

**A Biodegradable Microparticle Vaccine Platform using Femtomole Peptide Antigen  
Doses to Elicit T-cell Immunity against *Chlamydia abortus***

by

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## Abstract

Successful vaccination against *Chlamydia* spp. has remained elusive, largely due to a lack of vaccine platforms for the required Th1 immunization. Modeling of T helper cell immunity indicates that Th1 immunity requires antigen concentrations that are orders of magnitude lower than those required for Th2 immunity and antibody production. We hypothesized that the *C. abortus* vaccine candidate proteins that we identified earlier, DnaX2, GatA, GatC, Pmp17G, and Pbp3, mediated protection in an A/J mouse model of *C. abortus* lung infection if administered each at low, 1-20 femtoMole doses per mouse. This immunization significantly protected the mice from lethal challenge with  $10^8$  *C. abortus* organisms.

Additional experiments proved that particulate delivery of antigens was required for optimum immunity. As a delivery vehicle, we constructed spray-dried microparticles of 1-3  $\mu\text{m}$  diameter that were composed of biodegradable poly-lactide-co-glycolide polymers and the poloxamer adjuvant Pluronic L121. These microparticles, when administered at 10  $\mu\text{g}$  per mouse dose, were effectively phagocytosed by macrophages and protected C3H/HeJ mice from lethal challenge with *C. abortus*, and thus were effective immune stimulators (biological response modifiers).

We further hypothesized that i) 20-mer peptides overlapping by 10 amino acids could substitute for whole protein antigens when embedded in a 1-3  $\mu\text{m}$  diameter microparticulate vaccine; ii) such phagocytosed biodegradable microparticles would

intracellularly release peptides and adjuvant from such microspheres in antigen presenting cells and would enable controlled generation of Th1 immunity; and iii) inclusion of Q-VD-Oph, an inhibitor of apoptosis, could suppress a co-emerging inflammatory Th17 response and enhance a protective Th1 response.

A dose of 2.00 femtoMoles of each peptide per mouse significantly reduced disease after lethal *C. abortus* challenge inoculation, but failed to effectively eliminate chlamydiae. In contrast, the inclusion of Q-VD-Oph in the subcutaneously or intranasally administered 0.20 femtoMoles peptide vaccine resulted in effective elimination of *C. abortus* and completely reversed the disease outcome to a fully protected healthy phenotype. We hypothesized that the 50:50DL-PLG-PEG lactide-co-glycolide polymer, used as rapidly degrading vaccine carrier, had released substantial acidity intracellularly in antigen presenting cells that induced an immunosuppressive apoptotic signal. Thus, the enhanced apoptosis of antigen presenting cells required release of Q-VD-Oph from vaccine microparticles to abolish apoptosis and Th1 immunosuppression. Therefore, we investigated the use of an alternative polymer carrier, the slowly degrading and minimally acid-releasing polylactide polymer DL-PL R202S. This carrier, when used with 0.5-1.25 femtoMole dosage of *C. abortus* peptide antigens, induced highly significant protection against chlamydial challenge without triggering an unwanted Th1 suppressive or inflammatory immune response in the *C. abortus* respiratory mouse disease model.

In summary, we have developed a fully synthetic biodegradable microsphere vaccine for controlled release of adjuvant and ultralow doses of peptide antigens. This vaccine platform may be commercially useful for discovery and production of vaccines.

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## List of Abbreviations

$\mu\text{g}$	Microgram; one millionth of a gram; $10^{-6}$ gram
$\mu\text{m}$	Micrometer; one millionth of a meter; $10^{-6}$ meter
APC	Antigen Presenting Cells
BRM	Biological response modifier
<i>C.</i>	<i>Chlamydia</i>
COMC	Chlamydial Outer Membrane Complex
<i>Cp</i>	<i>Chlamydothila</i>
CPAF	Chlamydial Protease-like Activity Factor
DC	Dendritic cell
DCM	Dichloromethane
DL-PL	Poly (DL-lactide)
DL-PLG	Poly (DL-lactide-co-glycolide)
DL-PLG-PEG	Methoxy poly (ethylene glycol) - DL-poly (lactide-co-glycolide)
Dnax2	DNA polymerase III subunits gamma and tau
DTH	Delayed Type Hypersensitivity
EB	Elementary bodies
fM	femtoMoles
GatA	Glutamyl-tRNA amidotransferase subunit A
GatC	Glutamyl-tRNA amidotransferase subunit C
i.n.	intranasal
i.p.	intraperitoneal
IFN	Interferon
IL	Interleukin

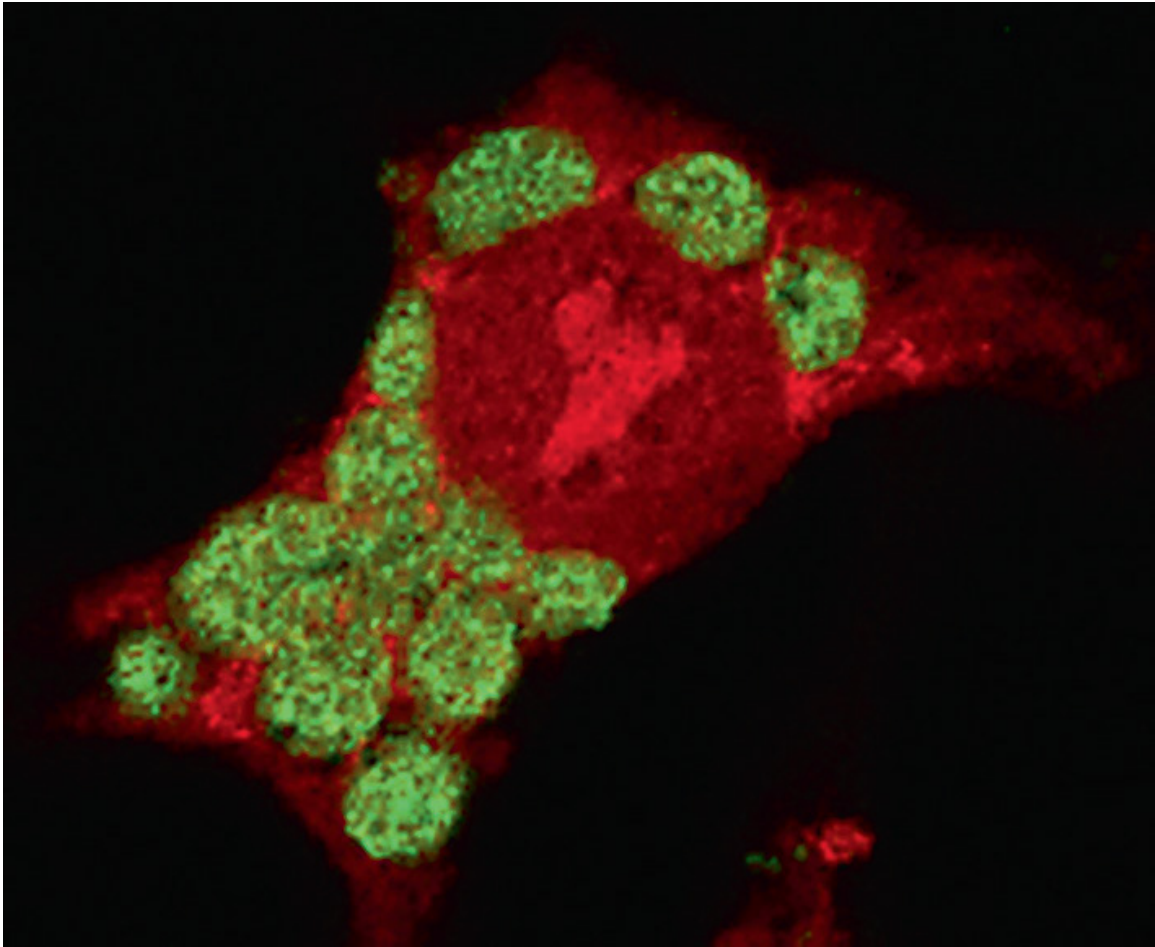
IV	Intrinsic Viscosity
LPS	Lipopolysaccharides
MHC	Major Histocompatibility Complex
mg	milligram
mL	milliliter
Mw	Molecular weight
NF- $\kappa$ B	Nuclear Factor $\kappa$ B
ng	nanogram; one billionth of a gram; $10^{-9}$ gram
OmpA	Outer membrane protein A
PAMP	Pathogen Associated Molecular Pattern
Pbp	Penicillin-binding protein
PBS	Phosphate Buffered Saline
pg	picogram; one-trillionth of a gram; $10^{-12}$ gram
Pmp90A	Polymorphic outer membrane protein 90A
PRR	Pattern Recognition Receptors
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
qPCR	quantitative Polymerase Chain Reaction
RB	Reticulate bodies
s.c.	subcutaneous
Tg	Glass transition temperature
Th	T helper cells
TLR	Toll-like Receptor

