plan for long-term storage of vaccines without a loss of protective efficacy is important. In this regard, the stability of the immunogen, the choice of adjuvant, and the method of vaccine delivery must all be evaluated. Stability of protein antigens in the presence of polyanhydride monomers or during encapsulation conditions has been previously evaluated. Induction of antigen-specific immunity following single dose vaccination with tetanus toxoid (TT) loaded polyanhydride microspheres has been previously shown. In order to evaluate the shelf-life of TT loaded microspheres, mice were vaccinated with TT-loaded microspheres that had been stored for four years at -20 °C. Using a single dose immunization regimen, TT-specific antibody and lymphocyte proliferative response were evaluated 12 weeks later. By comparing serum samples obtained from mice that had been immunized with the TT-loaded microspheres

immediately after fabrication with serum from mice immunized after four years of storage there was no statistical difference in the anti-TT response. Lastly, the magnitude of the TT-specific proliferative recall response of mice immunized with stored microspheres was similar when compared to responses previously observed. Polyanhydride microspheres show promise for single dose vaccination by not only preserving protein structure during encapsulation and release but as shown by this study, stability under common storage conditions.
Keywords: Polyanhydrides, Tetanus Toxoid, Single-Dose Vaccination, Shelf-life

1 Introduction

Even under the best of circumstances, there is an extensive time lag between manufacture of a vaccine formulation and actual administration to a patient. Therefore, pharmaceutical preparations of novel vaccine formulations must remain antigenicly stable over time. For example, stockpiles of foot-and-mouth disease vaccine are part of the prevention and control program of several countries [1]. These vaccines, with or without an oil adjuvant are stable for 18-24 months when stored at 4°C, and potency is lost when stored at temperatures higher than 4°C. More than 60% of vaccine formulations currently on the market consist of refrigerated solution suspensions [2]. These suspensions are also susceptible to degradation during both freeze-thaw and lyophilization procedures [2]. Oil emulsions, widely used in veterinary vaccines, show a reduced potency when stored at -20°C [1]. Longer storage times can be achieved by storing vaccines at lower temperatures. Thus a vaccine adjuvant that preserves critical antigens during lyophilization or storage at -20°C is needed.

Antigens of novel vaccines are derived from rationally designed engineered proteins [3]. Preservation of primary and secondary structure of protein epitopes is necessary to achieve immunity as degraded and altered proteins have been shown to lessen the immune response [4]. Previous studies have evaluated the protein stability in conjunction with novel polyanhydride polymers. Studies by Determan et. al., showed enhanced preservation of primary and secondary structure, and retention of antigenic epitopes of various proteins incubated in the presence of monomers of 1,6-bis(*p*-

carboxyphenoxy)hexane (CPH) and sebacic acid (SA), versus proteins incubated in the presence of polyesters (lactic and glycolic acids) [5, 6]. Studies conducted by Lopac et. al., showed preservation of these same key measurements (primary structure, secondary structure and antigenicity) for proteins that were encapsulated and released from polyanhydride microspheres (Manuscript in Preparation). These studies were conducted within short time frames following the fabrication of the polyanhydride microspheres. As was demonstrated by Kipper et. al., TT-loaded microspheres induced high titer serum antibody responses and demonstrable lymphocyte proliferation at 12 weeks following a single immunization [7].

The opportunity arose that would allow for the testing of antigen-loaded microspheres four years from the date of manufacture. This study allowed for the evaluation of the immune response to stored microspheres as compared to the immune response elicited from the same microspheres within a month of their manufacture.

2 Materials and Methods

2.1 Polymer synthesis and characterization

Poly(CPH-SA) (20:80) and poly(CPH-SA) (50:50) were synthesized by melt polycondensation from acetylated prepolymers as described previously [7]. Gel permeation chromatography was performed on a Waters GPC system (Milford, MA) using PL Gel columns (Polymer Laboratories, Amherst, MA). The 20:80 copolymer had an average molecular weight (M_w) of 21,000 and a polydispersity index (PDI) of 2.2. The 50:50 copolymer had an M_w of 13,000 and a PDI of 2.0. Polymers were stored desiccated flooded by dry argon gas.

2.2 Microsphere fabrication and storage

Polyanhydride polymer and TT-loaded microspheres were prepared and evaluated as described in Kipper et al [7]. A portion (< 10 mg) of the batch used in that manuscript was placed in a microcentrifuge tube inside a small airtight container (~2 oz) with approximately 20 g DriRite desiccant and stored at -20 C.

2.3 Animals

Adult mice, strain C3H/HeN, ranging 16 to 30 weeks of age, were used for these experiments. Groups were arranged so that both males and females were distributed across all the treatment groups. Mice were obtained from the breeding colony maintained at Iowa State Laboratory Animal Resource Facility (Ames, IA). All bedding, caging, water, and feed were sterilized prior to use. All animal procedures were undertaken with prior approval from the Iowa State University Committee on Animal Care and Use.

2.4 Immunizations and blood collection

To evaluate the effectiveness of polyanhydride microspheres to induce an antibody response, mice (5 per group) received a single intramuscular (right quadriceps) injection of microspheres composed of 20:80 CPH:SA which had been stored for four years as indicated above. For comparison, a separate group of 5 mice received 10 μg soluble TT in 100 μL saline and a third group received 100 μL saline only. The soluble TT had been stored solublized in saline at 4°C for two years. Following immunization, blood samples were collected weekly for 13 weeks via the saphenous vein [8]. Blood was allowed to clot, and serum was collected and stored at –20 °C until assayed.

2.5 TT-specific enzyme linked immunosorbent assay (ELISA)

Ninety-six well microtiter plates (Costar high protein binding) were coated overnight with 100 μl PBS containing 1 μg/mL TT. To remove unbound TT, plates were washed with PBS (pH 7.0) containing 0.05% Tween 20 (PBST) and then blocked for two hours at room temperature with PBST containing 2% gelatin and 2% fetal bovine serum (FBS). Serum samples (100 μL/well) from individual mice were serially diluted in PBST supplemented with 2% FBS (PBST-FBS). The plates were then incubated overnight (18 h) at 4 °C. The plates were again washed three times with PBST followed by addition of 100 μL of PBST-FBS containing alkaline phosphatase-conjugated goat anti-mouse IgG (H&L) (0.5 mg/mL diluted 1:1000) (KPL, Gaithersburg, MD). After a two hour incubation period, the plates were washed three times with PBST followed by the

addition of 100 µL of sodium carbonate buffer (pH 9.3) containing phosphatase substrate (Sigma 104, Sigma-Aldrich, St. Louis, MO) at a concentration of 1 mg/mL and allowed to react for one hour at room temperature. The optical density (OD) of the reaction was measured at 405 nm using a Spectramax 190 Plate Reader (Molecular Devices, Sunnyvale CA). Serum titers are reported as the reciprocal of the highest dilution giving an OD equal to or greater than the average OD of all background wells (PBST-FBS alone) plus two times the standard deviation of these samples. Of note, the original value for the group mean titer published in Kipper et. al., was 12,800 indicating that the stored serum from the original study has not undergone significant degradation [7].

2.6 In vitro antigen-specific recall proliferation assay

To evaluate the antigen-specific recall response induced by vaccination, mice that were immunized 13 weeks previously were euthanized by CO_2 asphyxiation and draining lymph nodes (popliteal and inguinal) were removed using aseptic technique. Single cell suspensions were prepared from each animal, cellular debris was removed by settling, and the cells were then washed via centrifugation. The cells were resuspended in culture medium (cRPMI) consisting of RPMI 1640 containing L-glutamine (Mediatech, Herndon, VA) and supplemented with 1 % nonessential amino acids (Mediatech), 1 % sodium pyruvate (Mediatech), 2 % essential amino acids (Mediatech), 25 mM HEPES buffer (Mediatech), 100 units/mL penicillin, 0.1 mg/mL streptomycin (Mediatech), 0.05 mg/mL gentamicin (Sigma), 1 % L-glutamine (Mediatech), 5 x 10^{-5} M 2mercaptoethanol (Sigma), and 5 % heat-inactivated FBS. Round-bottomed 96-well microtiter plates were seeded with 2.5 x 10^5 cells in cRPMI at a total volume of 200 µL per well. Wells also contained either concanavalin A (Con A, 5 µg/mL, Sigma), TT (5 or 25 µg/mL), or cRPMI alone (i.e., no stimulation). Plates were then incubated for 3 days at 37 ^oC in 5 % CO_2 in air. After 3 days, 0.5 µCi of methyl- [^{3}H]-thymidine (specific activity 6.7 Ci mmole^{-1}, Amersham Life Science, Arlington Heights, IL) at a concentration of 50 µCi/mL in 10 µL of complete media was added to each well, and the plates were incubated for an additional 18 h. The contents of each well were harvested onto glass fiber filters, and the incorporated radioactivity was measured by liquid scintillation

counting. Treatments were run in triplicate. Stimulation index (SI) is the counts per minute (CPM) of stimulated wells divided by the CPM of background wells.

3 Results

TT-loaded microspheres or soluble TT was suspended in saline and injected intramuscularly into the hind leg of adult C3H/HeN mice. Blood was drawn at weekly intervals and serum antibody titers were chronologically evaluated. As shown in Figure 1, the total IgG TT-specific antibody titer for the serum samples obtained from mice immunized with soluble TT reached > 400,000 by six weeks post-vaccination. At three weeks post-vaccination, the TT-specific antibody titer for the mice immunized with TT-loaded microspheres was similar to that induced by soluble TT (76,900). By eight weeks post-vaccination, the titer for the microsphere vaccinated group reached it maximal level (105,700) and this waned by 13 weeks 38,500. When comparing the serum anti-TT titer of the week 13 samples to a pool of serum collected after 12 weeks from mice immunized with the TT-loaded 20:80 CPH:SA microspheres when they were originally prepared, the stored microspheres induced an equivalent serum antibody titer than the freshly prepared TT-loaded microspheres (Figure 2) [7].

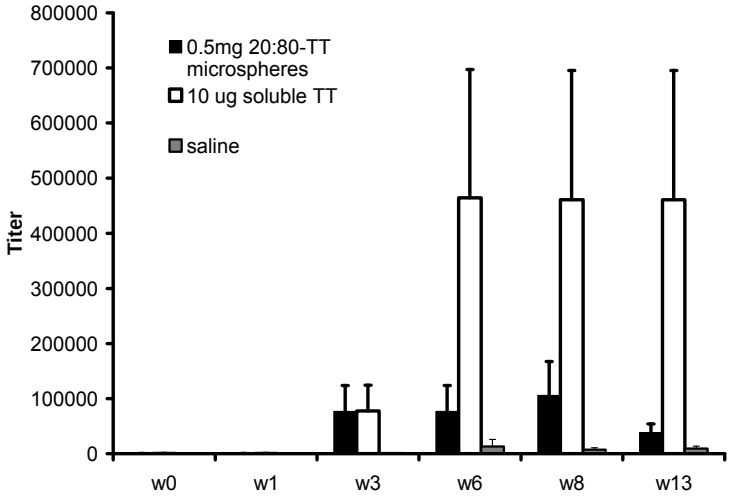

Figure 1: Anti-tetanus toxoid (TT) serum antibody titer of mice immunized with TT-loaded 20:80 CPH:SA microspheres that were stored for four years, 10 µg soluble TT, or saline alone. Mice received a single immunization at week 0 (w0) and samples collected at intervals over 13 weeks (w13). Data is presented as the mean $\pm$ SEM titer of serum samples from five mice per group. Error bars represent the standard error of the mean.

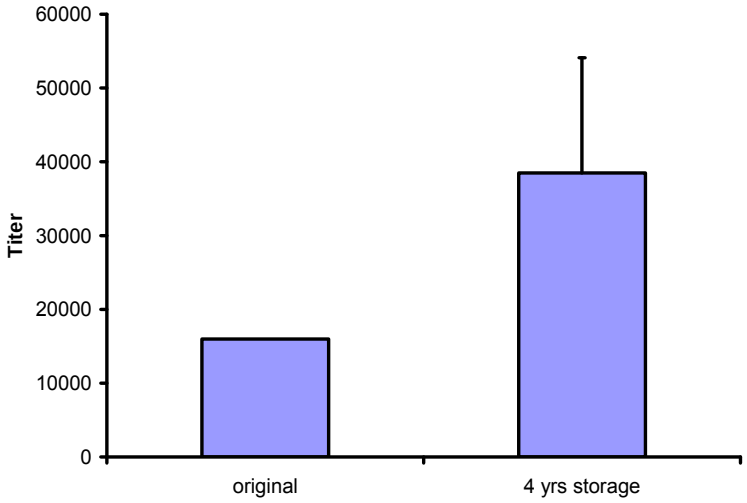

Figure 2: Effect of storage on the ability of TT-loaded 20:80 CPH:SA microspheres to induce serum antibody. Comparison of serum antibody titer of a pooled serum sample collected four years earlier to that of mice immunized with TT-loaded 20:80 CPH:SA microspheres that had been stored for four years. The serum samples were collected from the vaccinated mice at either 12 or 13 weeks post-immunization for the original immunization study (original) or the current study (4 yrs storage), respectively. Error bar represents SEM (n=5)

In addition to serum antibody, an effective vaccine regimen should induce cell-mediated immune responses. At 12 weeks post-immunization, the poplietal and inguinal lymph nodes draining the injection cite were excised from the euthanized mice. The data depicted in Figure 3 depicts the TT-specific recall response of lymphocytes stimulated with 1 µg/mL TT. The data indicates that the mice immunized with the TT-loaded microspheres (four years old) had greater proliferative responses than mice immunized with TT alone.

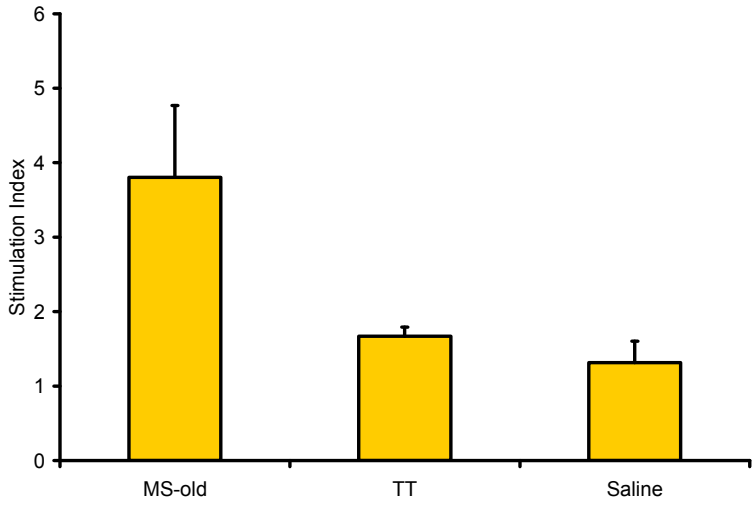

Figure 3: Induction of antigen-specific lymphocyte proliferation. At 12 weeks post-immunization, the proliferative response of lymphocytes recovered from the draining lymph was evaluated. Specifically, the treatment groups consisted of mice that had been immunized with the aged-microspheres (MS-old), soluble TT (TT), or saline. Single cell suspensions of lymphocytes were stimulated with 1 µg/mL TT in vitro for 72 h at which time ^{3}H-thymidine was added and the cultures were incubated for another 18 h. Stimulation index was calculated from counts per minute of TT-stimulated cells divided by the counts per minute of unstimulated cells. Data is presented as the mean stimulation index $\pm$ SEM (n=5).

4 Discussion

As vaccine stockpiles are part of many disease outbreak control programs (i.e., foot-and-mouth disease in UK and Anthrax in the US), there is a need for the development of vaccines with long-term storage capabilities [1]. Currently used alum-adjuvants and many oil-emulsion adjuvants undergo irreparable separation and destabilization of the emulsion during freeze-thaw cycles [2].

While the stored sample of TT-loaded microspheres was too small to allow for extensive analysis (e.g., protein stability, release kinetics), there was sufficient sample to immunize five mice [7]. When serum samples were compared with respect to the anti-TT titer there was no statistical difference ($p<0.05$) between that induced by mice that had been immunized with the TT-loaded microspheres one week or four years after microsphere fabrication. The simple storage conditions, glass jar with desiccant at -20ºC, were able to preserve the immunogenicity of TT-loaded polyanhydride microspheres. One method that has been employed in the effort to extend the shelf life of foot-and-mouth disease, involves storing the non-emulsified vaccine at -80ºC [1]. These specialized freezers can be costly, consume considerable energy, and are susceptible to power outages. This study showed that polyanhydride adjuvants maintained the immunogenicity of TT during long term storage in a regular lab freezer, in a ready to use state.

Protein degradation can occur at several steps in the process of microsphere fabrication. Protein can be altered or rendered non-immunogenic during encapsulation in reaction to polymer monomers and organic solvents. Previously published reports from the Narasimhan group reported that the tertiary conformation of a protein is altered by interactions with polyanhydride monomers in a chemistry dependent manner [5, 6, 9]. Other immunization studies have shown that immunogenic protein is released from CPH:SA microspheres as evidenced by the induction of antigen-specific antibody and proliferative responses [7] (Wilson-Welder, manuscript in preparation). This is the first study undertaken to evaluate the immunogenicity of protein encapsulated in polyanhydride microspheres and held for long term storage.

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CHAPTER 6

Evaluation of Single Dose Swine Dysentery Vaccine Based on Novel Biodegradable Polyanhydride Microspheres

[1]Jennifer H. Wilson Welder, [2]Brenda Carrillo, [2]*Balaji Narasimhan, [1]Michael J. Wannemuehler

[1]Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011

[2]Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

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1. Industry Summary

As commodity markets fluctuate and producer profit margins diminish, economic loss due to infectious disease become even more important to the survivability of operations. Vaccination continues to be the most economic method for controlling infectious disease, especially ones which are difficult to control without prophylactic antibiotics. As consumer acceptance of current livestock practices change (e.g., use of antibiotics), as well as the increase in organic and antibiotic free niche markets, control of disease by preventive vaccination becomes more important. Single dose vaccines have long been sought in human medicine to improve vaccine efficacy. The same advantage applies to animal health – a single dose vaccine would improve vaccine compliance, reduce labor costs, and, in the end, result in higher producer profits due to prevention of disease. A single dose vaccine could be readily integrated into current livestock management systems. The goal of this project was to evaluate a novel biodegradable polymer adjuvant as single dose vaccine carrier. In many cases, it is impractical in terms of labor and animal stress to immunize more than once. For most vaccines, including

swine dysentery, two or three doses of a vaccine administered over several weeks are needed for complete protection. While the disease studied in this case was swine dysentery, the concept could be applied to other infectious disease agents. Using a mouse model of swine dysentery, a single dose microsphere delivered vaccine containing pepsin-digested *Brachyspira hyodysenteriae* antigen (PD) induced immune response to *Brachyspira* antigen and ameliorated inflammatory cytokine production associated with disease. A single dose vaccine containing co-polymers of CPH:SA microspheres encapsulating PD along with some unencapsulated PD was administered to crossbred grower pigs. No tissue reactivity at the injection site of polymer containing vaccine pigs was observed, whereas most of the animals receiving PD antigen incorporated into incomplete Freund's adjuvant (a common mineral oil based carrier) had granulomatous masses at the injection site. While all of the PD-vaccinated pigs were protected from challenge or did not develop dysentery, only one out of five pigs receiving microsphere based vaccine were protected from developing any signs of clinical dysentery. Further study is needed to characterize the nature of the immune response (immune regulation and/or inflammatory cytokine profile) of the microsphere vaccine. While no injection site reactivity was observed, further evaluation of antigen stability, antigen loading of the microspheres and dosing regimens are needed to show that single dose vaccines based on polyanhydride microspheres will be beneficial/efficacious for use in livestock animals.

2. Scientific Abstract

Single dose vaccination has long been sought as one of the key hallmarks for increasing vaccine efficacy. Biodegradable polyanhydrides possess many properties that facilitate the development of single dose vaccines, including ability to enhance protein stability, tailorable release kinetics and surface erosion. This study evaluated the use of polyanhydride encapsulated pepsin-digested *Brachyspira hyodysenteriae* antigen (PD) as a vaccine regimen to protect pigs from the development of swine dysentery. In comparison to previously studied microspheres containing a single purified protein antigen, microspheres containing the complex antigen performed as expected with respect to morphology and release kinetics of the encapsulated antigens. Prior to

challenge, mice vaccinated with PD encapsulated into microspheres developed demonstrable immune responses, both serum antibody and cellular proliferation, to *B. hyodysenteriae* antigen. Upon challenge with *B. hyodysenteriae* organisms, the cytokine profile of cecal tissue recovered from microsphere vaccinated animals and the antibody isotype profile was qualitatively different than those of mice vaccinated with PD or from non-vaccinated *B. hyodysenteriae* infected mice. In swine, animals vaccinated with PD in Freund's incomplete adjuvant (FIA) or PD loaded microspheres showed a reduction in disease severity upon challenge with *B. hyodysenteriae,* 100 % and 60%, respectively. While pigs vaccinated with PD-loaded microspheres exhibited lower serum antibody titers than the pigs receiving PD in FIA prior to challenge, post-challenge serum antibody titers were equal and greater than that of sham vaccinated pigs indicating immunological priming. Furthermore, lymphocytes recovered from the colonic lymph node of pigs vaccinated with the PD-loaded microspheres exhibited greater in vitro antigen-specific recall responses than cells recovered from pigs receiving the PD-FIA vaccine. In addition, the proliferation of peripheral blood mononuclear cells recovered from the microsphere vaccinated pigs was lower than that for cells recovered from the PD-FIA vaccinated pigs, suggesting differential immune modulation. The results of these studies demonstrate that the use of polyanhydride microspheres is safe, induced no detectable tissue reaction at the site of injection. However, pigs vaccinated with PD-loaded microspheres were not protected from disease. Taken together, further refinement in antigen loading, and dosing regimens is needed in order to demonstrate that polyanhydride microspheres can be used as a single dose vaccine carrier that can be used in livestock species.

3. Introduction

As commodity markets fluctuate and producer profit margins diminish, economic loss due to infectious disease become even more important to the survivability or operations. Vaccination continues to be an effective method for controlling infectious disease, especially ones such as swine dysentery (SD). Single dose vaccines have long been sought after in human medicine to improve vaccine efficacy and patient compliance.

The same advantages apply to animal health – a single dose vaccine would improve vaccine compliance, reduce labor costs, and, in the end, result in higher producer profits due to prevention of disease. A single dose vaccine could be easily integrated into current livestock management systems.

Studies evaluating the use of controlled-release, single dose vaccines in large animals (sheep, mini-pigs, cattle, and horses) have shown promise when employing protein antigens [1-3]. Biodegradable polyanhydride delivery systems for vaccines offer attractive features such as improved adjuvanticity, antigen stabilization, and enhanced immune responses [4-7]. The specific polyanhydrides of interest in this proposal copolymers of 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) and sebasic acid (SA). This class of hydrophobic polymers degrades into biocompatible, water-soluble carboxylic acids which are quickly metabolized, leaving no tissue residue. Studies have shown that polyanhydrides are capable of stabilizing polypeptides and sustaining their release without the inclusion of potentially reactive excipients or stabilizers [8-11]. In addition, polyanhydrides have characteristics that make them suitable carriers for vaccine delivery, including a beneficial environment for protein stabilization by preventing aggregation, enhanced adjuvant effect (controlled by polymer chemistry), and potential immunomodulatory capabilities (Th1/Th2 balance) [8, 12].

There are many different swine diseases where both effective humoral immunity (Th2) and cell-mediated immunity (Th1) are desired [13, 14]. One instance where protection seems to depend on activation of both is swine dysentery (SD) [15]. SD is a severe diarrheal disease of swine and the etiologic agent is an anaerobic spirochete, *Brachyspira hyodysenteriae,* which colonizes the cecum and colon [16-18]. The disease characterized by the presence of mucohemorrhagic diarrhea, weight loss, dehydration and shedding of spirochetes in the stool. Any age of pig can be affected, however, most severe losses occur during the grower/finisher stage [19]. The acute phase of the disease appears to be driven by leukocytes responding to translocation of luminal commensal bacteria into the lamina propria following epithelial erosion due pathogenic factors secreted by *B. hyodysenteriae* [20]. The chronic phase is mediated by $CD4^+$ T cell infiltrate into the colonic mucosa [20]. The disease is endemic in most pig producing

countries, where infection prevalence can be as high as 35% of the swine herds [21, 22]. Efforts to maintain herds free of SD can be complicated by the fact that wild rodents and waterfowl are natural hosts of the bacterium [23, 24]. Control measures include antibiotic therapy, however, recently, antibiotic resistant strains have emerged [25]. With the increase in demand for organic and/or antibiotic free pigs, producers are encountering an increase in incidence in SD. Currently in the United States, there is no licensed vaccine in use for SD. Efforts to produce both recombinant and whole cell vaccines have met with varying success [16, 26-28]. Outer membrane preparations and other recombinant vaccines yielded only partial protection [16, 26-28]. Using a squalene/pluronic acid adjuvant, protection was conferred by a pepsin digest preparation of whole cell *B. hyodysenteriae* [16, 29, 30]. Non-mineral water/oil emulsions such as this are capable in inducing cell-mediated immunity (Th1) with lower dosage of protein than can be induced using an alum based vaccine [31]. The current study was undertaken to provide proof-of-concept that a single dose polyanhydride based vaccine can induce protective immunity in pigs. A single-dose microsphere-based vaccine was compared to an efficacious vaccine regimen for SD in a grower pig model.

4. Materials & Methods

4.1 Pepsin digest preparation

Pepsin digest of *Brachyspira hyodysenteriae* antigen was prepared as described previously in Waters et al [15, 16]. *Brachyspira hyodysenteriae* strain B204 frozen lyophilized bacterial stock was rehydrated with distilled water, briefly sonicated and pH adjusted to 2.0 with 1 N HCl. Lysate was mixed with 0.5 μg Pepsin (Sigma) per mg of dry weight bacteria. The solution was placed on rotary mixer at 37°C for 24 hrs. The pH was then adjusted to 7.2 with 1N NaOH. PD was sterilized by UV irradiation. Protein was quantified by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) and re-lyophilized. All measurements were based on dry weight of lyophilized pepsin digest (PD). Whole cell sonicate (WCS) was prepared from the same lot of frozen lyophilized stock. Briefly, lyophilized bacteria were rehydrated to give 2 mg/ml suspension in sterile

PBS, subjected to two freeze thaw cycles and briefly sonicated. WCS was sterilized by UV irradiation and stored at -20 until needed for ELISA or cellular re-stimulation.

4.2 Polymer synthesis and microsphere fabrication

Prepolymers for both CPH and SA were synthesized using a method outlined by Shen et al. [32]. Poly(CPH-SA) (20:80) and poly(CPH-SA) (50:50) were synthesized by melt polycondensation from acetylated prepolymer solutions as described previously [32, 33]. Gel permeation chromatography was performed on a Waters GPC system (Milford, MA) using PL Gel columns (Polymer Laboratories, Amherst, MA). The 20:80 copolymer had an average molecular weight (M_w) of 21,000 and a polydispersity index (PDI) of 2.2. The 50:50 copolymer had an M_w of 13,000 and a PDI of 2.0. Polymers were stored desiccated under dry argon until needed. Microspheres encapsulating PD were fabricated by a solid/oil/oil double emulsion method as reported elsewhere [12, 33]. Polymer (100 mg) was dissolved in methylene chloride (2 mL) and PD (10 mg) was added to the dissolved polymers in a 50 ml centrifuge tube and immediately emulsified by agitation at 30,000 rpm for 20:80 CPH:SA and 20,000 rpm for 50:50 CPH:SA with a handheld homogenizer (Tissue-Tearor^{TM}, Biospec Products Inc., Bartlesville, OK) for one minute. With the homogenizer running at 10,000 rpm, 3 ml of Dow Corning Fluid (silicon oil), saturated with methylene chloride (4 mL), was added drop-wise to form the microspheres. Homogenization at 30,000 rpm was continued for an additional minute. To precipitate the microspheres, the double emulsion was transferred to a 400 mL Berzelius beaker containing 200 mL *n*-heptane on an ice water bath. The heptane was stirred at 300 rpm using a Caframo overhead stirrer (Warrington, Ontario) with a three-inch impeller for two hours to extract the methylene chloride. Heptane was periodically added during the solvent removal to replace the volume lost due to evaporation. The PD-loaded microspheres were isolated by filtration using Whatman #50 filter paper. The beaker and impeller were rinsed several times with fresh heptane to maximize recovery. The microspheres were washed at least three times with 50 mL of heptane to remove residual Dow Corning fluid, and dried for 24 h under vacuum. This procedure yielded a free-flowing powder with about 80% of the input polymer mass being recovered. Blank and

WCS containing microspheres were fabricated by a similar techniques, without the addition of PD or the inclusion of *B. hyodysenteriae* WCS respectively.