## CHAPTER 1

### REVIEW OF LITERATURE

#### 1.1. *CHLAMYDIA* - BACKGROUND AND TAXONOMY

The genus *Chlamydia* of the family *Chlamydiaceae*, order *Chlamydiales*, and phylum *Chlamydiae* is comprised of coccoid, gram negative-like obligate intracellular bacteria. Chlamydiae are characterized by a specialized biphasic developmental cycle that lasts 30–72 hours and is unparalleled among prokaryotes. Due to their such a unique nature, chlamydiae were initially not only misclassified as protozoa or viruses, but also received various designations such as *Miyagawanella*, *Bedsonia*, Ornithosis-, Trachoma inclusion conjunctivitis (TRIC)- and psittacosis-lymphogranuloma venereum (LGV) trachoma (PLT)- agents, either in honor to the scientists who first described them or referring to the type of disease they caused (Nunes and Gomes, 2014). The current term ‘*Chlamydia*’ has its root in the Greek word “*Chlamys/khlamus*”, meaning mantle or the short cloak worn by Greek military men draped around their upper shoulders and secured with a brooch on the right shoulder (Black, 2013). The term derived from a misconception dating back to 1907, when Halberstaedter and von Prowazek (1909,1907) thought they had discovered “‘mantled protozoans’”, to designate intracytoplasmic vacuoles containing numerous microorganisms clustered around (are ‘draped’ around) the nucleus of the cell (Fig. 1.1) , in Giemsa-stained conjunctival scrapings from trachoma cases (reviewed by Nunes and Gomes, 2014).



**Figure 1.1. Photomicrograph of fluorescently stained chlamydial inclusions (green) clustered around the nucleus of a host cell given appearance of a chlamys. (From Black, 2013).**

The taxonomy of *Chlamydiales* has been controversial during the past decade. There are two recognized types of taxonomy in the classification of chlamydial species, the ‘old classification’ and ‘new classification’. The ‘old’ classification is based on distinct biochemical characteristics, morphological features, developmental forms, and host range. In 1966, immediately after Moulder definitively reported the bacterial nature of chlamydiae (Moulder, 1966), the genus *Chlamydia* was established (Page, 1966) and classified into two species: *Chlamydia (C.) trachomatis* and *C. psittaci* (Page, 1968). *C. pneumoniae*

(Grayston *et al.*, 1989) and *C. pecorum* (Fukushi and Hirai, 1992), formerly known as strains of *C. psittaci*, were designated as distinct species, in 1989 and 1992, respectively.

However, in 1999, a combination of 16S, 23S ribosomal RNA (rRNA), and ribosomal intergenic spacer genes were used by one group to propose a new classification based exclusively on the phylogeny derived from sequence polymorphisms of these genes (Everett *et al.*, 1999). This new molecular knowledge led to a reclassification of the *Chlamydiales* into one order comprised of four families (*Parachlamydiaceae*, *Simkaniaceae*, *Waddliaceae*, and *Chlamydiaceae*), with the *Chlamydiaceae* family being subdivided into two separate genera: *Chlamydia* and *Chlamydophila*. The division of the *Chlamydiaceae* was based on sequence polymorphisms as well as the fact that *Chlamydophila* spp. do not produce any detectable glycogen and have only a single ribosomal operon. The new classification further split the genetically heterogeneous species of *C. trachomatis* and *C. psittaci* into three and four species, respectively. Thus, this revision resulted in the definition of nine species within *Chlamydiaceae*, i.e. *C. trachomatis*, *C. muridarum* and *C. suis*, as well as *Chlamydophila (Cp.) abortus*, *Cp. caviae*, *Cp. felis*, *Cp. pecorum*, *Cp. pneumoniae*, and *Cp. psittaci*.

However, a majority of researchers in the field opposed the new classification and advocated a re-unification of the genus *Chlamydia* (Bavoil *et al.*, 2013; Greub, 2013; Schachter *et al.*, 2001; Stephens *et al.*, 2009). The main issues of concerns are that the 16S rRNA sequence identity thresholds do not consistently separate *Chlamydia* from *Chlamydophila* species, the genomes of all members of the *Chlamydiaceae* are highly similar, and most importantly, clear phenotypic features distinguishing *Chlamydia* from *Chlamydophila* species are missing (Sachse *et al.*, 2015).

Recently a ‘compromise’ taxonomy has been suggested, which puts a single genus (*Chlamydia*) into the family *Chlamydiaceae* which contains now eleven chlamydial species since the addition of *C. gallinacea* and *C. avium* (Sachse *et al.*, 2015). This compromise taxonomy has already been published in the current edition of Bergey’s Manual of Systematic Bacteriology (Kuo *et al.*, 2011), and is the classification that will be adhered to throughout this thesis (Bavoil *et al.*, 2013). One more species, *C. ibidis*, has recently been proposed based on the isolation of a single strain from the cloaca of a feral sacred ibis (*Threskiornis aethiopicus*), but this species has not yet been accepted (Vorimore *et al.*, 2013). Therefore, the single genus *Chlamydia* currently comprises 11 species (Table 1.1) based on broadened criteria that encompass biological as well as molecular properties.



**Table 1.1. Current major members of the order *Chlamydiales*** (Reviewed by: \*Sachse *et al.*, 2015; † Vorimore *et al.*, 2013; \* Corsaro and Greub, 2006; \*Greub, 2009; ♥ Lamoth *et al.*, 2015).

Family	Genus	Species	Natural Host	Other Host	Site of Infection
<i>Chlamydiaceae</i>	<i>Chlamydia</i>	(i) <i>C. abortus</i> *	Sheep, goat	Cattle, swine	Genital, respiratory
		(ii) <i>C. psittaci</i> *	Birds	Mammals	Respiratory
		(iii) <i>C. caviae</i> *	Guinea pig	Horse	Ocular, genital
		(iv) <i>C. felis</i> *	Cat		Ocular, respiratory
		(v) <i>C. avium</i> *	Pigeon, parrots		Respiratory
		(vi) <i>C. gallinacea</i> *	Chicken	Other poultry	Respiratory
		(viii) <i>C. pecorum</i> *	Cattle, koala	Sheep, goat, swine	Gastro-intestinal, respiratory, urogenital
		(ix) <i>C. pneumoniae</i> *	Human, horse, koala	Amphibians, reptiles	Respiratory, cardiovascular
		(x) <i>C. muridarum</i> *	Rodents		Gastro-intestinal
		(xi) <i>C. suis</i> *	Swine	Ruminants	Gastro-intestinal, genital
		(xii) <i>C. trachomatis</i> *	Human		Ocular (Trachoma), urogenital (STD), lymph node (LGV)
		<i>Parachlamydiaceae</i>	<i>Parachlamydia</i>	<i>P. acanthamoebae</i> **	Amoeba
<i>Simkaniaceae</i>	<i>Simkania</i>	<i>S. negevensis</i> *	Amoeba	Human	Respiratory in humans
<i>Waddliaceae</i>	<i>Waddlia</i>	<i>W. chondrophila</i> **♥	Amoeba	Ruminants	Urogenital in ruminants

## 1.2. BIOLOGY AND MOLECULAR PATHOGENESIS

**Chlamydial structure.** Despite profound differences in host range, chlamydiae display a remarkable similarity in their genome sequences (Carlson *et al.*, 2005; Read *et al.*, 2000 and 2003; Stephens *et al.*, 1998). They also possess a conserved intracellular growth cycle with distinct biphasic developmental cycle comprising two morphologically and functionally distinct infectious and reproductive forms: the elementary bodies (EBs) and the reticulate bodies (RBs) (Bedson and Bland, 1932; Bedson and Gostling, 1954).

The EBs are characterized by a small size (around 0.3  $\mu\text{m}$ ), a rigid cell wall, densely packed DNA, with the ability to survive in the harsh extracellular environment (AbdelRahman and Belland, 2005; Elwell *et al.*, 2016). Although earlier they were considered to be metabolically inert, recent studies conducted in a host-free (axenic) system demonstrated that EBs have high metabolic and biosynthetic activities and rely on D-glucose-6-phosphate as an energy source (Omsland *et al.*, 2014). Electron microscopic examination of EBs revealed the presence of a hexagonally organized surface projections, arranged regularly with a center to center spacing of approximately 50 nm, which correspond to a Type III secretion system “needle” structure (Nichols *et al.*, 1985; Matsumoto, 1982; Fields *et al.*, 2003).

In contrast to EBs, RBs are comparatively larger (1–2  $\mu\text{m}$ ), the cytoplasm appears granular with diffuse, fibrillar nucleic acids, and are bounded by an inner and outer-membrane, resembling other gram-negative eubacteria (AbdelRahman and Belland, 2005). They are specialized in nutrient acquisition and replication (Bastidas *et al.*, 2013). They also highly express proteins that are required for ATP generation, protein synthesis and

nutrient transport (Saka *et al.*, 2011). They probably depend on ATP scavenged from the host as a source of energy (Omsland *et al.*, 2014).