4.3 Determination of microsphere size and morphology

A JEOL 840A scanning electron microscope (SEM) was used to determine the size and shape of the microspheres. Microspheres were smeared onto carbon stubs, sputter coated with 200 Å of gold, and imaged. Size distribution analysis was performed using Image J software (NIH, Bethesda, MD).

4.4 Antigen release and antigenicity

Polyanhydride microspheres (10 mg) fabricated by S/O/O were suspended in 1 mL of phosphate buffered saline (PBS, pH 7.4) containing 0.01% sodium azide and placed in an incubator at 37 °C while stirring at 100 rpm. Samples of the buffer were collected at two hours later, then daily for one week, and every other day for 35 days. An aliquot of 750 μL was sampled each time and subsequently replaced with 750 μL of fresh PBS to ensure perfect sink conditions; the microcentrifuge tubes containing the microsphere suspensions were centrifuged before sampling to ensure that no microspheres were removed along with the sampled PBS. In order to quantify the amount of protein released, BCA was performed on each sample, in duplicate, as described by the manufacturer (Pierce, Rockford, IL). The release data is presented as cumulative fraction of protein released, in which the amount of protein released is normalized by the total protein loaded into the microspheres.

4.5 Mice

C3H/HeOuJ mice of either sex and 8 to 16 weeks of age were used for these studies. Mice were obtained from the breeding colony maintained at Laboratory Animal Holding Facility, Iowa State University (Ames, IA) or were purchased from Harlan Sprague Dawley. Animals were free of any observable diarrheal disease at the beginning of each experiment. Mice were given autoclaved conventional rodent diet, water, and bedding. During the infection phase of the experiment, mice were house in isolation

cages with HEPA filtered positive pressure air. All procedures were approved by the Iowa State University Committee on Animal Care. Mice were weighed and observed for any clinical signs of disease every other day during infection. All mice were euthanized by CO_2 asphyxiation 30 days post-infection, tissues were scored for gross lesions, and samples were collected for bacteriological, histopathological, serological, and cell-mediated immune analyses.

4.6 Mouse vaccinations

Mice were immunized with a single vaccination intramuscularly in the right quadriceps with 100 µL pyrogen-free saline containing one of the following: 0.5 µg WCS, 25 µg PD, 0.5 mg WCS-loaded 20:80 CPH:SA microspheres (20:80-WCS), 0.5 mg PD-loaded 20:80 CPH:SA microspheres (20:80-PD), 0.5 mg PD-loaded 50:50 CPH:SA microspheres (50:50-PD), or 0.5 mg PD-loaded microspheres (20:80+PD or 50:50+PD) along with 2.5 µg unencapsulated PD. Suspensions of microspheres were sonicated briefly to disperse aggregates before delivery via 25 gauge hypodermic needle.

4.7. Bacterial inoculation

At 30 days post-vaccination, mice were challenged with *B. hyodysenteriae*, and four to seven mice were sham inoculated with broth alone in each experiment. Mice were orally colonized with a bacterial inoculum (0.3 mL, approximately 10^8 cells/mL) of B. *hyodysenteriae* administered by orogastric intubation on two consecutive days. Spirochete infection confirmed bacteriologically at necropsy by demonstration of β-hemolytic spirochetes grown on anaerobic selective media [34].

4.8 Gross inflammatory scores of murine ceca

At necropsy, gross pathological changes to the cecum were scored using a modification of a previously reported scoring system [34, 35]. Lesions were scored from 0 to 6, giving one point for each of the following parameters when present: cecal blunting, cecal atrophy, cecal emptying (excessive mucus), watery or mucoid cecal contents, enlarged lymphoid aggregates, and observed blood in the cecum.

4.9 Histology of murine cecal tissue

Formalin-fixed cecal tissues were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E), and scored for microscopic inflammation [34, 35]. Tissue sections were coded and presented blind to the pathologist. Histological scores were based on the severity of mucosal epithelial damage (erosions, edema), degree of lamina propria cellular infiltrate, and architectural distortion (crypt length, cellular hyperplasia). These parameters were added together to give a relative histologic score index.

4.10 Murine antigen-specific serum antibodies

Serum samples were obtained every two weeks via saphenous vein blood collection [36]. Blood was refrigerated overnight and serum separated via centrifugation. Sera samples were stored at -20ºC until assays were performed. Measurement of *B. hyodysenteriae* antibodies in sera was performed by ELISA. Briefly, 96-well plates were coated with 5 μg/mL WCS of *B. hyodysenteriae*. After blocking the plates (2% gelatin with 1% fetal calf serum) for two hours, serial dilutions (1:100 to 1:256,000) was added to each well and incubated at 4 ºC overnight. Then, alkaline phosphatase conjugated goat anti-mouse IgG (H&L), IgG1, or IgG2a (Southern Biotechnology, Birmingham AL) was added and incubated for 2 h at room temperature. Wells were developed using p-nitrophenyl phosphate (Sigma 104) in sodium carbonate/bicarbonate buffer (pH 9.3) at room temperature. Optical densities were measured at 405 nm using a microtiter plate reader.

4.11 Antigen-specific proliferation responses of murine lymphocytes

In order to assess the cellular response induced by a single vaccination, lymphocytes isolated from the mesenteric lymph nodes and lymph nodes draining the injection site (right side popliteal and inguinal). Single cell suspensions were prepared from each individual animal, cellular debris was removed by settling, and the cells were then washed via centrifugation. The cells were resuspended in complete culture medium

(cRPMI) consisting of RPMI 1640 containing L-glutamine and supplemented with 1 % nonessential amino acids, 1 % sodium pyruvate, 2 % essential amino acids, 25 mM HEPES buffer, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 0.05 mg/mL gentamicin, 1 % L-glutamine, 5 x 10^{-5} M 2 mercaptoethanol, and 5 % heat-inactivated FBS. Round-bottomed 96-well microtiter plates were seeded with 2.5 x 10^5 cells in cRPMI at a total volume of 200 μL per well. Wells also contained either concanavalin A (Con A, 0.5 μg/mL, Sigma), *B. hyodysenteriae* WCS (5 or 25 μg/mL), or cRPMI alone (i.e., no stimulation). Plates were then incubated for 3 days at 37 ^oC in 5 % CO_2 in air. After 3 days, 0.5 μCi of methyl- [^{3}H]-thymidine (specific activity 6.7 Ci mmole^{-1}) at a concentration of 50 μCi/mL in 10 μL of cRPMI was added to each well, and the plates were incubated for an additional 18 h. The contents of each well were harvested onto glass fiber filters, and the incorporated radioactivity was measured by liquid scintillation counting. Treatments were run in triplicate and data is presented as mean counts per minute (CPM) or stimulation index (SI) calculated by dividing the CPM of treated wells by the CPM of non-stimulated (background) wells.

4.12 Cytokine secretion from murine lymphocytes and cecal tissue

Single cell suspensions of lymphocytes were prepared by tissue homogenization from the mesenteric lymph nodes and were incubated in cRPMI at 2.5 x 10^5 cells/well with or without WCS of *B. hyodysenteriae* for 72 h. Cells were stimulated with specific bacterial antigens at a concentration of 25 μg/mL or with ConA (0.5 mg/mL). Cell-free supernatants were harvested and analyzed for the presence of TNF-α, IFN-γ, IL-4, IL-5, IL-6, IL-10 and IL-12 cytokines using a multiplexed flow cytometric assay (FlowMetric System; Luminex, Austin TX). Additionally, at time of necropsy, two 4 mm biopsy punches were taken from cecal tissue and placed in 200 μL cRPMI with additional antibiotics (200 μg/mL Kanamycin, 200 units/mL penicillin, 0.2 mg/mL streptomycin, 0.1 mg/mL gentamicin). Punches were incubated for 48 h at 37°C, 5% CO2. Cell free supernatants were collected and stored at -20°C until assayed for IL-1β, IL-6, IL-10, IL-12p40, IL-17a, IFN-γ and TNF-α by multiplexed flow kilometric assay.

4.13 Pigs

Twenty-seven mixed bred pigs (both genders) were purchased from Northwood Farms, a herd known to be free of dysentery and respiratory mycoplasma disease. Pigs were 10 days of age upon arrival and randomly split into pens. Pigs were treated with 25 mg EXCENEL (ceftiofur hydrochloride, Pfizer) three times, 24 h apart starting day of arrival and 50 mg Baytril (enrofloxin, Bayer) two times, on days 5 and 7 after arrival. Pigs were fed nursery diet free-choice for the first two weeks and gradually switched to a standard corn-soybean grower ration (14% crude protein) (Heartland Co-op, Des Moines, IA) hand fed once daily. Animals were maintained to preserve the high-health, pathogen free status. Pigs were weighed weekly to monitor growth rate and enrichment provided in the pens. Pigs remained healthy and free of any clinical signs of disease (respiratory or enteric) prior to challenge with *B. hyodysenteriae*. All procedures involving pigs were approved by the Iowa State University Committee on Animal Care.

4.14 Experimental design

At 45 days of age, pigs were randomly assigned to their respective treatment groups. The first group was vaccinated in order to assess the immune response to the vaccine designated NI or non-infected and the second group was vaccinated and challenged with *Brachyspira hyodysenteriae* strain B204, 4 weeks following initial vaccination, designated INF for infected. Pigs were vaccinated once with PD loaded microspheres or blank microspheres on day 0 and a separate group of pigs was immunized twice with PD in incomplete Freund's adjuvant (IFA) on day 0 and day 14. On day 28 and day 29 following initial vaccination, pigs were challenged orally with 100 mL of culture broth containing *B. hyodysenteriae* (10^8 cells/mL). Animals were euthanized on day 42 (14 days post-challenge) via administration of Fatal-Plus followed by exsanguination. All injection sites were examined for presence of granuloma formation or other adverse tissue reactions. Colonic lymph nodes and lymph nodes draining the injection site were excised for evaluation of in vitro lymphocyte responses. Ceca and spiral colon were examined for gross lesions. Tissue samples of colon, ceca and

injection site tissue and lymph node were preserved in formalin for histological evaluation.

4.15 Pig vaccinations

Vaccines were prepared and administered as follows: PD vaccinated pigs: 1.25 mg dry weight PD (40% protein) dissolved in 1 mL pyrogen-free saline was then emulsified with 1 mL IFA. Total volume of 2 mL delivered intramuscularly (IM) into neck muscle with 20 gauge hypodermic needle on days 0 and 14. MS vaccinated pigs: 7 mg 50:50 CPH:SA PD-loaded (10 % w/w), 7 mg 20:80 CPH:SA PD-loaded (10% w/w) and 0.8 mg dry weight PD (40% protein) were suspended in 2 mL of saline, sonicated briefly to suspend the microspheres and injected IM with 20 gauge hypodermic needle on day 0. Non-loaded (i.e., blank) microsphere vaccinated pigs: 7 mg 50:50 CPH:SA (blank) microspheres and 7 mg 20:80 CPH:SA (blank) microspheres were suspended in 2 ml of saline, sonicated briefly to suspend particles and were injected IM using a 20 gauge hypodermic needle on day 0.

4.16 Porcine PBMC proliferation assay

To evaluate the in vitro antigen-specific recall response induced by vaccination, peripheral blood was collected at days 0, 14, 28 and 35 from the jugular vein into heprinated vaccutainer tubes. Peripheral blood mononuclear cells (PBMC) were isolated by diluting peripheral blood 1:3 in phosphate buffered saline (PBS, pH 7.2). Diluted blood was layered over Lymphocyte Separation Medium (density 1.077) (Mediatech Inc, Manassas VA), and centrifuged at 500 x g for 40 minutes. PBMC were obtained from the medium/plasma interface (buffy coat), washed three times in sterile PBS, and the cells were enumerated for use in proliferation assays.

The cells were resuspended in culture medium (cRPMI) consisting of RPMI 1640 containing L-glutamine and supplemented with 1% nonessential amino acids (Mediatech), 1% sodium pyruvate, 2% essential amino acids, 25 mM HEPES buffer, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 0.05 mg/mL gentamicin, 1% L-glutamine, 5×10^{-5} M 2 mercaptoethanol, and 10% heat-inactivated FBS. Flat-bottomed 96-well

microtiter plates were seeded with 2.5 x 10^5 cells in cRPMI at a total volume of 200 µL per well. Wells also contained either Con A (5 µg/mL, Sigma), 5 or 25 µg/mL *B. hyodysenteriae* WCS antigen or cRPMI alone (i.e., no stimulation/background). Plates were then incubated for 3 days as described above. Treatments were run in triplicate and data are presented as mean counts per minute (CPM) ± SEM.

4.17 Lymphocyte proliferative response of porcine lymph nodes(injection site and colonic)

Injection site lymph nodes were identified by administering diluted India ink in PBS to a test pig in the same site and manner as vaccines were administered eight hours prior to sacrifice. Upon necropsy, muscles and tissue were carefully dissected until lymph nodes containing the ink were found. Two small lymph nodes located between the ear and shoulder, deep to the trapezius and longissimus muscles were removed from each pig. Additionally, colonic lymph nodes located within the fold of the spiral colon were analyzed. Lymph nodes were homogenized and single cell suspensions were passed through a 40 µm mesh cell filter (BD) to remove fibrous material and washed twice by centrifugation. Flat-bottomed 96-well microtiter plates were seeded with 5 x 10^5 cells in cRPMI at a total volume of 200 µL per well. Wells also contained either Con A (5 µg/mL, Sigma), 5 or 25 µg/mL *B. hyodysenteriae* WCS antigen or cRPMI alone (i.e., no stimulation/background) and were cultured as described above. Stimulation indices were calculated by dividing the CPM of treated wells by the CPM of non-stimulated (background) wells.