**Developmental cycle.** The development cycle of chlamydiae, as shown in Fig. 1.2, is completed in three phases: (i) binding and invasion of the EBs into the host cell, (ii) establishing an intracellular niche and reorganization of EBs to RBs, and (iii) transition of a large fraction of the reticulate body population into a new generation of elementary bodies and their exit from the host cell (Elwell *et al.*, 2016).

The chlamydial life cycle is initiated when an infectious EB binds to a susceptible target cell, promoting entry into a host cell-derived phagocytic vesicle. Binding of EBs with the host cells is thought to be a two-step process, involving an initial reversible and electrostatic interaction between the EB and the host cell mediated by heparan sulfate containing glycosaminoglycans, followed by high-affinity irreversible binding to a secondary receptor (Dautry-Varsat *et al.*, 2005; AbdelRahman and Belland, 2005). Both chlamydial and host proteins may act synergistically to promote invasion. Immediately upon contact, pre-packaged T3SS effectors from EBs are injected to the host cell (Saka *et al.*, 2011), and EBs are internalized within a membrane bound inclusion (Bastidas *et al.*, 2013). The nascent inclusions then quickly dissociate from the endolysosomal pathway, are transported along microtubules to the microtubule-organizing center, which facilitates interactions with nutrient rich compartments and helps to avoid fusion with lysosomes (Richards *et al.*, 2013).

After 6 to 8 hours post-internalization, EBs develop into RBs and early genes are transcribed (Tan, 2012). These early effectors remodel the inclusion membrane, redirect

exocytic vesicles to the inclusion membrane and promote host-pathogen interaction (Moore and Ouellette, 2014). In mid-cycle (approximately 8 to 16 hours post-internalization), essential genes are expressed which include effectors that mediate nutrient acquisition and maintain the viability of the host cells. During this stage, the RBs replicate by binary fission and secrete additional effectors that promote substantial expansion of the inclusion.

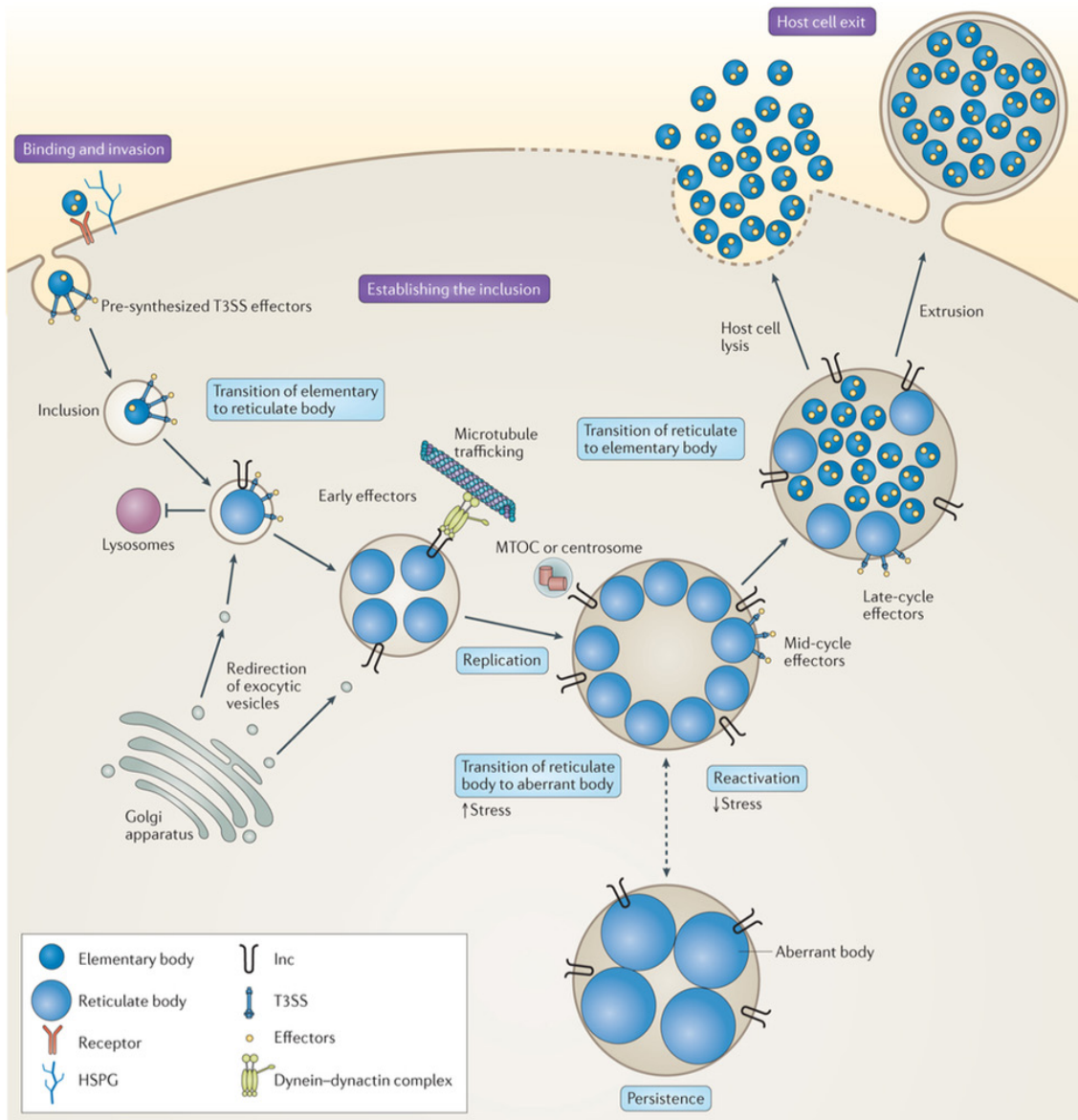
At the late stage of the development cycle, ~24 to 72 hours post-internalization of the EBs, RBs begin differentiating back to EBs in an asynchronous fashion. These EBs then detach from the inclusion membrane and accumulate in the lumen of the inclusion as the remainder of the RBs continue to multiply (Fields, 2012). The genes that are expressed at this late stages encode mainly the outer membrane complex and the DNA binding histone H1-like and H2-like proteins, Hc1 and Hc2, which condense DNA and switch off the transcriptions of many genes (Tan, 2012). Moreover, some of these late cycle effectors are packaged in progeny EBs to be discharged in the next cycle of infection (Fields, 2012; Saka *et al.*, 2011). Finally, forty-eight to 72 h after completing the development cycle, depending primarily on the infecting chlamydial species, infectious EB are released from the host cell by two mutually exclusive mechanisms: host cell lysis or the extrusion of the inclusion through fusion of the inclusion membrane with the plasma membrane (Hybiske and Stephens, 2007; Hackstadt, 1999; Moulder, 1991).

Under certain conditions, however, such as depletion of essential nutrients (iron, tryptophan, essential amino acids), exposure to host cytokines, or antibiotics that target cell synthesis, the life cycle of *Chlamydia* can be reversibly arrested (Byrne and Beatty, 2012). In response to these stresses, *Chlamydia* transitions into metabolically quiescent

noninfectious aberrantly enlarged structures termed the “persistent” form (Beatty *et al.*, 1994; Schoborg, 2011). Due to their reduced or negative ribosomal cell activities, these persistent bacteria present no adequate targets for the known *Chlamydia*-targeting antibiotics (Gieffers *et al.*, 2001; Kutlin *et al.*, 1999; Wang *et al.*, 2009). They also represent characteristic gene and protein expression profiles with reduced levels of outer membrane proteins like the major outer membrane protein OmpA or OmcB, and significantly higher levels of Heat shock protein 60 (Hsp 60) (Beatty *et al.*, 1993). *Chlamydiae* may be reactivated from persistence by removal of the inducing stimulus.

**Virulence Factors.** *Chlamydiae* encode a large number of virulence factors that represent ~ 10% of their genome (Elwell *et al.*, 2016). As identified by proteome-analysis of *Chlamydia* spp., proteins of the chlamydial outer membrane complex (COMC), chlamydial lipopolysaccharide (cLPS), chlamydial heat-shock-proteins (e.g. chsp60/GroEL-1), a type III secretion apparatus (TTS), the “chlamydial protease- or proteasome- like activity factor” (CPAF) or peptidoglycans and peptidoglycan-like structures are likely candidates as possible virulence factors (Krüll and Suttorp, 2007).

The chlamydial COMC is a disulfide cross-linked network of three proteins: the outer membrane protein A, OmpA (formerly termed as major outer membrane protein or MOMP), the large cysteine-rich outer membrane complex B protein (OmcB) and the small cysteine-rich outer membrane complex A protein (OmcA) (Hatch, 1999). OmpA, OmcA, and OmcB are present in characteristic molar ratio (5:2:1) in the COMC (Everett and Hatch, 1991).



**Figure 1.2. The life cycle of *Chlamydia trachomatis*.** The binding of elementary bodies to host cells is initiated by the formation of a trimolecular bridge between bacterial adhesins, host receptors and host heparan sulfate proteoglycans (HSPGs). Next, pre-synthesized type III secretion system (T3SS) effectors are injected into the host cell, some of which initiate cytoskeletal rearrangements to facilitate internalization and/or initiate mitogenic signaling to establish an anti-apoptotic state. The elementary body is endocytosed into a membrane-bound compartment, known as the inclusion, which rapidly dissociates from the canonical endolysosomal pathway. Bacterial protein synthesis begins, elementary bodies convert to reticulate bodies and newly secreted inclusion membrane proteins (Incs) promote nutrient acquisition by redirecting exocytic vesicles that are in transit from

the Golgi apparatus to the plasma membrane. The nascent inclusion is transported, probably by an Inc. along microtubules to the microtubule-organizing centre (MTOC) or centrosome. During mid-cycle, the reticulate bodies replicate exponentially and secrete additional effectors that modulate processes in the host cell. Under conditions of stress, the reticulate bodies enter a persistent state and transition to enlarged aberrant bodies. The bacteria can be reactivated upon the removal of the stress. During the late stages of infection, reticulate bodies secrete late-cycle effectors and synthesize elementary-body-specific effectors before differentiating back to elementary bodies. Elementary bodies exit the host through lysis or extrusion. (From Elwell *et al.*, 2016).

The gene *ompA* encodes OmpA and possesses four highly variable domains (VD1 to VD4) that contain subspecies- and serovar-specific antigenic determinants (Millman *et al.*, 2001). OmpA is found as highly immunodominant in all chlamydial strains except *C. pneumoniae* (Christiansen *et al.*, 1997). In the EBs, it is the main component for chlamydial protection against environmental stress outside the host, binding to the host cell, and defense against the host immune response (Hackstadt, 1999). In contrast to OmpA, OmcB, encoded by *omcB*, is not surface exposed, rather it forms a supramolecular lattice in the periplasm, and highly conserved among *Chlamydia* species (Frikha-Gargouri *et al.*, 2008). OmcB has been shown to act as an adhesin for chlamydial invasion into host cells (Fadel and Eley, 2007 and 2008). It is also thought to be involved in the RBs to EBs transition and contribute to cell wall rigidity and osmotic stability of the EBs (Newhall, 1987; Mygind *et al.*, 1998). Like OmpA, it is also found as an immunodominant antigen with both B-cell and T-cell epitopes (Gervassi *et al.*, 2004; Goodall *et al.*, 2001a; Mygind *et al.*, 1998; Sarén *et al.*, 2002; Wang *et al.*, 2010) and has been considered and used as a candidate for developing both serodiagnostics (Bas *et al.*, 2001; Frikha-Gargouri *et al.*, 2008; Rahman *et al.*, 2015) and subunit vaccines (Olsen *et al.*, 2010; Eko *et al.*, 2004; Penttilä *et al.*, 2004). OmcA serves as the functional equivalent of peptidoglycan and provides the structural integrity of the outer envelope through disulfide cross-links with OmcB and OmpA (Everett and Hatch, 1995).

The 12-, 60-, and 75-kDa heat shock proteins of *Chlamydiae* are closely related to their counterparts in *Escherichia coli* (GroEL, GroES and DnaK) and also to related human mitochondrial proteins (Hsp10, Hsp60, and Hsp70) (Furrows and Ridgway, 2006; LaVerda, 1999). They are highly conserved chaperonins involved in protein folding. Hsp60



is highly immunogenic and thought to play a major role in pathogenicity. *C. trachomatis* Hsp60 has been shown to stimulate production of pro-inflammatory cytokines in endothelial and smooth-muscle cells and macrophages, and it can also promote the activation of specific immune cells via a Toll-like receptor (Cappello *et al.*, 2009). In fact, elevated anti-Hsp60 IgA antibody-titers are considered biomarkers of chronic chlamydial infections, such as, bronchial asthma, chronic obstructive pulmonary disease (COPD), arteriosclerosis or pelvic inflammatory disease, pelvic inflammatory disease (PID) (Sävykoski *et al.*, 2004; Huittinen, *et al.*, 2001; Kol *et al.*, 1998; Wagar *et al.*, 1990).

Like other Gram-negative bacteria, *Chlamydia* have LPS antigen (cLPS). Although chlamydial LPS has been considered as genus-specific, a monoclonal antibody that recognized the LPS and neutralizes the infectivity of *C. pneumoniae* strain TW183 (Peterson *et al.*, 1998), failed to neutralize other strains of *C. pneumoniae* suggesting the presence of more than one genus-specific epitope on cLPS (Krüll and Suttorp, 2007). cLPS is similar to the rough LPS of certain salmonellae, however, it differs significantly from LPS of other Gram-negative pathogens at least in two different ways: 1) the core trisaccharide 3-deoxy-D-manno-octulosonic acid (KDO) structure of chlamydial LPS contains a 1-8 linkage, a genus specific epitope as well as a 1-4 linkage similar to that of other bacteria (Belunis *et al.*, 1992; Brade *et al.*, 1987), and 2) the chlamydial LPS has low endotoxic activity, although it induces some cytokines, such as TNF- $\alpha$  (Ingalls *et al.*, 1995; Ivins and Wyrick, 1978). cLPS is synthesized by a single multifunctional KDO transferase (Löbau *et al.*, 1995). During the developmental cycle of chlamydiae, cLPS can be released from intracellular, intra-inclusion chlamydiae to the inclusion membrane, and diffuse through the host cell cytoplasm and surface to cells surrounding the infected cells

(Campbell *et al.*, 1994; Karimi *et al.*, 1989; Richmond and Stirling, 1981). Although such release might have an impact on the chlamydial pathogenesis and the host's immune disposition of infected cells, studies have demonstrated that cLPS plays only a minor role for target cell activation (Netea *et al.*, 2002; Löbau *et al.*, 1995), which may explain the prevalence of asymptomatic nature of chlamydial infection.

Chlamydial protease-like activity factor (CPAF) is encoded on the chlamydial genome, produced by the chlamydial RBs, secreted out of the inclusion, and localizes predominantly to the cytosol of the infected cell and released into the extracellular milieu upon cellular lysis (Zhong *et al.*, 2001). It has been first identified to function in the degradation of host cell transcription factors necessary for MHC-I (RFX5) and -II ("upstream stimulatory factor 1", USF-1) antigen presentation which suggested a specific immune evasion mechanism of *Chlamydia*-mediated by CPAF (Zhong *et al.*, 2000; Zhong *et al.*, 1999). Recently, CPAF has been found to cleave the pro-apoptotic Bcl2 family proteins (Pirbhai *et al.*, 2006), which may contribute to the strong anti-apoptotic effect exerted by *Chlamydia* in the infected host cell (Fan *et al.*, 1998), thereby prolonging survival and allowing successful completion of the developmental cycle. Furthermore, CPAF has been shown to degrade cytoskeletal elements, including keratin-8 (Dong *et al.*, 2004), keratin-18, vimentin and  $\beta$ -tubulin (Savijoki *et al.*, 2008), within infected cells and is thought to allow the expansion of the inclusion and subsequent release of EBs. Collectively, the protease-like activity of CPAF interacts with major host proteins to manipulate the host immune response in various ways and thus keep the developing inclusion less detectable by immune surveillance. CPAF was also found to be one of the seven highly immunodominant proteins in an array of 156 fusion proteins cloned from

ORFs in the chlamydial genome (Sharma *et al.*, 2006). It is highly conserved among different chlamydial serovars and species (Dong *et al.*, 2005), hence seems to be a potential candidate for a pan-serovar anti-chlamydial vaccine.