4.18 Porcine antigen-specific serum antibody (ELISA)

Ninety-six well microtiter plates (Costar high protein binding) were coated overnight with 100 µL PBS containing 5 µg/mL *B. hyodysenteriae* WCS antigen. To remove unbound WCS, plates were washed with PBS containing 0.05% Tween 20 (PBST) and then blocked for two hours at room temperature with PBST containing 2% gelatin and 2% fetal calf serum (FCS). Serum samples (100 µL/well) from individual pigs were serially diluted in PBST supplemented with 1% FCS (PBST-FCS). The plates

were then incubated overnight (18 h) at room temperature (25°C). The plates were again washed three times with PBST followed by addition of 100 µL of PBST-FCS containing alkaline phosphatase-conjugated goat anti-swine IgG (H&L) (0.5 mg/mL diluted 1:1000) (KPL, Gaithersburg, MD). After a two hour incubation period, the plates were washed three times with PBST followed by the addition of 100 µl of sodium carbonate buffer (pH 9.3) containing phosphatase substrate (Sigma 104, Sigma-Aldrich, St. Louis, MO) at a concentration of 1 mg/mL and allowed to react for 1 h at room temperature. The optical density (OD) of the reaction was measured at 405 nm using a Spectramax 190 Plate Reader (Molecular Devices, Sunnyvale CA). Serum titers are reported as the reciprocal of the highest dilution giving an OD equal to or greater to a value calculated from the average of all background (d0) wells plus one standard deviation.

4.19 Statistical analysis:

Where appropriate, one-way analysis of variance (ANOVA) was conducted on group data with the aid of various computer software packages. Pair-wise comparisons were done for significant ANOVA using Bonferroni's Correction for multiple comparisons.

5. Results

5.1 Encapsulation and release of PD in microspheres

Pepsin digest (PD) whole cell lysate of *Brachyspira hyodysenteriae* was encapsulated into either 20:80 CPH:SA or 50:50 CPH:SA microspheres. Scanning electron microscopy revealed spherical particles between 5 and 10 µm in diameter as seen in Figure 1. Resulting microspheres were a gray free-flowing powder which suspended in PBS following brief sonication. The data in Figure 2 depicts the in vitro release kinetics of the encapsulated protein. The large initial burst was consistent with previous reports using ovalbumin which clustered near the outer surface of the microsphere during solid-oil-oil solvent emulsification [11]. For microspheres made from 20:80 CPH:SA formulation, 50% of the material was released within the first two hours, with over 80% of the total protein encapsulated released in approximately 12 days. The

release profile of 50:50 CPH:SA formulations was more consistent with previous results evaluating the release of ovalbumin [11, 37] or tetanus toxoid [12], the release profile indicated that 40 to 60 % of the material was released within 20 days and material continued to be released for at least 35 days (the length of this release kinetic study).

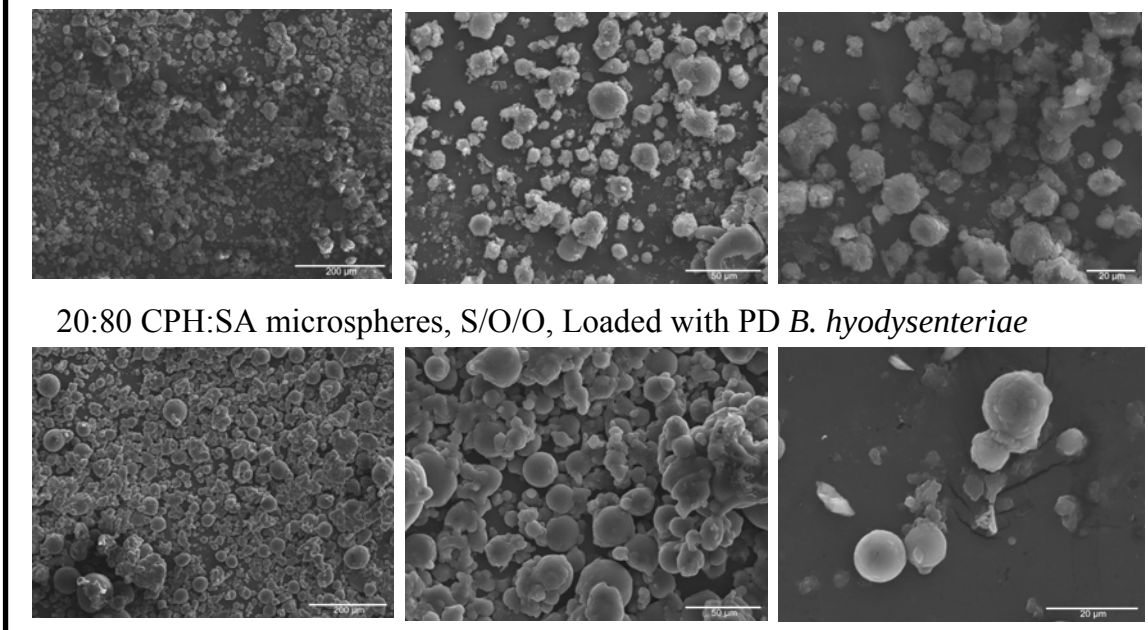

Figure 1: Scanning electron photomicrographs of pepsin-digest loaded microspheres. Photomicrographs are increasing in magnification, as shown by the scale bar in the bottom right of each image (200 µm, 50 µm, 20 µm from left to right, respectively). Top panel depicts 20:80 CPH:SA formulation, and the bottom panel depicts the 50:50 CPH:SA formulation. Microspheres were spherical in shape, non-aggregated and had a size distribution between 5 and 10 µm.

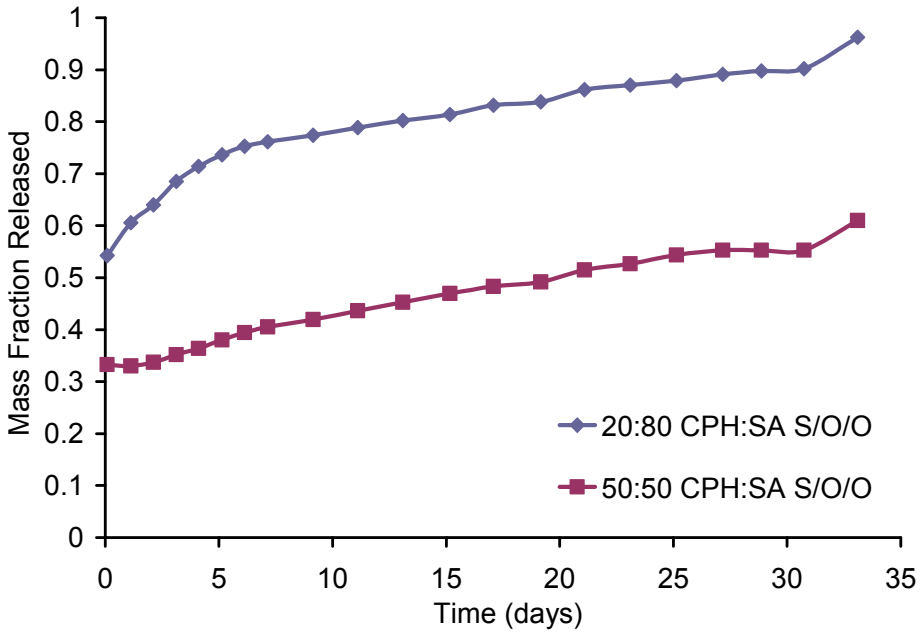

Figure 2: In vitro release kinetics of encapsulated *B. hyodysenteriae* pepsin-digest from polyanhydride microspheres. Following encapsulation, microspheres were incubated in buffer for up to 35 days. Samples of the released materials were analyzed for protein content. Data depicts the accumulated fractional release of the protein from the microspheres. The *B. hyodysenteriae* PD antigen preparation was encapsulated into one of two polyanhydride formulations: 20:80 CPH:SA or 50:50 CPH:SA.

5.2 Immune response and protection against B. hyodysenteriae *in mice immunized with PD-loaded microspheres*

In order to assess the immune response and efficacy of pepsin digest-loaded microspheres, C3H/HeOuJ mice were immunized with various microsphere formulations. Four independent experiments were performed. Results presented are from a single replicate but are representative of trends present in each independent experiment. Rarely do mice exhibit clinical signs of chronic *B. hyodysenteriae* infection. In order to more closely monitor the overall health status, mice were weighed every two days during the infection period. Infection with *B. hyodysenteriae* alone did not cause a significant decrease in weight as compared to non-infected mice (Figure 3). Vaccination had no discernible impact on weight gain. Four weeks following infection, mice were euthanized and evaluated for protection or attenuation of the typhlocolitis induced by *B.*

hyodysenteriae infection of mice. Unlike the swine, vaccination in mice does not protect from the acute phase of disease but does facilitate recovery from the chronic or immune mediated phase of infection [38]. Gross cecal scores between the individual vaccinated groups were not significantly different (Figure 4), but there was a trend toward less severe cecal lesion scores in the mice receiving either the single dose of PD or PD-loaded 50:50 microspheres administered along with a small bolus (0.5 µg) of free PD. In previous studies in our lab, mice were protected (60%) when given two doses of PD given 10 to 14 days apart.

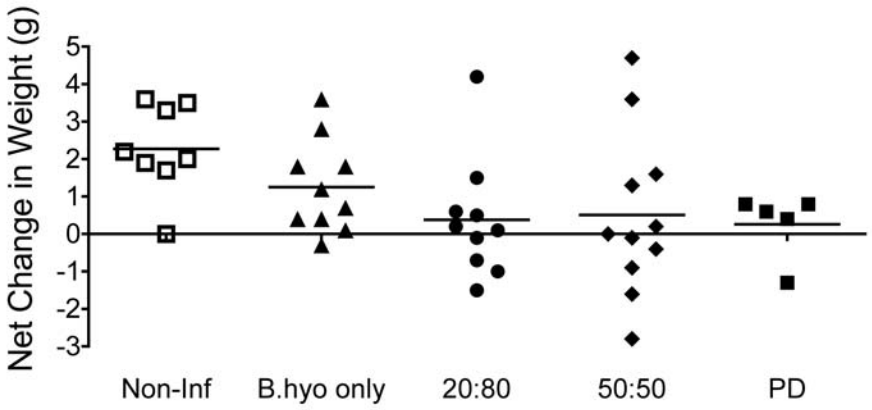

Figure 3: Net weight change of C3H mice vaccinated with pepsin digest (PD), 50:50 CPH:SA PD-loaded, 20:80 CPH:SA PD-loaded microspheres and challenged with *Brachyspira hyodysenteriae*. Weight changes during the four week infection period were monitored. Individual mice are depicted by symbols, the horizontal bar represents the group mean.

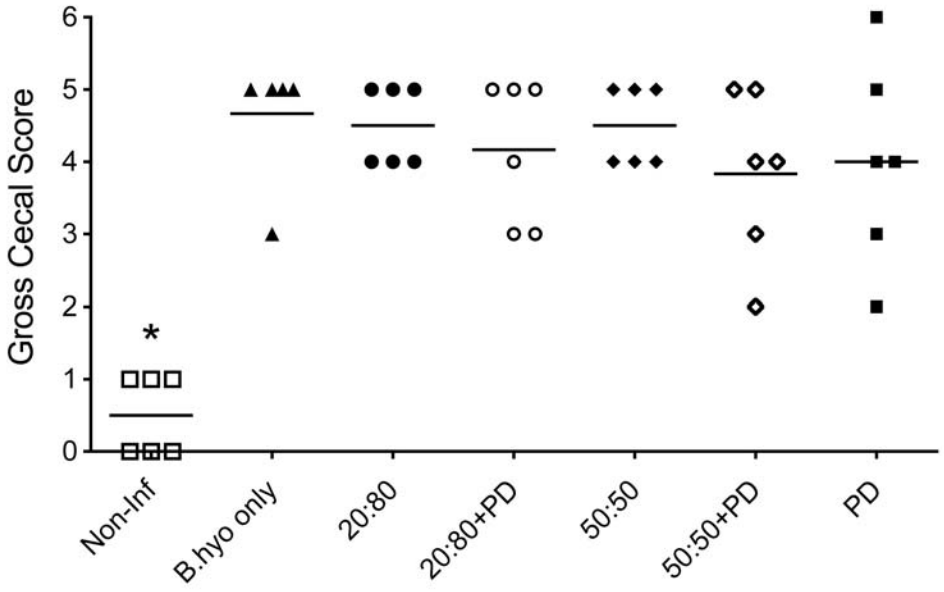

Figure 4: Four weeks following infection with *B. hyodysenteriae*, cecal lesions were evaluated using a modification of a previously established scoring system. Lesions scores of non-infected mice were significantly different ($p \leq 0.05$) from all other groups but none of the vaccinated groups were significantly different from each other.