Type III secretion systems (T3SSs) are complex structures composed of several bacterial proteins that are designed to promote the discharge of effector proteins after contact with the host cell (Cornelis *et al.*, 2000). Like most other Gram-negative pathogens, *Chlamydia* encodes a T3SS (cT3SS) which is thought to form projections on the chlamydial cell surface as detected with electron microscopy (Gregory *et al.*, 1979; Matsumoto, 1982). The genomes of *Chlamydiae* encode multiple conserved proteins of the T3SS injectisome, a molecular ‘nanosyringe’ made of ~20–25 proteins, the translocator apparatus and chaperone subclasses, which together are required for the assembly and functioning of the T3SS pathway (Ghosh, 2004). With the delivery of pathogen effector molecules at the different developmental stages (Mueller *et al.*, 2013), cT3SS appears to play multiple roles throughout the chlamydial developmental cycle (Peters *et al.*, 2007; Fields, 2007). Some of the important Chlamydial T3SS effectors include translocated actin-recruiting phosphoprotein (Tarp), Ser/Thr protein kinase (Pkn5), negative regulator of TTS (CopN), and inclusion membrane proteins IncA-C (Peters *et al.*, 2007). Tarp is ‘preloaded’ in the T3SS needle of the EB so that it can mediate early cytoskeletal changes, by recruiting actin during internalization to the site of EB internalization (Clifton *et al.*, 2005, 2004; Jewett *et al.*, 2006). Inc proteins have been suggested to subvert host cell vesicular and nonvesicular transport (Derre *et al.*, 2011; Rzomp *et al.*, 2006). CopN is involved both in downregulation of T3SS and physical shutoff of the injectisome as RBs transition into EBs (Peters *et al.*, 2007). The importance of chlamydial T3SS has been

proven by the abnormal intracellular chlamydial development as the result of inhibition of the T3S or the effectors (Bailey *et al.*, 2007; Muschiol *et al.*, 2006; Wolf *et al.*, 2006).

**Detection of and Response to Chlamydial Infection by the Host.** Similar to most other bacterial infection, pattern recognition receptors (PRRs) of the host recognize chlamydial LPS through TLR4 (Heine *et al.*, 2003; Ingalls *et al.*, 1995; Prebeck *et al.*, 2001, 2003) and Hsp60 via TLR2 and TLR4 (Bulut *et al.*, 2009, 2002; Costa *et al.*, 2002; Kol *et al.*, 1999, 2000; Vabulas *et al.*, 2001). TLR2 has been demonstrated as the predominant receptor required for an inflammatory response to infection (Darville *et al.*, 2003; O’Connell *et al.*, 2006; Prebeck *et al.*, 2001). Interestingly, TLR2 and its downstream adaptor myeloid differentiation primary response protein 88 (MYD88) have been reported to localize on the periphery of the chlamydial inclusion during active infection, suggesting that TLR2 may signal intracellularly during infection (Mackern-Oberti *et al.*, 2006; O’Connell *et al.*, 2006).

The intracellular nucleotide sensors cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP-AMP, cGAMP) synthase (cGAS) and stimulator of interferon genes (STING) also detect chlamydial infection and induce the expression of type I interferons (Zhang *et al.*, 2014). The intracellular peptidoglycan-binding molecule nucleotide-binding oligomerization domain-containing 1 (NOD1) is also activated, probably by trace amounts of chlamydial peptidoglycans (PGNs) (Bastidas, 2013). Instead of abundant PGNs, the cell envelope of EBs is comprised of a highly crosslinked latticework of outer membrane proteins (OmpA, OmcA, OmcB, and others) (Hatch, 1996),

many of which are highly immunogenic (Wang *et al.*, 2010) and could possibly be recognized as PAMPs by cytosolic receptors.

Certain antimicrobial molecules such as reactive oxygen species (ROS) are synthesized in response to infections (Bastidas, 2013), and K<sup>+</sup> efflux triggered by *Chlamydia* infection activates ROS production (Abdul-Sater *et al.*, 2009; Boncompain *et al.*, 2010; He *et al.*, 2010). ROS production activates caspase 1 through several response proteins that are aggregated into an inflammasome composed of Nucleotide-binding Oligomerization Domain (NOD) and Leucine-Rich Repeat (LRR) proteins, and all associated with the Pyrin domain-3 containing Apoptosis-associated Speck-like protein that contains a Caspase activation and recruitment domain (NLRP3-ASC-inflammasome). This NLRP3-ASC-inflammasome proteolytically processes the pro-inflammatory cytokines IL-1 $\beta$  and IL-18 in *Chlamydia* infected cells (Abdul-Sater *et al.*, 2009; Entrican *et al.*, 1999; He *et al.*, 2010; Gervassi *et al.*, 2004; Lu *et al.*, 2000; Nagarajan, 2012; Rothermel *et al.*, 1989). Intriguingly, under certain circumstances activation of the inflammasome promotes infection, may be due to an increase in lipid acquisition or utilization (Nagarajan, 2012; Itoh *et al.*, 2014).

At the beginning of chlamydial infection, an acute localized inflammatory response largely mediated by the recruitment of polymorphonuclear leukocytes (PMNs) and mononuclear leukocytes take place, mainly due to the cytokines and chemokines secreted by infected epithelial cells (Huang *et al.*, 1999; Kelly and Rank 1997; Rank *et al.*, 2008, 2000). Studies conducted in the *C. muridarum* mouse model of genital tract infections have demonstrated that as early as 3 h post-infection, genes encoding chemokines (CCL20, CCL3, and CCL24) that recruit immature dendritic cells (DCs) are up-regulated, as well as

those encoding for TNF- $\alpha$  and C3 (alternative complement). Therefore, early responses prime the site of infection for immature DC infiltration, which is an important step of the adaptive response (Rank *et al.*, 2010). By 12 h postinfection, expression of chemokines that are chemotactic for natural killer (NK) cells (CXCL9-11, CCL2-4, CCL7, CCL8, and CCL12) is significantly induced (Rank *et al.*, 2010; Tseng and Rank, 1998). The pro-inflammatory cytokines IL-1 $\alpha$ , IL-1 $\beta$ , IL-1F6, and IL-1F8 are also expressed, as are PMN-recruiting chemokines (CXCL1, CXCL5, and CXCL15) (Rank *et al.*, 2010). The recruitment of immune cells that mediate innate and adaptive immune responses to *Chlamydia* infections are, under most circumstances, sufficient for bacterial clearance during primary infections, which may explain why most infections are asymptomatic. However, inflammatory responses resulting from recurring infections or when bacteria access sterile sites (i.e., the upper genital tract for *C. trachomatis* infection) contribute to the detrimental scarring and pathology (Darville and Hiltke, 2010; Stephens, 2003).

**Chlamydial Modification of Host Response.** *Chlamydia* has evolved several mechanisms so that it can avoid the host defense systems and prolong its survival in the host. The organism can limit the recognition of PAMPs by ensuring the stability of the inclusion, during the intracellular stage of infections, which is achieved through reorganizing actin and intermediate filaments at the periphery of the inclusion (Kumar and Valdivia 2008). Chlamydial infection inhibits multiple pro-apoptotic pathways as well as potential necrotic cell death to ensure survival within host cells (Fan *et al.*, 1998; Fischer *et al.*, 2001; Rajalingam *et al.*, 2001; Yu *et al.*, 2010). One of the prominent mechanisms that *Chlamydia* utilizes to prevent cell death is the CPAF mediated degradation of the pro-

apoptotic Bcl-2 (B-cell lymphoma 2) Domain 3 Homology (BH3)-only proteins Bik, Bim, and Puma which are essential for initiating apoptosis (Bouillet and Strasser, 2002; Dong *et al.*, 2005; Fischer *et al.*, 2004; Pirbhai *et al.*, 2006; Ying *et al.*, 2005). *Chlamydia* is also thought to modulate host progression through cell cycle using multiple mechanisms to maximize nutrient acquisition at specific stages of development (Kokes and Valdivia, 2012; Sun *et al.*, 2015). It has also been speculated that by perturbing cell survival and regulating the cell cycle, *Chlamydia* may favor malignant transformation of the host cell (Kokes and Valdivia, 2012).

*Chlamydia* has also evolved with the mechanisms that may potentially subvert the host-immune responses, and under some conditions, prevent clearance. Chlamydial infection can suppress the production of interferon (IFN) or counteract downstream gene products that are involved in cell-autonomous immunity (Chen, *et al.*, 2014; Nagarajan, 2012; Wolf and Fields, 2013). The organism also uses a wide range of strategies to evade or dampen the transcription of nuclear factor  $\kappa$ B (NF- $\kappa$ B) (Bastidas *et al.*, 2013; Hackstadt, 2012), a central regulator of immune responses. It has been shown that *C. trachomatis* T3SSs effector ChlADub (also known as CT686) deubiquitylates and stabilizes NF- $\kappa$ B inhibitor- $\alpha$  (I $\kappa$ B $\alpha$ ) in the cytosol (Misaghi *et al.*, 2006), whereas *C. pneumoniae* Inc Cp0236 binds to and sequesters NF- $\kappa$ B activator 1 to the inclusion membrane (Wolf *et al.*, 2009), thereby obstructing NF- $\kappa$ B signaling.

### 1.3. CHLAMYDIAL INFECTIONS AND SIGNIFICANCE

The bacterial species of the genus *Chlamydia* cause a broad spectrum of diseases in a wide variety of hosts, approximately 32 species of mammals, including humans, have been shown to carry chlamydial infections (Longbottom and Coulter, 2003; Schachter, 1999; Storz, 1988; Vlahović *et al.*, 2006). Therefore, they are clinically and epidemiologically important throughout the world, both in human and in veterinary medicine.