PD was considered successful if 60% of the mice were protected. In the current study, all mice received only a single dose of PD, thus confirming the need for multiple antigen exposures to generate a protective immune response. When the ceca were evaluated microscopically for lesions (Figure 5), there was a trend toward less severe epithelial erosions, restitution of the crypt architecture, and less inflammatory cell infiltrate even though statistical difference between vaccinated groups was not observed. Evaluation of cytokines released from cecal tissue, differences in local inflammatory milieu were observed (Figure 6A-D)

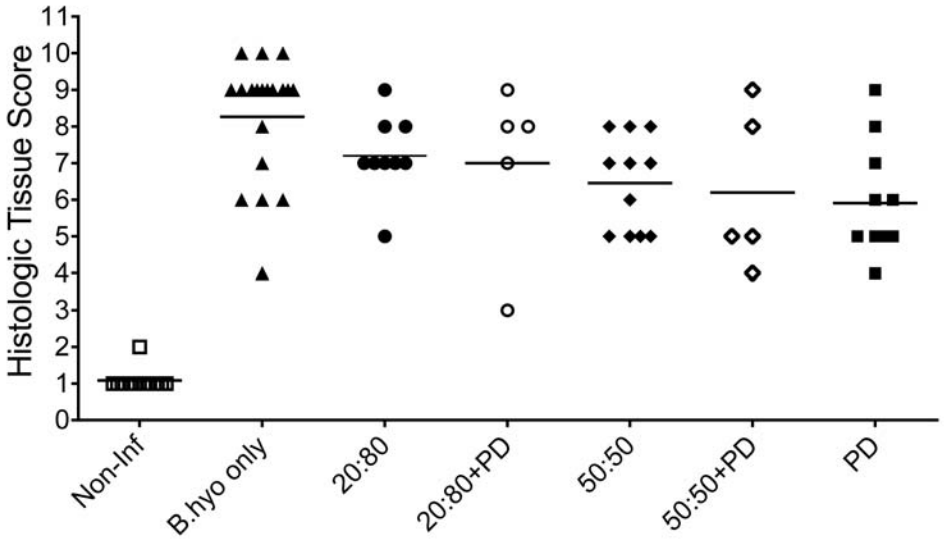

Figure 5: Evaluation of microscopic erosions, cellular infiltrate, and inflammation in cecal tissue from mice vaccinated as described above and challenged with *B. hyodysenteriae*. Four weeks following infection with *B. hyodysenteriae*, ceca were placed in formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin. Slides were evaluated in a blinded fashion by a pathologist for epithelial erosion, inflammatory cell infiltrate, and changes in cecal tissue architecture. Tissue sections from non-infected mice were significantly different from all other groups ($P<0.05$) and *B. hyodysenteriae* infected only group was significantly different from 50:50 and PD vaccinated groups.

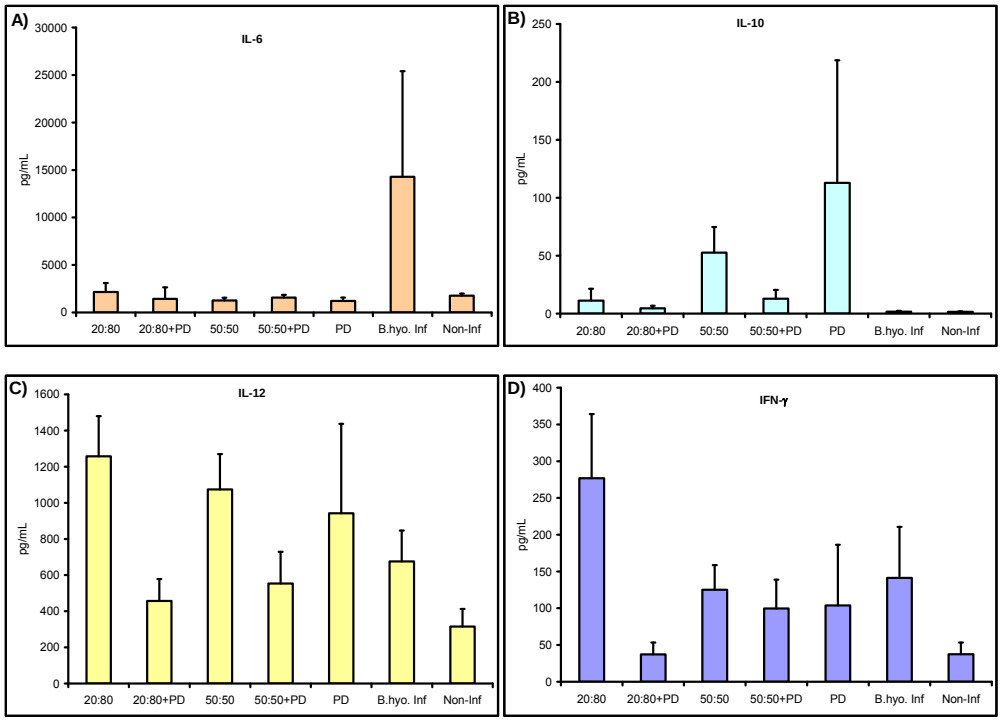

Figure 6: Cytokines released from murine cecal tissue explants collected four-weeks post-infection. Mice were immunized and infected as described in Materials and Methods. Cytokines IL-1β, IL-17a, and TGF-β were also assayed but values were either below detection or there were no observable differences between vaccinates and non-Infected groups. Data represented is mean $\pm$ SEM of the amount of cytokine released from one experiment, (n = 4 – 6 samples per treatment group).

Despite lack of protection from typhlocolitis, the PD-loaded microspheres either primed the mice or induced antigen-specific immune response prior to infection. As seen in Figure 7, the serum antibody response mice vaccinated with microspheres (20:80 or 50:50) with or without free PD greater than that of non-vaccinated mice. The highest antibody responses were observed in mice receiving the PD-loaded 50:50 CPH:SA plus free PD, and PD alone groups. Post-challenge, all challenged mice demonstrated nearly the same level of serum antibody response (Figure 8). There was a trend for a greater antibody response in the 50:50 microsphere vaccinated group, indicating that the mice were primed by the antigen-loaded microspheres prior to challenge. However, the only statistical difference was observed between the non-infected mice and the challenged groups as a whole. Antibodies can be induced to switch to different IgG isotypes

depending on immune modulating signals from the $CD4^+$ T cells. To evaluate the ability of the microspheres to modulate the antibody response, the induction of antigen-specific IgG1 (Th2) and IgG2a (Th1) antibody were measured. Mice immunized with 20:80 CPH:SA microspheres, PD or WCS, with or without bolus, exhibited an antigen-specific antibody response suggesting a dominant Th2 immune response (IgG1:IgG2a ratio greater than one, Figure 9). A ratio less than one would indicate the presence of higher amounts of IgG2a which is indicative of a dominant Th1 immune response. The differential IgG1:IgG2a ratios induced by vaccination with the PD-loaded microspheres supports the immune modulation observed with other antigens [12]. To evaluate the cellular immune response induced by microsphere and PD vaccination, lymph nodes draining the injection site (popliteal and inguinal) were removed and evaluated for proliferation in response to in vitro stimulation with WCS antigen. While there was demonstrable proliferation of cells recovered from all of the challenged groups (Figure 10), the highest amount of proliferation was the group receiving 50:50 CPH:SA microspheres (significantly different from *B. hyodysenteriae* challenged only and Non-infected). In contrast, proliferation from the mesenteric lymph node (Figure 11) was elevated in all challenge groups with no significant difference between them. To further analyze the in vitro recall response of the cells recovered from the mesenteric lymph node, culture supernatants were harvested and levels of IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ and TNF-α were evaluated in order to assess the Th1/Th2 bias of the immune response. There was no evidence that there was a measurable in vitro cytokine response following antigenic stimulation. However, the 20:80 + PD regimen appeared to modulate the IgG response towards a Th2 response (Figure 9).

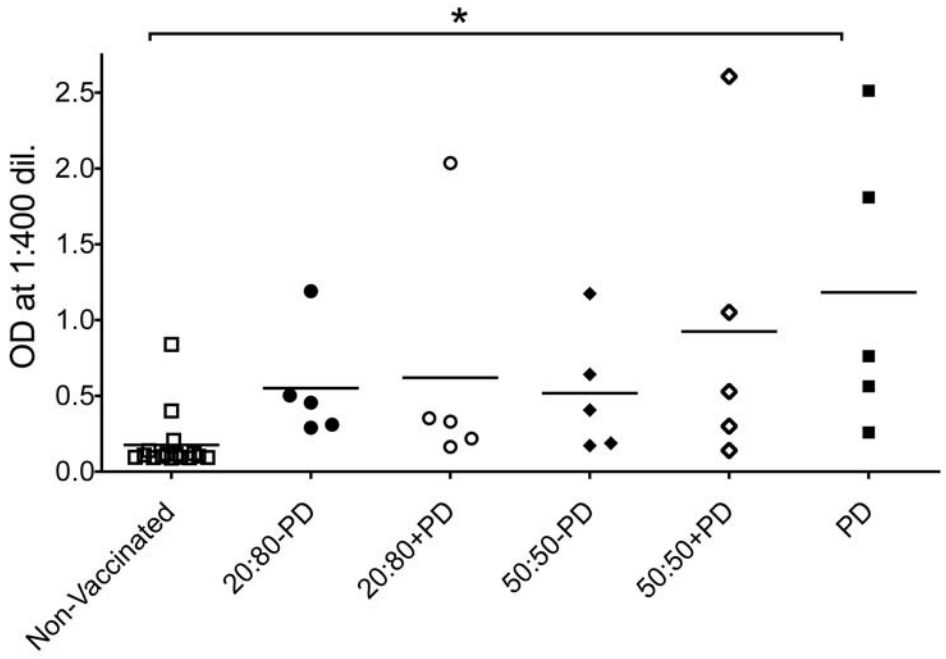

Figure 7: Analysis of serum antibody responses (total IgG) from mice following a single immunization with PD or PD-loaded microspheres. Mice were immunized as described in Materials and Methods. Data depicted is the ELISA results four weeks after immunization, but before *B. hyodysenteriae* challenge. Asterisk indicates that the antibody response of the PD vaccinated group was significantly different only from non-vaccinated mice ($P < 0.05$)

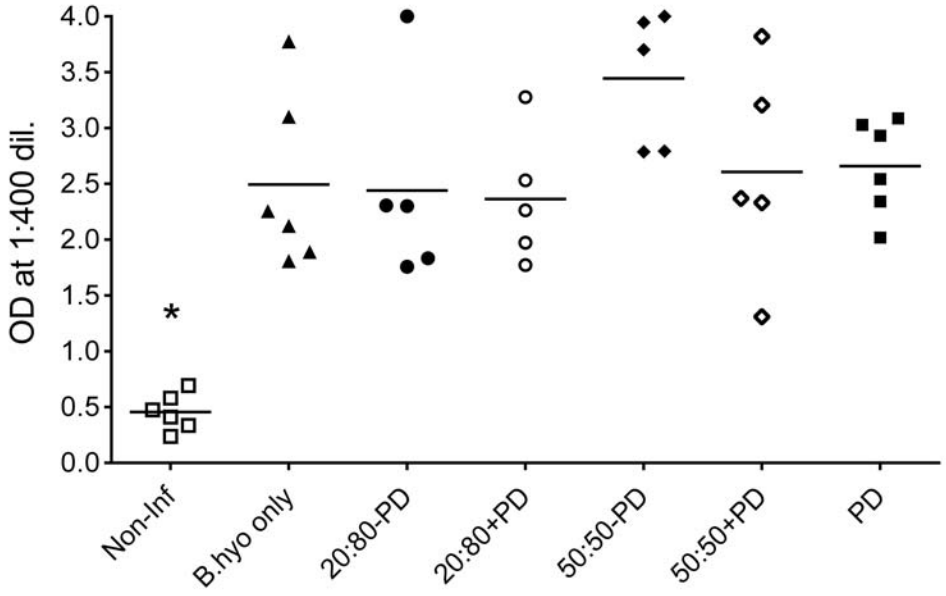

Figure 8: Analysis of serum antibody responses (total IgG) following challenge with *B. hyodysenteriae*. Mice were immunized as described in materials and methods. Serum antibody responses in mice vaccinated with PD-loaded 20:80 and 50:50 microspheres (with and without free antigen bolus) increased compared to controls and to pre-challenge serum antibody responses (Figure 7) indicating that the lower serum antibody responses observed prior to challenge were not due to the induction of immune tolerance. The asterisk indicates that the antibody response of the non-infected group was significantly different ($P < 0.05$) from all other groups.

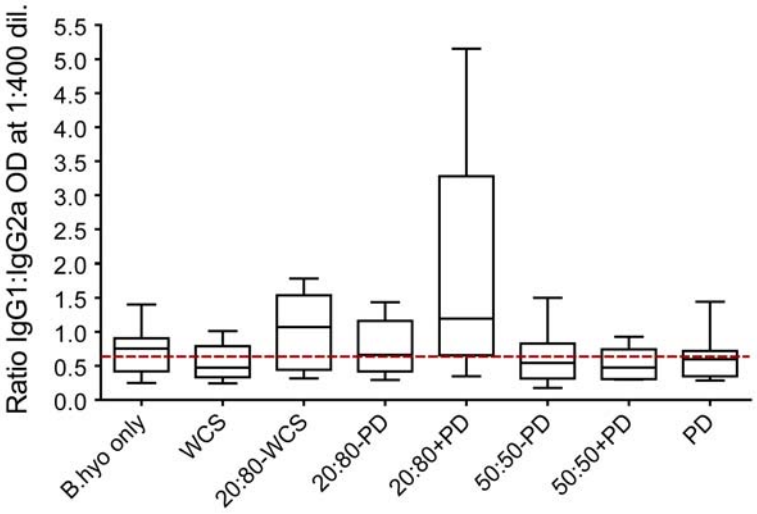

Figure 9: Assessment of the immunological balance in the IgG isotype response induced by vaccination with PD-loaded microspheres followed by challenge with *B. hyodysenteriae*. A ratio of one would suggest a similar amount of IgG1 and IgG2a were induced against *B. hyodysenteriae*. Ratios below one would suggest a Th1-bias in the antibody response while ratios above one would suggest a Th2-bias in the antibody response.

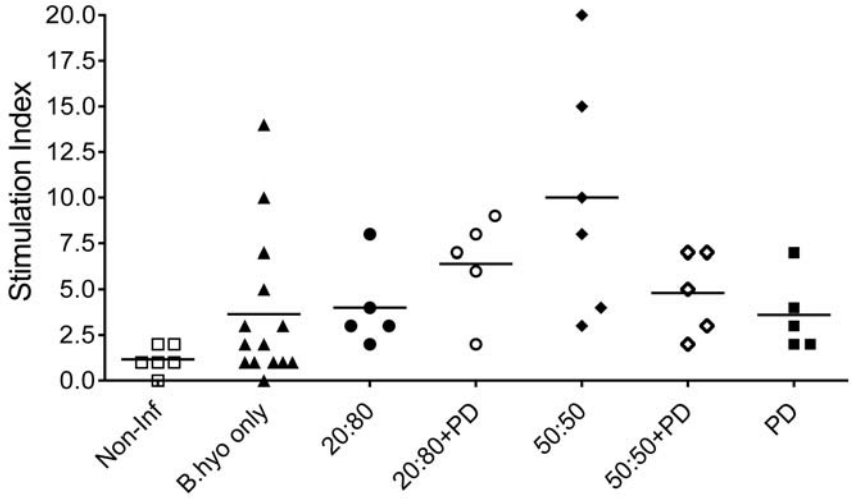

Figure 10: Antigen-specific proliferation of lymphocytes recovered from lymph nodes draining the injection. Mice were immunized once with PD digest-loaded CPH:SA microspheres of the given formulations as described in Materials and Methods. Four weeks after challenge, mice were euthanized and the lymph nodes draining the injection site were collected and analyzed in vitro for antigen-specific recall responses. Enhanced responses induced by 50:50 CPH:SA PD-loaded microspheres suggests that the predicted persistent release of antigen from this formulation differentially effected lymphocyte proliferation.