**Infections in Humans.** Sexually transmitted *C. trachomatis* strains infect the endocervical epithelia of women and the urethral epithelia in men and cause multiple syndromes, including cervicitis, endometritis, salpingitis, and urethritis (Peipert, 2003; Miyairi, 2010). However, the infection remains asymptomatic in a large proportion of infected individuals (70–90% of women; 30–50% of men) (Peipert, 2003; Gonzales *et al.*, 2004; Stamm, 1999). If the infection remains untreated, the persistent infection may lead to pelvic inflammatory disease (PID) in women with serious consequences of chronic pelvic pain, tubal factor infertility, and potentially fatal ectopic pregnancy, and epididymitis in men (Ibrahim *et al.*, 1996; Taylor-Robinson and Thomas, 1980; Westrom *et al.*, 1992). In pregnant women, untreated chlamydial infection has been associated with pre-term delivery as well as ophthalmia neonatorum (conjunctivitis) and pneumonia in the newborn (Frommel *et al.*, 1979; Rours *et al.*, 2011; Zar, 2005). Reactive arthritis, also called Reiter's syndrome, is also a complication of genital chlamydial infection (Barth and Segal, 1999). It has also been reported that genital chlamydial infection may predispose to



HIV-related AIDS (Thior *et al.*, 1997; Kilmarx *et al.*, 2001) and human papilloma virus-associated cervical dysplasia (Zhu *et al.*, 2016).

Besides genital infection, *C. trachomatis* also infects eyes and may cause a disease known as “trachoma” – meaning “roughness of the conjunctiva” (Schachter, 1999). It is the leading cause of preventable infectious blindness globally and endemic to the poorest countries of the world where people live in overcrowded conditions with limited access to water and health care (Thylefors *et al.*, 1995; World Health Organization (WHO), 2001b). The infection transmits easily from person to person and frequently spreads from child to child and from child to mother within the family. Active trachoma occurs most frequently in children and is clinically characterized by signs of follicles and papillae on the conjunctival epithelium of the upper lid due to the inflammatory response following infection with *C. trachomatis* (Thylefors *et al.*, 1987). The disease progresses over years as repeated episodes of re-infection may cause in scarring leading to distortion of the eyelids and upper lid entropion (in-turning of the eyelids), earning it the name of the “quiet disease” (Polack *et al.*, 2005; WHO, 2001a). The eyelashes eventually rub on the eye globe and cornea, a condition known as trichiasis, which may lead to corneal opacity and eventually blindness (Polack *et al.*, 2005).

*C. pneumoniae* is a common pathogen in acute human respiratory infections worldwide (Ward, 1995), with more than 60% of most American, European and Asian societies being exposed (Igietseme and Black, 2013). While the majority (around 70%) of *C. pneumoniae* infections cause mild to subacute respiratory diseases, such as pharyngitis and bronchitis, a minority results in more severe infections such as community acquired pneumonia and bronchitis (Hahn *et al.*, 2002; Kumar and Hammerschlag, 2007). The

organism has also been suggested to play a role in chronic inflammatory lung diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, as well as in atherosclerosis or clinical manifestation of coronary heart disease (Choroszy-Król *et al.*, 2014; Hahn *et al.*, 1991, 2002; Joshi *et al.*, 2013; Kuo and Campbell, 2003). In recent years, reports have suggested the association of *C. pneumoniae* with several neurologic diseases, such as multiple sclerosis, Alzheimer's disease, meningoencephalitis and neurobehavioral disorders (Stratton and Sriram, 2003; Contini *et al.*, 2010; Choroszy-Król *et al.*, 2014).

**Infections in Animals.** *C. abortus* infects the placenta and cause ovine enzootic abortion (OEA) in sheep and goats (Aitken, 2000; Longbottom and Coulter, 2003). It is recognized as a major cause of abortion and lamb loss throughout the world, especially in the lowland flocks intensively managed at lambing time (Kerr *et al.*, 2005; Longbottom *et al.*, 2013; Nietfeld, 2001). As the disease implies, *C. abortus* shows an enzootic nature by virtue of the abortions in primiparous animals that tend to annually re-occur in affected herds (Aitken and Longbottom, 2007). Experimental evidence strongly suggests that the annual re-occurrence of *C. abortus* abortions is due to the ability of the organism to escape elimination by the immune response and maintain itself in dormancy in the infected ruminant herd. This dormancy already been observed in initial studies on OAE (Wilsmore *et al.*, 1984; Papp *et al.*, 1994; Livingstone *et al.*, 2009; Gutierrez *et al.*, 2011; Longbottom *et al.*, 2013), and the re-occurrence does not result from the periodic re-introduction of *C. abortus* from a heterologous, e.g. avian, host (Van Loock *et al.*, 2003). Moreover, the deep endometrial location of the chlamydial inclusions in ewes with dormant infection strongly suggests that *C. abortus* exploits the immune-privilege of the uterus for its persistence

(Hunt, 2006; Niederkorn, 2006). The increased number of endometrial immune cells in the area of chlamydial inclusions point towards the presence of regulatory T cells (Tregs) that play a dominant role in peripheral immune tolerance. While the uterine immune privilege serves to maintain immune tolerance to fetal antigens (Hansen, 2014; Zenclussen, 2006), *C. abortus* may exploit this narrow zone of tolerance to establish a permanent habitat in a ruminant population. The existence of Tregs in sheep has been demonstrated (Rocchi *et al.*, 2011). On a side note, the exploitation of immune privilege by *C. abortus*, and therefore the absence of immunoselective pressure, may also explain why *C. abortus* has maintained itself with an essentially identical genome for more than 70 years (Siarkou *et al.*, 2015).

Animals infected prior to pregnancy, in most cases, remain asymptomatic when the organism enter into a latent phase. It is not until around day 90-95 of pregnancy that *C. abortus* can be first detected in the placenta (Buxton *et al.*, 1990). The actual, clinically observed disease, i.e. abortion or delivery of weak, infected lambs, represents a breakout from the primary endometrial habitat of *C. abortus* late in gestation (last 2-3 weeks of gestation), when the immune-privileged modified uterine endometrium (decidua) vastly expands due to growth of the fetus. A possible explanation may be placental dynamics and fetal immune response during ovine gestation. The synepitheliochorial placenta in sheep is characterized by the absence of fusion between maternal uterine epithelium and the fetal chorionic membrane, with placental structures called placentomes serving as exclusive sites of limited infiltration by maternal blood vessels (Wooding and Flint, 1984). At around 60 days of gestation, maternal haematomas begin to develop at the maternal-fetal interface in the hilus of each placentome. These haematomas allow direct contact between maternal

blood and fetal trophoblast, and thus provide opportunity for placental infiltration by *C. abortus* (Buxton *et al.*, 1990). However, *C. abortus* would break out from the permanent deep endometrial habitat only in the absence of vigorous systemic immunity of circulating anti-*C. abortus* T effector cells. This may most frequently occur after systemic and endometrial infection via contact with an aborted fetus, or, probably less frequently, after venereal transmission. In experimental inoculations, the earliest placental lesions can be observed at 90 days of gestation, in the hilus of the placentome where *C. abortus* can be demonstrated within chorionic epithelial cells (Buxton, 1990; Aitken and Longbottom, 2007). Simultaneously, antichlamydial maternal inflammatory cytokines such as interferon-gamma (IFN- $\gamma$ ) and tumor necrosis factor-alpha (TNF- $\alpha$ ) are down regulated at the trophoblast junction to avoid rejection of the fetus by the maternal immune system (Entrican, 2002). These factors potentially enhance chlamydial replication and invasion of the placenta and fetus during a time when the fetus becomes increasingly immunocompetent after approximately 80 days of gestation (Nettleton and Entrican, 1995; Buxton *et al.*, 2002) and responds with increased inflammation to *C. abortus*.

The organism establishes itself in the trophoblast cells of the fetal chorionic epithelium, spreading to the surrounding intercotyledonary membranes, where it gives rise to the typical thickened and necrotic placental lesions that are associated with the disease (Buxton *et al.*, 2002). Although lesions are mostly confined to the placental membranes (Buxton, *et al.*, 1990; McEwen, *et al.*, 1951), they may also occur in the brain and liver of foetuses (Buxton *et al.*, 2002). The typical histopathological changes in placental tissues in experimentally *C. abortus* infected ewes consisted of purulent placentitis with highly

disrupted chorionic epithelium and associated neutrophil aggregations along with arteritis (Longbottom *et al.*, 2013).

During an extended lambing period, naïve primiparous ewes may acquire the infection from other aborting ewes, and abort in the same pregnancy. However, infections acquired very late, after around 110 days of gestation (i.e. within the last 5 weeks of pregnancy), often fail to induce abortion and remain asymptomatic, although such animals may abort in the subsequent lambing (Aitken and Longbottom, 2007).