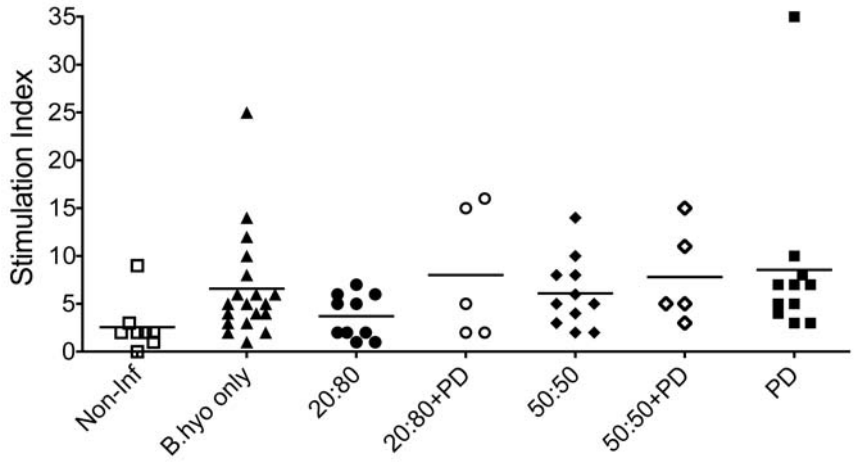

Figure 11: Lymphocyte proliferation from mesenteric lymph node cells following restimulation with *B. hyodysenteriae* WCS. Mice were immunized and challenged as described in materials and methods. Four weeks after challenge, mice were euthanized and the antigen-specific proliferation of mesenteric lymph node cells was analyzed.

5.3 Immune response and subsequent protection of a single dose SD vaccine based on PD loaded polyanhydride microspheres in grower pigs

In order to evaluate the efficacy of a single dose vaccine based on polyanhydride microspheres, a model of swine dysentery (SD) was chosen. The investigators have had success developing a vaccine for SD, and many years of experience with the model. 27 crossbred pigs were purchased at 10 days of age from a farm known to be free of both swine dysentery and *Mycoplasma*-induced respiratory diseases. Upon arrival at ISU, pigs were given several rounds of long lasting, broad spectrum antibiotics and housed to prevent acquisition of respiratory pathogens. When animals reached 45 days of age, vaccination for evaluation of microsphere based vaccines was initiated. A time line of the study is depicted in Figure 12.

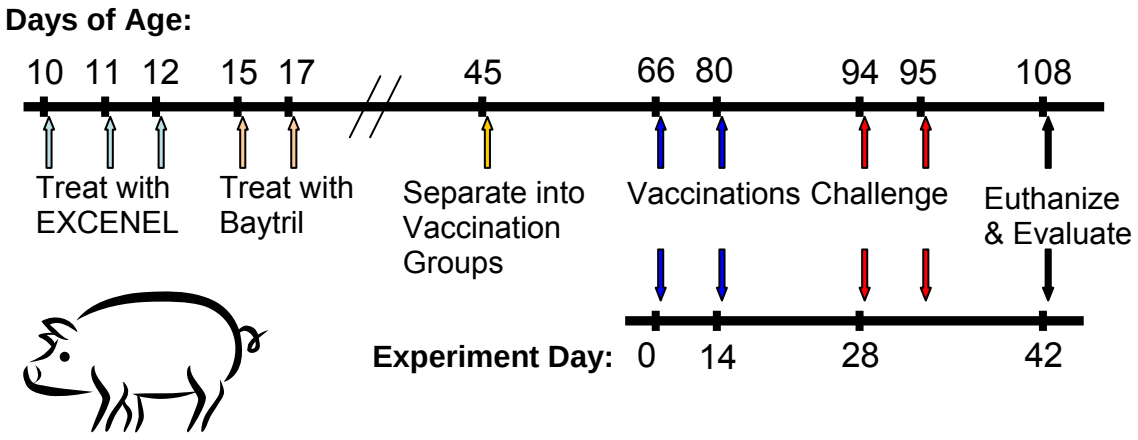

Figure 12: Timeline depicting experimental design for vaccinating and challenging pigs. Animals were given antibiotics upon arrival, at 45 days of age the pigs were separated into their respective vaccination groups, at day 66 (experimental day 0) all pigs were injected with vaccine or received a sham treatment (IFA-PD, PD-MS and blank), and at day 80 (experiment day 14) the IFA-PD group received the second vaccination. Pigs were challenged with *Brachyspira hyodysenteriae* on two consecutive days (28 and 29).

Pigs were weighed weekly in order to monitor growth. Growth rate is also an economically important clinical sign of *B. hyodysenteriae* infection. Prior to challenge, vaccination had no effect on growth rate. Upon challenge, vaccination had a definite effect on growth rate indicating protection from disease. The growth rate (average daily gain, ADG) for the group receiving two doses of pepsin digest *B. hyodysenteriae* antigen in incomplete Freund's adjuvant (IFA), a common mineral oil vaccine adjuvant, was significantly higher ($p \leq 0.05$) than the group receiving the sham vaccine (Figure 13). The variability of ADG in the pigs receiving the PD-loaded microspheres correlated with the severity of clinical disease of the animals in the group. For example, pigs presenting with little or no disease had an ADG of 1.6 to 1.8 pounds/day compared to pigs presenting with severe disease (ADG 0 to 0.4).

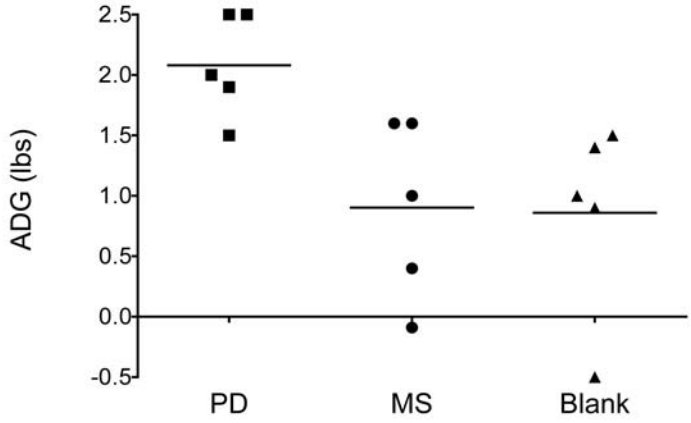

Figure 13: Average daily gain (ADG) of pigs following infection with *B. hyodysenteriae*. The ADG for the group receiving two doses of the IFA-PD vaccine was significantly different ($P<0.5$, $\alpha=0.05$) from the group receiving blank microspheres but neither was significant from the group receiving the single dose of PD-loaded microspheres (MS) (n=5). Non-infected (control) animals were housed in a separate room and had an ADG of 1.7 lbs (n=12).

Reduction of carcass loss due to adverse reactions and granuloma formation at the injection site is of economic importance to producers as well as important for adjuvants with potential use in human as well as livestock vaccines. At necropsy, injection sites were examined for any gross signs of adverse reactions. As summarized in Table 1, 6 of 8 pigs receiving PD in IFA developed granulomas in the muscle tissue of the injection site. The granulomas consisted of a sterile abscess (no bacterial growth from the exudate) with thick fibrous capsule and contained mostly inflammatory cells. In the pigs receiving either the blank and PD-loaded microspheres there was no evidence of tissue reactivity, granuloma formation or other adverse reactions. This is consistent with other observations from mice and sheep vaccinated with the polyanhydride microspheres in that there is little to no local inflammatory reaction or granuloma formation in response to microsphere vaccination.

Pigs were observed daily beginning day of challenge and evaluated for any clinical signs of dysentery including loose or watery stools, presence of blood and/or mucus, inappetence, and lethargic behavior. Observations were recorded for each individual pig and total number of diarrhea days for each individual are reported (Figure

14). The PD-IFA vaccinated group remained free of any clinical signs of dysentery. While there was no significant difference between the PD-microsphere vaccinated group and the sham (blank) vaccinated groups, there was animal-to-animal variability. All five of the animals in the sham vaccinated group displayed clinical signs of dysentery whereas one of the microsphere vaccinated pigs remained completely healthy. In contrast to all previous studies in this laboratory with the swine dysentery model, four out of 5 pigs in the sham vaccinated group spontaneously recovered from disease by 10 days following challenge. Likewise, two of the animals with disease symptoms in the PD-microsphere vaccinated group recovered between days 7 to 10 post-challenge. The spontaneous resolution of clinical signs was not due to clearance of *B. hyodysenteriae*. As shown in Table 1, there were animals in each group that were positive for β-hemolytic anaerobic spirochetes at time of necropsy.

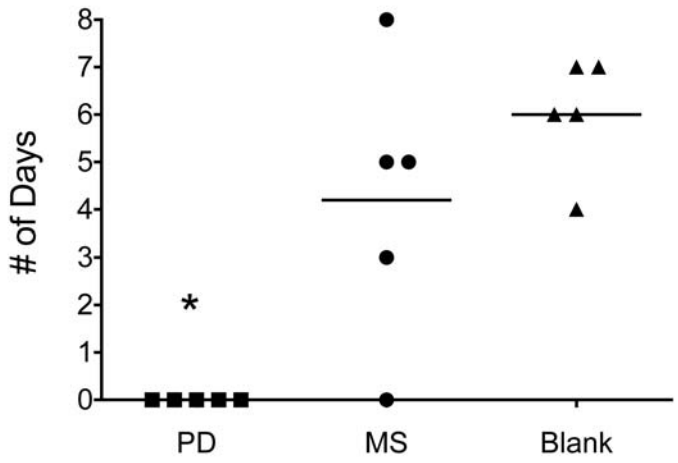

Figure 14: Total number of days that clinical signs of dysentery was observed in pigs infected with *B. hyodysenteriae*. Animals were observed daily during the 14 day infection period. Signs of dysentery included loose or watery stools, presence of blood or mucus in the stools, lethargy, and reduced appetite. The group receiving two doses of IFA-PD (PD) was significantly different (* $P < 0.05$) from either group receiving blank microspheres or the group receiving a single dose of the pepsin digest loaded microsphere vaccine (MS) ($n = 5$).

Table 1: Summary of clinical observations of infected and non-infected pigs following vaccination.

Treatment Group	No. pigs with Colonic Lesions[a]	Positive for β-hemolytic spirochetes[b]	No. pigs with clinical signs of dysentery[c]	No. pigs with injection site reactions[d]
IFA-PD Vaccinated	0/5	2/5	0/5	6/8
PD-loaded Microspheres	3/5	3/5	4/5	0/8
Blank Microspheres	1/5	3/5	5/5	0/8

[a]gross lesions at necropsy included mild to severe hyperemia, hemorrhage, mucus, or fibrin deposition.
[b]culture of colonic samples for β-hemolytic spirochetes on selective media.
[c]presence of clinical signs of dysentery during the 14 days post-challenge.
[d]presence of granulomatous reactions at the injection site at time of necropsy. Observations of injection site reactivity also include the vaccinated but non-challenged animals.

In order to assess the immune response induced by PD-loaded microspheres, peripheral blood was collected at day 0 (pre-vaccination), day 14 following vaccination, day 28 (prior to challenge) and day 38 (10 days post-challenge). Serum antibody responses to *B. hyodysenteriae* antigen were measured by ELISA. No group showed any appreciable antibody on day 14, but on day 28 the IFA-PD vaccinated groups had a serum antibody titer of 12800 and 6400 for the infected and non-infected groups (Figure 15). Following infection, the median titer for the IFA-PD vaccinated group increased from 12800 on day 28 to 25600 on day 38. The group receiving PD-loaded microspheres did not exhibit any appreciable antibody until day 38 and only those pigs that were challenged with *B. hyodysenteriae*. No appreciable antibody titer was observed after infection in the pigs receiving the blank microspheres, indicating that the PD-loaded microspheres primed these pigs for a secondary immune response.

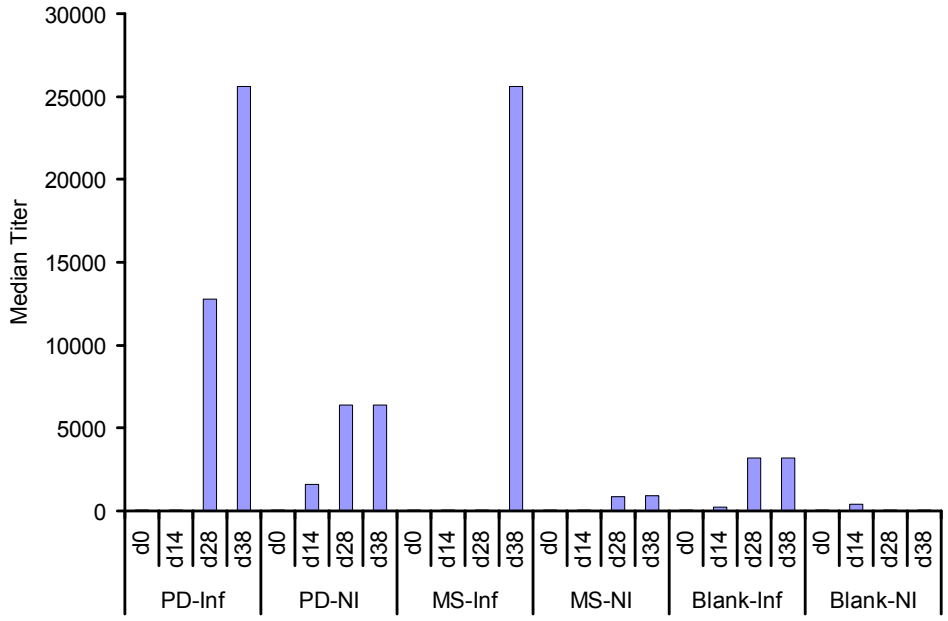

Figure 15: Serum antibody (IgG H&L) responses to B. *hyodysenteriae* antigen in pigs following vaccination and challenge. Peripheral blood was collected prior to vaccination (d0), at day 14 after initial vaccination, day 28 (prior to challenge), and day 38 (10 days after challenge). Pigs were vaccinated with either two doses of pepsin digested *B. hyodysenteriae* antigen in incomplete Freund's adjuvant (PD), a single dose of pepsin digest loaded microspheres (MS) or a single injection of blank microspheres containing no antigen (Blank). On day 28, some animals (n = 15, 5 per treatment group) were challenged with *B. hyodysenteriae* (-Inf), and the other animals (n = 4 per treatment group) remained unchallenged (-NI).

On days 0, 14, 28 and 38 mononuclear cells were isolated from peripheral blood and stimulated with *B. hyodysenteriae* antigen. The proliferation of these PBMC is depicted for the challenged groups in Figure 16. The IFA-PD vaccinated group's proliferative response increased after day 14, after the second PD immunization, and again between days 28 and 38, increasing after challenge. Similarly, IFA-PD vaccinated but non-challenged pigs' proliferative response increased from day 0 to 14 and again days 14 to 28 (data not shown). Peripheral blood mononuclear cells recovered from pigs vaccinated with the blank microsphere did not exhibit appreciable proliferation until after challenge (day 38). In contrast, the PD-loaded microsphere vaccinated group did not show an increase their proliferative response following challenge. This failure to increase proliferation in the PD-loaded microsphere vaccinated group in contrast to the blank

microsphere group further indicates immunological priming and differential immunological response induced by the microsphere delivered vaccine from PD delivered in Freund's incomplete adjuvant or the immune response induced by infection alone (blank microsphere group).

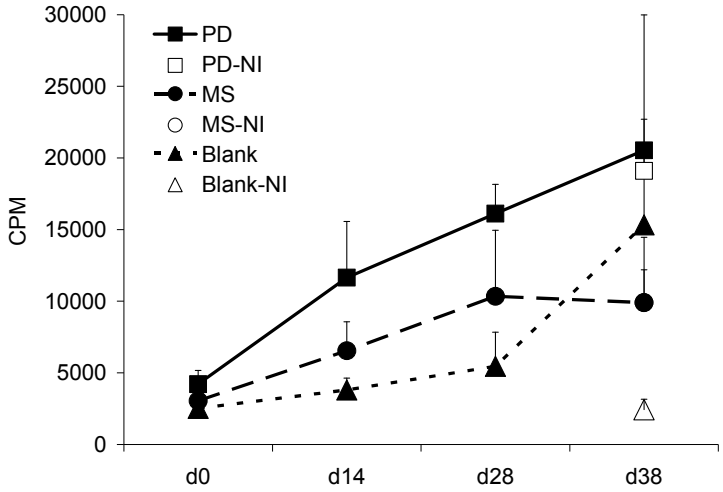

Figure 16: Peripheral blood mononuclear cell (PBMC) proliferative recall response to *B. hyodysenteriae* antigen. Peripheral blood was collected prior to vaccination (d0), at day 14, day 28 (prior to challenge), and day 38 (10 days after challenge). PBMC were isolated and stimulated as described in Materials and Methods. Pigs were vaccinated and challenged as described in Materials and Methods. Data points that represent the PBMC proliferation at day 38 for the corresponding non-infected pigs are represented by open symbols: IFA-PD (open square, n =5), PD-MS (open circle, n = 4 – superimposed with the closed circle) and blank-MS (open triangle, n = 3).

At necropsy, lymph nodes draining the injection site were excised, single cell suspensions were prepared, and stimulated in vitro with *B. hyodysenteriae* antigen. Very little antigen-specific proliferation was observed and was not statistically different between vaccination groups (data not shown). Unlike the vaccinated mice, antigen-specific cells were not detected in the lymph nodes adjacent to the injection site four weeks following vaccination. In addition, antigen-specific proliferative responses were also evaluated for cells recovered from the colonic lymph nodes. A robust proliferative response was observed in the colonic lymph node cell cultures from challenged pigs receiving the IFA-PD and PD-loaded microspheres (Figure 17). A significant

proliferative response was also observed in non-challenged animals receiving the PD-loaded microspheres. In contrast, colonic lymphocyte proliferation to *B. hyodysenteriae* antigen was not observed in infected only (blank microsphere vaccinated) animals. Colonic lymph node proliferation is in contrast to the PBMC response further indicating a different immune response induced by the two vaccine regimens.

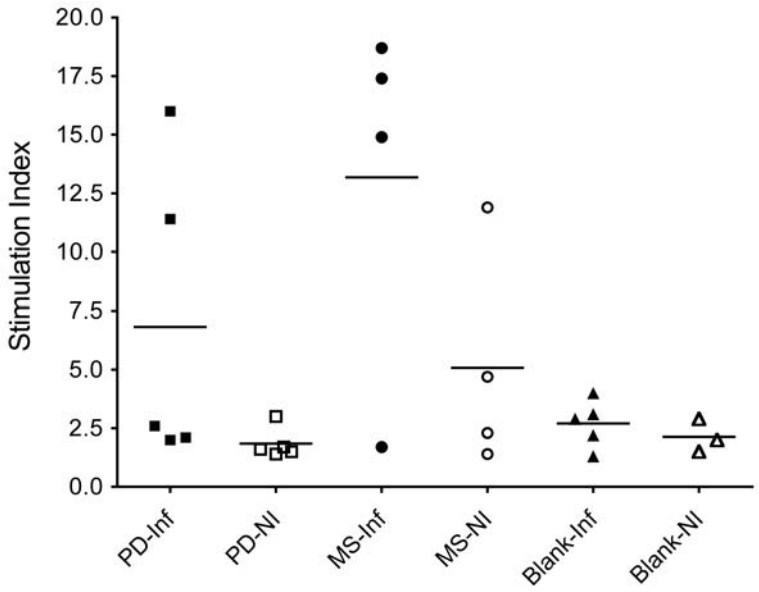

Figure 17: Antigen-specific proliferative response of lymphocytes recovered from porcine colonic lymph nodes. At necropsy, colonic lymph nodes were excised and single cell suspensions were prepared as described in materials and methods. Stimulation indices were calculated by dividing the counts per minute of antigen stimulated wells with the counts per minute from non-stimulated (background) wells. Statistical differences were found between PD-NI group and MS-Inf, and between MS-Inf and Blank-Inf but no other groups.

6 Discussion

To date, a majority of commercial vaccines used to induce protection against bacterial-induced diseases of swine employ two or more vaccinations increasing the burden on personnel needs and costs. Additionally, these vaccines are often whole cell bacterins and there is concern that adverse reactions at the injection site induced by TLR ligands can affect carcass quality. Previous studies from this laboratory had shown that the intramuscular administration to pigs of an enzymatic digest of *B. hyodysenteriae* (PD)

incorporated into IFA induced protection from swine dysentery. As was shown in the current study, two doses of the IFA-PD vaccine induced protection in 100 % of the pigs challenged with *B. hyodysenteriae.*

Consistent with previous observations, the IFA containing vaccine induced demonstrable antigen-specific serum antibody and proliferation of PBMCs that was accompanied by a characteristic granulomatous reaction at the injection site. In contrast to other laboratories using this model, there does not appear to be direct link between culture positive, highest number of symptom days, or severity of gross lesions at necropsy, as not all of these disease indices were seen in the each of the affected animals. All of the sham vaccinated pigs developed clinical swine dysentery following challenge. The majority of the pigs (4 of 5) receiving the single dose vaccine containing the PD-loaded polyanhydride microspheres (PD-MS) also developed swine dysentery. Relative to the sham vaccinated pigs, the PD-MS vaccinated pigs had slightly fewer diarrhea days (Figure 14) and were primed for the induction of a serum antibody response after challenge (Figure 15, day 38). There is little evidence that a serum antibody response alone provides protection from swine dysentery. However, these results suggested that pigs immunized once with a vaccine formulated with antigen-loaded MS would induce significant serum antibody following infectious challenge.

As cell-mediated responses may also be an important component of protective immunity, the results of these studies indicate that lymphocytes recovered from lymph nodes (LN) draining the colon of vaccinated pigs proliferated to a greater extent than those from the sham immunized pigs following challenge (Figure 17). In addition, the antigen-specific proliferative response of colonic LN cells recovered from the PD-MS vaccinated pigs showed greater proliferation than cells recovered from the IFA-PD group. Waters and Hontecillas showed that cells expressing CD8αα were increased following *B. hyodysenteriae* infection [15]. Vaccination with PD in a squalene-oil adjuvant increased proliferation and IFN-γ secretion by peripheral blood mononuclear cells but, at the colonic lymph node level, *PD* vaccination decreased numbers of proliferating cells [16].

While the original Th1/Th2 paradigm of CD4 T cell activation has evolved to include other induction pathways (Th17 and Treg) in laboratory rodents and humans,

these new T helper cells are poorly defined in livestock species and thus the Th1/Th2 differentiation still provides a suitable reference for nature of immune response. Aberrant immune responses can exacerbate many different inflammatory diseases. The lesions of *B. hyodysenteriae* have been likened to human inflammatory bowel disease. While the etiology of these diseases are not completely known, they are characterized by an increase of pro-inflammatory or Th1 cytokines. Previous studies have shown that PD vaccination increased IL-10 secretion by murine MLN [38] and comparatively reduced IFN-γ secretion by colonic lymph node cells of pigs [16]. In the present studies, increases in IL-10 secretion were observed from cecal tissue explants of vaccinated mice versus infected controls (Figure 6 B). The nature of the antigen-specific response (i.e., type of T cells proliferating or cytokines produced) induced by the various vaccination regimen was not evaluated. However, prior experience by the authors indicated that the clinical signs of swine dysentery were often exacerbated (earlier onset) by the induction of an inappropriate immune response. In this regard, clinical signs of swine dysentery appeared one to two days earlier in the PD-MS vaccinated pigs in comparison to the sham vaccinated group suggesting that vaccination with the PD-MS exacerbated disease induction. However, there were fewer total number of days with clinical disease in the PD-MS group compared to the sham vaccinated pigs (Figure 14).

Alteration in the colonic microbiota alters microbial activities (such as the inactivation of trypsin and conversion of bilirubin to urobiliogen, production of butyrate). The interactions between the microbiota and diet may predispose pigs to increasing severity of dysentery [39]. By treating the pigs in this study with several courses of broad spectrum antibiotics and housing them in very clean conditions, their intestinal microbiota may have been altered such as the organisms necessary for the induction of severe disease were not present. Oral administration of Baytril eliminated many Gram-negative anaerobe species resident in the gut of a wild rodent species [40]. Wiuff et. al., found that intramuscular administration of Baytril was effective at reducing the numbers of *Salmonella* and other coliform bacterial species in the intestinal tract of pigs [41]. As shown by Whipp et. al., gnotobiotic pigs only displayed the characteristic lesions of SD when also colonized with *Bacteriodes vulgatus*, a Gram-negative anaerobe [42]. The

authors speculate that the mild lesions and reduced immune response observed in this study may be attributed to alterations of the microbiota upon arrival at the facility and treatment with EXCENEL and Baytril in conjunction with to the clean environment, certain bacterial species were not reinoculated.

While the mouse studies did not provide evidence that the antigen-loaded MS induced protection from disease, there was evidence that the PD-loaded 50:50 microspheres enhanced antigen-specific antibody responses prior to and after challenge with *B. hyodysenteriae.* While the results were not statistically significant, there was also a trend toward less severe typhlocolitis and histopathological lesions in mice receiving the PD-loaded 50:50 polyanhydride microspheres. From the different replications (shown in this report as one experiment), there is a definite effect of vaccine regimen on resultant immune response. When free-antigen is included with the 50:50-PD loaded microspheres, the response more closely mirrors the PD vaccinate responses and there is a trend toward greater protection. This is consistent with other studies performed in this laboratory using ovalbumin as a model antigen in mice. Mice receiving Ova-encapsulated in 50:50 CPH:SA microspheres show an increased immune reactivity (antibody and cellular proliferation) as compared to mice receiving Ova-loaded 20:80 CPH:SA microspheres. The sustained release predicted for the 50:50 CPH:SA microspheres by model antigens in vitro, may be important in vivo for the development of sustained immune responses. In a separate replicate of the mouse immunization studies, the phenotype of cells in the mesenteric lymph node was analyzed by flow cytometry. While the percentage of $CD19^+$ and $CD4^+CD25^+$ in the draining lymph node was not different than those from non-immunized mice, there was a definite increase in the total number of cells recovered from the MLNs of infected with *B. hyodysenteriae* compared to those from mice infected and vaccinated or non-infected.

In a pilot study, sheep were injected with 20:80 CPH:SA microspheres loaded with *B. hyodysenteriae* whole cell sonicate (WCS). At 14 days post-immunization, the serum antibody response and peripheral blood proliferative responses were similar to sheep receiving WCS in Freund's incomplete adjuvant (data not shown). These responses, while still greater than their pre-immunization responses, waned by 45 days

post-immunization. It is possible that the microspheres did not sustain release of WCS antigens as was predicted by in vitro release of other model proteins. Also, while the dose of microspheres administered was based on a weight comparison of doses administered to mice and previous PD vaccination studies, the microsphere dose given to the sheep may have been insufficient for sustained response in the absence of *Brachyspira* species in the ovine microbiota or subsequent to an infectious challenge. This is relevant to the current study in pigs in that many of the same issues could explain the weak immune response (e.g., insufficient antigen dose) of pigs immunized with PD-loaded microspheres.