*C. pecorum* is identified as one of the most widely distributed chlamydial species, with a diverse host range that includes production animals such as cattle, sheep, goats, and pigs, as well as important wildlife species (Giovannini *et al.*, 1988; Francesco *et al.*, 2011; Polkinghorne *et al.*, 2013). It is typically associated with polyarthrititis and keratoconjunctivitis in ruminants (Anderson *et al.*, 1996; Cutlip *et al.*, 1972; Fukushi and Hirai, 1992; Meagher *et al.*, 1992; Walker *et al.*, 2015), as well as encephalomyelitis in cattle (Kessell *et al.*, 2011; Jelocnik *et al.*, 2014; McNutt, 1940). There are some reports of *C. pecorum* causing pneumonia and respiratory disease (Wheelhouse *et al.*, 2013; Wilson and Thomson, 1968), and enteritis in sheep and cattle (Doughri *et al.*, 1974; Reggiardo *et al.*, 1989). The organism has also been implicated in abortion, vaginitis, endometritis, and mastitis in cattle and sheep (Kaltenboeck *et al.*, 2009; Rønsholt and Basse, 1981; Wittenbrink *et al.*, 1988; 1993).

Until recently, *C. psittaci* was considered to be the sole pathogenic chlamydial species in birds, however, evidence suggests that avian chlamydiosis may also be associated with *C. gallinacea* and *C. avium* (Sachse *et al.*, 2013; Sachse and Laroucau, 2014) as well as *C. abortus* (Pantchev *et al.*, 2009), *C. pecorum* or *C. trachomatis* (Sachse

*et al.*, 2012). Although most avian chlamydial infections remain asymptomatic (Kaleta and Taday, 2003), depending on the chlamydial strain and the avian host, chlamydiae cause pericarditis, air sacculitis, pneumonia, lateral nasal adenitis, peritonitis, hepatitis, and splenitis (Harkinezhad *et al.*, 2008; Harrison, 1989; Knittler *et al.*, 2014; Vanrompay *et al.*, 1995). In the case of avian chlamydioses, appropriate control measures during importation of birds and uses of antibiotic, especially tetracycline in feed, are the only available tools in the fight against this disease (Smith, *et al.*, 2010). However, sick birds may require a more rigorous form of treatment such as intramuscular injection with oxytetracycline, which may cause severe local tissue damage (Vanrompay *et al.*, 1995), and hence has economic implications for the poultry industry.

Feline chlamydiosis, caused by *C. felis*, is most frequently associated with rhinitis and conjunctivitis in cats (Longbottom and Livingstone, 2006). Antibiotics are routinely used to treat the clinical infections. However, such treatment may require the daily use of oral antimicrobials for a long period of time, which often results in treatment failure as pet owners fail to adhere to such regimes. Moreover, a recent study showed that the organism could still be isolated following treatment with azithromycin (Owen *et al.*, 2003). Since antibiotic treatments fail to eliminate the disease, and recent studies have shown *C. felis* infection to be common in stray cats (Yan *et al.*, 2000), the possibility of transmission to humans is greater than generally thought.

*C. suis* is a pathogen that is widespread in pig herds and is often associated with several chronic diseases, such as conjunctivitis, keratoconjunctivitis, enteritis, pneumonia and genital tract infections (Rogers and Andersen, 1996; Rogers *et al.*, 1996, 1993).

**Socio-economic Importance of Chlamydial Infections.** In humans, *C. trachomatis* accounts for the most common bacterial cause of sexually transmitted diseases (STDs) worldwide (Shim, 2011). In 2013 it was estimated that approximately 147 million people were infected with sexually transmitted *Chlamydia trachomatis* worldwide (Global Burden of Disease Study 2013 Collaborators), but an average of 131 million new infections usually occur annually worldwide as estimated by the World Health Organization (WHO, 2015). It is the most common notifiable infectious illness in the European Union (European Centre for Disease Prevention and Control, 2013; Katz, 2014) and United States (Katz, 2014). In 2014, 1.4 million *C. trachomatis* STD cases were reported to the Centers for Disease Control and Prevention (CDC) from 50 states and the District of Columbia (CDC, 2014). However, an estimated 2.86 million new cases occur every year in the US (Satterwhite *et al.*, 2013) that cause an estimated tangible costs over USD 2.4 billion annually (Johnson *et al.*, 2002). *C. trachomatis* induced trachoma is responsible for 15 % of the world's blindness (WHO, 1997). It is essentially an epidemic in some of the world's poorest countries in Africa, south-east Asia, the Indian subcontinent, the western Pacific and some areas of Oceania (Schachter, 1999). An estimated 146 million people are infected worldwide with trachoma, of which 6 million are visually impaired or irreversibly blinded (Schachter, 1999; WHO 1997). Although, *C. pneumoniae* mostly causes mild acute respiratory disease, its association with COPD, asthma, cardiovascular (atherosclerosis, coronary heart disease), and neurological diseases (multiple sclerosis, Alzheimer's disease) underscores its great impact on public health.

The significance of animal chlamydioses can be attributed from the economic losses due to their effect on production and growth in farming animals as well as their

zoonotic potential. The impact of *Chlamydia* on animal in some cases is enormous. *C. abortus* is recognized as a primary cause of reproductive loss in sheep and goats worldwide, except Australia and New Zealand (Kauffold, 2014; Stuen and Longbottom, 2011). Although an initial outbreak of *C. abortus* infection within a flock that may result only a few abortions, over 30% of the ewes abort or give birth to stillborn or weak offspring in the following year (Aitken, 2000). In the United Kingdom, approximately 44% of all diagnosed infectious cases of abortions are caused by *C. abortus* (Stuen and Longbottom, 2011) which costs an estimated £15 million (25 million Euros) per annum. *C. pecorum* is also an important animal chlamydial species and accounts for notable economic losses in the dairy cattle industry. It has been reported that subclinical *C. pecorum* infection of dairy cattle might have a significant impact on herd performance by contributing to reduced body weight gain (Reinhold *et al.*, 2008; Poudel *et al.*, 2012), potentially reducing growth rates by up to 48% (Poudel *et al.*, 2012). Its presence in the dairy herds has also been associated with reduced milk yield (Kemmerling *et al.*, 2009), subclinical low-grade vaginitis (DeGraves *et al.*, 2003), increased somatic cell counts (Biesenkamp-Uhe *et al.*, 2007), and reduced fertility (Kaltenboeck *et al.*, 2005; Wehrend *et al.*, 2005). In Europe, economic losses due to decreased milk production and milk quality, along with abortions and reduced fertility rates, were an estimated of 40,000 Euros per annum at an average farm of 60 dairy cows and 20 heifers.

Moreover, most of the animal *Chlamydia* spp. can cause disease in humans. *C. psittaci* is one of the most common zoonotic agents and the infection in humans is usually associated with respiratory disease, although other organs can become infected resulting in endocarditis, myocarditis, hepatitis, encephalitis or meningitis (Vanrompay *et al.*, 1995;



Williams and Sunderland, 1989). *C. abortus* is reported as a zoonotic pathogen and can cause severe, life-threatening disease in pregnant women (Longbottom and Coulter, 2003). The consequence of *C. abortus* infection in pregnant women is a spontaneous abortion within the first three months of pregnancy, while later infections cause still or premature births, which are typically preceded by several days of acute influenza-like illness, as well as renal failure, hepatic dysfunction, disseminated intravascular coagulation, and possibly death (Buxton, 1986; Hyde and Benirschke, 1997; Jorgensen, 1997). There are some reports that identified *C. felis* infection in humans and such infection is associated with follicular conjunctivitis (Ostler *et al.*, 1969), functional disorders of the liver (Griffiths *et al.*, 1978), endocarditis and glomerulonephritis (Regan *et al.*, 1979), as well as atypical pneumonia (Cotton and Partridge, 1998).

#### 1.4. HOST IMMUNITY AGAINST CHLAMYDIAL INFECTION

**Innate Immunity.** The first line of defense from chlamydial infection is the genital, respiratory, or ocular mucosal epithelium barrier. However, following of epithelial cell or the breach of the mucosal lining and subsequent establishment of a successful infection, the innate immune system provides the next stage of defense against the bacteria.

Epithelial cells. Although epithelial cells are not considered part of the classical innate immune system, they are important and early components of the host response to chlamydial infection (Quayle, 2002; Rasmussen *et al.*, 1997). An active infection of epithelial cells induces the production of pro-inflammatory cytokines such as interleukin 1 (IL-1), Il-6, and tumor necrosis factor alpha (TNF $\alpha$ ), and chemokines such as Il-8 (Buchholz and Stephens, 2006; Johnson *et al.*, 2004; Rasmussen *et al.*, 1997). These inflammatory mediators are most likely responsible for the recruitment of classical innate immune cells such as neutrophils, macrophages, natural killer (NK), and dendritic cells (DCs) seen during acute infection (Buchholz and Stephens, 2006; Darville 2006). Neutrophils and NK cells are the first immune cells that are recruited to the site of chlamydial infection. These cells play important role in innate immunity and have been implicated in the initial control of chlamydial infections.