The blend of microspheres consisting of 20:80 and 50:50 CPH:SA nanospheres was used in the current swine immunizations with the hypothesis that this would provide for a sustained release of antigens from the 50:50 CPH:SA microspheres while taking advantage of the more rapid release of antigen from the 20:80 CPH:SA microspheres. However, in other studies, the blend of these two polymer chemistries actually suppressed subsequent immune responses as compared to animals that received either 20:80 CPH:SA or 50:50 CPH:SA alone. This unpredicted result could also explain the low immune responses in pigs receiving the PD-MS vaccine.

In conclusion, an enzymatic digestion of a whole cell antigen preparation was successfully encapsulated into polyanhydride microspheres. The antigen was released from the MS with the expected kinetic rate, and the released material was immunogenic as well as antigenic. In addition, administration of the PD-loaded MS to both pigs and mice induced antigen-specific immune responses; however, the PD-MS formulations used in these studies did not induce protection from clinical dysentery. Future studies will be required to optimize the immunization regimen or to evaluate the ability of antigen-loaded MS in order to become an efficacious vaccine carrier with benefit to livestock health.

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CHAPTER 7

General Conclusions

The induction of antigen-specific immune responses, in general, follows fairly predictable patterns. Pathogen-derived stimuli activate antigen presenting cells via pattern recognition receptors (e.g., TLRs). Activation in APCs such as dendritic cells (DCs) is characterized by the increased surface expression of antigen presenting molecules (MHC I or MHC II), increased in surface expression of costimulatory molecules (CD80, CD86, and/or CD40) and secretion of cytokines (IL-6, IL-12 and TNF-α) necessary for induction and activation of antigen-specific effector T cells . Activated T cells differentiate into either effector or central memory T cells, and these effector T cells can affect the differentiation of memory B cells into antibody secreting plasma cells. Thus, there are three keys to induction of a long-lived efficacious immune response to a pathogen: i) activation of the antigen presenting cell, ii) a sufficient dose of antigen to induce and sustain T cell activation, and iii) sufficient activation signals to ensure the induction of long term T cell and/or B cell memory. The last step in the evolution of a pathogen-specific immune response involves induction of the critical effector molecules or cells that are required for pathogen clearance with minimal host damage. For example, an antibody to block viral or toxin binding may be sufficient to prevent disease and will induce a lot less cellular damage than a plethora of armed $CD8^+$ T cells. The studies presented in this dissertation were undertaken to address specific aspects of these three key steps in the process of immune activation; in this regard, the final study evaluated the induction of protective immunity against *Brachyspira hyodysenteriae* using antigen-loaded polyanhydride microspheres in the context of an infectious disease.

First, a mechanistic study was undertaken to evaluate the activation of murine bone marrow derived DCs by incubating these cells in the presence of varying concentrations and differing compositions of CPH:SA and CPTEG:CPH microspheres. To this end, experiments were performed to evaluate the expression of cell surface markers on DCs and cytokine secretion from DCs that had been incubated with microspheres. All of the microsphere formulations tested, in general, increased the MFI

of MHC II expression, increased the MFI and percentage of cells expressing both CD86 and CD40. In addition, microspheres composed of CPTEG enhanced surface expression of CD209. There was little evidence that this observed activation of DCs occurred in either a dose- or chemistry-dependent manner as evidenced by poor R^2 values obtained by following the linear regression analysis ($R^2 = 0.2$ or lower). The activation of DCs by microspheres did enhance antigen-specific T cell proliferation as was observed using cells derived from transgenic OT I ($CD8^+$ T cell responder) and OT II ($CD4^+$ T cell responder) mice which carry an clonotypic T cell receptor that recognizes Ova-specific peptides in the context of the appropriate MHC molecule. The studies outlined in Chapter 3 demonstrated that CPTEG containing polymers and PLGA polymers enhanced OT I or $CD8^+$ T cell proliferation to a greater extent than they enhanced OT II or $CD4^+$ T cell proliferation. $CD8^+$ T cells have the ability to cross prime each other and respond to a lower threshold of stimuli than do $CD4^+$ T cells. Activation of $CD8^+$ T cells does not require a prolonged interaction between the peptide-MHC I complex on the DC with the T cell receptor on the $CD8^+$ T cell in order to initiate immune activation, antigen-specific clonal proliferation, and induction of memory cells. The observed differential activation of CD209, the differing magnitude of cytokine secretion, and the inconsistent induction of lymphocyte proliferation all suggest that CPH:SA and CPTEG:CPH copolymers are not activating the same pathways in DC. The differing chemistries were shown to have an effect on cellular uptake (phagocytosis) [1] and may also effect pathways of antigen processing and presentation (i.e., cytosolic for MHC I presentation and $CD8^+$ T cell activation, phagolysosome for MHC II presentation and CD4+ activation). In contrast, the role of DC activation for B cell activation is poorly defined and as APCs themselves, the B cells may interact directly with the microspheres in vivo leading to differentiation into antibody producing plasma cells. Future studies will need to be conducted to determine the effects of microspheres on antigen processing and presentation by DCs.

Studies evaluating the role of Toll-like receptors (TLRs) on innate and adaptive immunity have flooded the immunology literature of late [2-27]. One article that has relevance to our work with polyanhydrides, was Polly Matzinger's hydrophobicity hypothesis [25]. Simplified, what Janeway described as non-self receptors may actually

be more like danger receptors that bind hydrophobic ligands derived from both pathogenic invaders and damaged host cells [25]. The most promiscuous of these receptors appears to be TLR2 and TLR4. Starting with non-activating blocking antibodies and moving on to TLR2 and TLR4 deficient mice, DC activation by microspheres and immune response to Ova co-injected with microspheres was evaluated. While that data is still being analyzed and are not a part of this dissertation, these studies indicated that there is no difference in activation or in vivo immune response between $TLR2^{-/-}$, $TLR4^{-/-}$, and wild type C57BL/6 mice in the presence of the polyanhydride microspheres.

While the microspheres enhanced the expression of cell surface markers and increased cytokine secretion of DCs derived from C3H or C57BL/6 mice over non-stimulated or background levels, the magnitude of the responses was much less than when DCs were stimulated with TLR ligands (LPS, lipotecholic acid from *S. aureus*, and MPLA). It is important to note that microsphere stimulated DCs were still capable of responding to LPS when it was added to the medium 8 to18 hours after addition of microspheres to the DC culture indicating that the TLR-MyD88 activation pathway was not exhausted following exposure to the microspheres. Thus, the responses observed were not aberrant activation of DC, a mechanism induced by many pathogens in order to evade or inhibit adaptive immunity, but just low levels of activation. In comparison, it has been shown to be necessary to include MPLA in polyester microspheres in order to enhance DC activation in similar studies clouding the ability to conclude that the polyester microspheres directly activate DCs [28]. In this regard, future work with polyanhydrides should evaluate the inclusion of TLR ligands for enhancement of DC activation and possible enhancement of subsequent immune activation.

A study by Thompson et. al., showed that transfer of DO11.10 transgenic (Tg) T cells into naïve donors proliferated well when Ova was administered to the mice at the same time; but these Tg T cells did not accumulate in the lymph nodes if TLR agonists are not co-administered with Ova [29]. It is possible that we observed a similar phenomenon in the present studies. Specifically, we were able to detect and recover an expanded population of $CD4^{+}KJ1.26^{+}$ T cells from recipient BALB/c mice that were co-injected with Ova and blank microspheres employed as adjuvants; these cells received

sufficient survival signals such that they were still demonstrable after day 5. However, it is hypothesized that these cells did not receive sufficient signal to differentiate into effector or memory cells. Furthermore, as T cells mature from a naïve cell into an antigen experienced, effector/memory T cell pool, $CD4^+$ T cells require more time engaged with APCs in order to complete replication rounds and differentiate into effector cells [30]. Successive rounds of APC engagement, T cell proliferation followed by inactive "rest", constitutes one theoretical model of T cell activation where at each successive engagement-proliferation stage the T cells are maturing into effector and/or memory T cells [30-32]. If sufficient antigen and/or "danger-signal"-activated APC are available at each of these successive rounds of stimulation and replication, progress toward effector/memory cells stops and $CD4^+$ T cells undergo death by neglect [30-32]. From the present studies, it is evident that: 1) the time points chosen as part of the experimental design to observe these events was critical (in terms of days PI and length of stimulation ex vivo); and 2) the administration of soluble Ova (without replenishment from a depot or a replicating pathogen) did not remain in the lymph node long enough for complete $CD4^+$ T cell activation to occur. Both of these outcomes are consistent with the poor proliferative responses observed and the phenotype ($CD44^{hi}CD62L^{hi}$) of antigen-specific T cells recovered from mice receiving the Ova-responsive Tg T cells and Ova co-injected with microspheres. Had there been sufficient Ova and/or APC activation, we would have expected to demonstrate the presence of $CD44^{hi}CD62L^{lo}$ effector $CD4^+$ T cells.

In contrast, B cells that reside in the follicles of tissue draining lymph nodes can obtain soluble antigen that directly enters the lymph node via the afferent lymphatic vessels [33]. B cells can also interact with activated DCs and receive co-stimulation from them, but the B cell response (e.g., isotype switching) is limited in the absence of sufficient $CD4^+$ T cell activation. It has been shown by others that an antibody response may be limited to IgG1 production without the appropriate $CD4^+$ T cell signals [34, 35]. Therefore, it is likely that the IgG1 dominant Ova-specific antibody responses measured arose as a consequence of similar mechanisms.

After evaluating the results from mice immunized with Ova-loaded microspheres 12 weeks following a single immunization, there appeared to be a definite affect on the

immune response based on fabrication method of the microspheres. Lopac et. al., showed that there was no difference in release kinetics of Ova encapsulated by the two different fabrication methods [36]. That study showed a chemistry dependent decrease in protein stability. The current studies demonstrated that the immunogenicity of Ova was also affected by fabrication method as evidenced by the differences observed in mice immunized with Ova-loaded microspheres fabricated by S/O/O and CA. Differences were also observed in groups of mice receiving *Brachyspira*-derived antigen-loaded into microspheres fabricated by S/O/O and CA (data not shown). There was a trend in the profile of the immune response that suggested that the CA fabricated microspheres induced lower antibody responses and recall proliferative responses, and increased clinical signs of disease upon challenge with *B. hyodysenteriae*, but these results were not significantly different (data not shown). Even though the encapsulated bacterial antigen induced an immune response, this did not result in protective immunity. These studies were undertaken in an attempt to evaluate the ability of the polymer delivery system to induce protective immunity using a single dose immunization regimen. While the mice and pigs were primed, protective immunity was not induced. This was in contrast to the protective immunity induced in pigs using a more traditional two-dose oil-in-water emulsion.

Ovalbumin is susceptible to heat, chemical, and enzymatic degradation resulting in reduced immunogenicity [37-39]. One might conclude that the polymer induced degradation of Ova during encapsulation and/or release reduced the immunogenicity of the protein sufficiently such that the immune responses approximated those induced by a small dose (25 μg) of soluble protein administered without an adjuvant. The observed responses were actually enhanced over soluble Ova-alone in that the mice were primed sufficiently to respond with an anamnestic antibody response and modest, but detectable, Ova-specific proliferative response following in vivo boost with an antigenic challenge.

The present studies also demonstrated that there were strain-related differences the Ova-specific immune response. As these common mouse strains (C3H/HeN, C57BL/6, and BALB/c) possess different MHC haplotype genes that likely affect or restrict the epitope repertoire of their respective MHC II molecules. In this regard, the

testing of a single antigen in several strains of mice has become a good predictor of an antigen's immunogenicity in an out-bred population [40-42]. Similarly, a mixed epitope vaccine should show varying magnitude of efficacy in an outbreed (mixed haplotype) population. Thus, the variation observed in the levels of protection of the PD-loaded microsphere vaccinated pigs may be linked to mixed genetic background. The pigs used in this study, like humans, represent a heterogeneous population. Two out of the five pigs receiving PD-loaded microspheres were protected from challenge, thus strongly suggesting that while the entire protein may not have been destroyed, key epitopes for some MHC configurations may have been lost. It was an interesting observation that the three pigs that exhibited the most severe clinical signs of dysentery (one from the blank-MS group and two from the PD-loaded MS group) were thin, dark skinned red pigs, a unique phenotype as compared to the rest of the group, but unfortunately records were not able to be obtained from the supplier that could confirm genetic linkage (e.g., littermates).

It is not insignificant that a key underlying observation in all of these studies was biocompatibility of these novel polyanhydrides. High concentrations of polymer were tolerated by a variety of cell types in vitro [43]. In all of the murine studies (comprising well over 1,000 mice) and preliminary studies in two livestock species (sheep and pigs), no injection site reactivity (severe fibrosis or granuloma formation) was observed. Good vaccine adjuvants walk a fine line between efficacy and toxicity (i.e., LPS and MPLA). As evidenced by the DC activation studies and the lack of tissue reactivity in vivo, the polymers themselves may not be inducing much of a "danger" signal. There is a correlation between tissue site reactivity and the magnitude of antibody production [44]. However, in humans, injection site reactivity following vaccination is cited as a key reason for poor patient compliance in westernized countries [45]. In livestock species, granulomas and tissue site abscesses contribute to economic losses at harvest [44, 46, 47]. Future studies must find a balance of danger signal and biocompatibility in order for these polyanhydrides to make effective vaccine adjuvants.

As for the effect of long-term storage on antigen loaded microspheres, the results described herein suggest that there is preservation of immunogenicity during storage.

There are several unique features of tetanus toxoid that make it a very successful model antigen. TT is a very potent immunogen in laboratory animals. Small doses induce high titer antibody responses and long term TT-specific memory responses [48, 49]. Finally, TT is generated by formalin fixation of tetanus toxin and this chemical modification may improve the antigenic stability of TT. There were quite possibly other sites within the TT that were stabilized by fixation besides just linking the A and B subunits. This formalin fixation could have given the protein additional stability during encapsulation and subsequent release from CPH:SA microspheres that was not a consideration for the proteins studied as part of this dissertation (i.e., Ova, or, *Brachyspira* pepsin digest).

Further studies will need to consider the protein-polymer interactions that are occurring and how these affect antigen processing and presentation. Taken together, these studies indicate that the protein stability and dose of delivered protein over time were key factors in the inability to induce robust antigen-specific immune responses or protective immunity.

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