Neutrophils. Neutrophils are the most predominant form of leukocytes with the functions of both immune surveillance and *in situ* elimination of microorganisms (Witko-Sarsat *et al.*, 2000). Infiltration with neutrophils has been observed in the uterine horns and oviducts of guinea pigs shortly after vaginal inoculation of *C. psittaci* (Rank and Sanders, 1992). Human neutrophils have also been found to effectively inactivate *C. trachomatis* in vitro (Register *et al.*, 1986; Yong *et al.*, 1982). Moreover, approximately 10-fold greater

burden of *C. muridarum* was isolated from the mice depleted of neutrophils than the wild type controls. However, both groups of mice in that experiment were able to effectively resolve the infection within the same time frame (Barteneva *et al.*, 1996), suggesting that neutrophils may be helpful in the early control of chlamydial infection, but are not critical for the clearance of chlamydial infection. In fact, due to their first recruitment in the site of infection and short life-span (Kolaczkowska *et al.*, 2013), the primary role of neutrophils is more likely to reduce the infection and to limit the spread of chlamydiae. However, recent evidence indicates that chlamydial multiplication may delay the spontaneous apoptosis of neutrophils (van Zandbergen *et al.*, 2004). As neutrophils are a major source of tissue-damaging cytokines, such as matrix metalloproteinase 9 (MMP9), during an acute inflammation (Bradley *et al.*, 2012), their prolonged life span may contribute to tissue pathology associated with a chlamydial infection (Darville, 2006; Lee *et al.*, 2010; Huang, 1999).

Natural killer cells. NK cells are a type of cytotoxic lymphocytes that play a similar role to that of neutrophils (Vivier *et al.*, 2011). They primarily kill viral-infected cells, cancer cells, and cells that have lost expression of MHC class I molecules. They have also been shown to be important in the early elimination of intracellular bacteria (Cheng *et al.*, 2013; Shegarfi *et al.*, 2009). In a *C. muridarum* mouse model study, recruitment of interferon gamma (IFN- $\gamma$ ) producing NK cell was observed to the site of chlamydial infection as early as 12 to 24 hours after challenge (Tseng and Rank, 1988). With the production of IFN- $\gamma$ , NK cells not only plays role in inhibiting the growth of *Chlamydia* (Beatty *et al.*, 1994) but also promote the induction of an adaptive CD4<sup>+</sup> T helper 1 (CD4<sup>+</sup> Th1) immune response. In fact, depletion of NK-cells by anti-NK-cell antibody treatment

resulted a significant increase in the Th2-associated antibody IgG1 in mice after *C. muridarum* infection. In contrast, mice that were not treated with an anti-NK-cell antibody demonstrated a Th1-associated IgG2a as the dominant antibody (Tseng and Rank, 1998). A more recent study indicated that NK cells may influence Th1 immunity by modulating DC function. This investigation demonstrated that adoptively transferred of DC from NK cell knockout (KO) mice into naive mice failed to induce a Th1- mediated immune response after intranasal challenge with *C. muridarum* (Jiao *et al.*, 2011). Therefore, it is more likely that early IFN- $\gamma$  production by NK cells modulates DC to downregulate the Th2 response and thereby promote a strong Th1-mediated immunity, essential for the resolution of chlamydial infection.

Natural killer T cells. Natural killer T (NKT) cells represent a unique population of innate lymphocytes that express the typical NK cell marker (NK1.1 and NKR-P1C) and a semivariant T-cell receptor ( $\alpha\beta$  T-cell receptor; TCR) (Zhao *et al.*, 2011). NK T cells are termed as CD1d-restricted due to their ability to recognize lipids and glycolipids presented by antigen-presenting cells on CD1d molecules rather than antigens from the classical major histocompatibility complex (MHC) (Godfrey *et al.*, 2010). Chlamydial glycolipid exo-antigen (GLXA) has been found to act as a specific ligand for NKT-cell activation (Peng *et al.*, 2012). NKT cells usually destroy infected and cancerous cells without prior sensitization and also secrete cytokines that are important in both innate and adaptive immunity (Shekhar *et al.*, 2015). They also play important role in regulating both innate and adaptive immune cells (Carnaud *et al.*, 1999; Joyee *et al.*, 2008). NK cells from NK T cells depleted mice showed decreased IFN- $\gamma$  production and proliferation in a *C. muridarum* lung infection model (Zhao *et al.*, 2011). NKT cells have also been shown to

modulate the functions of CD8 $\alpha$ <sup>+</sup> DC (promoting proliferation, CD40 upregulation, and production of IL-12) to generate protective Th1 immunity against *C. pneumoniae* infection (Joyee, 2010). Recently it has been demonstrated that alpha-galactosylceramide-stimulated NKT cells promote protective Th1 immunity against *C. muridarum* genital tract infection (Wang *et al.*, 2012).

Macrophages. Macrophages migrate to chlamydial infection sites nearly as rapidly as neutrophils, phagocytose bacteria, produce proinflammatory cytokines, and play an important role in the resolution of the infection (Bas *et al.*, 2008; Beagley *et al.*, 2009; Morrison and Morrison, 2000; Yilma *et al.*, 2012). Studies have shown that through autophagy (a process by which cells degrade cytoplasmic proteins and organelles) macrophages destroy phagocytosed *Chlamydia* and also facilitate antigen presentation to T-cells (Al-Zeer *et al.*, 2013; Crotzer and Blum, 2009; Sun *et al.*, 2012; Yasir *et al.*, 2011). Interestingly, macrophages may also have an effect on chlamydial infection by inducing TNF- $\alpha$  mediated T cell apoptosis and perpetuating a persistent infection (Jendro *et al.*, 2004, 2000).

Dendritic cells. Dendritic cells (DC) are the typical professional antigen presenting cells (APC) and play a key role in induction of chlamydial immunity. They have been shown to activate T cells through MHC class I/II presentation and secrete Th1 cytokines in chlamydial infection both in *in vitro* and *in vivo* studies (Jiang *et al.*, 2008; Matyszak *et al.*, 2002; Morrison *et al.*, 1995; Ojcius *et al.*, 1998; Su *et al.*, 1998). In an early study it has been shown that adoptively transferred bone marrow-derived DC pulsed with heat-killed *C. trachomatis* induced a Th1 response and protected the recipient mice following the subsequent nasal challenge with live *C. trachomatis* (Lu and Zhong, 1999). DCs are

the critical linker between innate and adaptive immunity, and therefore are important for vaccine research.

**Adaptive Immunity.** It is well established that T cells are critical for clearance of chlamydial infections. Almost 30 years ago Rank and his group first demonstrated that athymic nude mice developed chronic *C. muridarum* infection after intravaginal inoculation, while their wild-type counterparts were able to eliminate the infection within 20 days (Rank *et al.*, 1985). Conversely, when polyclonal *Chlamydia*-specific T cells were transferred into *Chlamydia*-infected T cell-deficient mice, these mice effectively clear the infection (Ramsey and Rank, 1991; Thoma-Uszynski *et al.*, 1998). Numerous studies have shown that both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells are present at the site of chlamydial infection (Penttila *et al.*, 1998; Rank *et al.*, 2000; Van Voorhis *et al.*, 1996). Both types of T-cells have also been found to recognize *C. trachomatis* antigens, including outer membrane protein 2 (OMP2) (Goodall *et al.*, 2001a), polymorphic outer membrane protein D (POMP-D) (Goodall *et al.*, 2001b), major outer membrane protein (MOMP), heat shock protein 60 (Hsp60) (Holland *et al.*, 1997; Kim *et al.*, 2000; Ortiz *et al.*, 2000), chlamydial protease activating factor (CPAF) (Li *et al.*, 2011), polymorphic membrane protein G (PmpG), PmpF, and ribosomal protein L6 (RpIF) (Olive *et al.*, 2011; Johnson *et al.*, 2012).

Data from mouse models strongly support a dominant role for CD4<sup>+</sup> T-helper cells in protective immunity against chlamydial infection. Adoptive transfer of CD4<sup>+</sup> T cells of immune mice, but not of CD8<sup>+</sup> T cells, confers protective immunity to naive mice against chlamydial infection in the genital tract (Su and Caldwell, 1995). Moreover, mice deficient in major histocompatibility complex (MHC) class II molecules were unable to clear

primary *C. muridarum* genital tract infections, and mice with a disrupted CD4 gene resulted in significantly delayed clearance (Morrison *et al.*, 1995). These studies clearly demonstrate the necessity of CD4<sup>+</sup> T cells to the clearance of chlamydial infection. However, the critical issues for understanding protective host immunity are: (1) which CD4 T cell subset(s) mediate protection and (2) by what mechanisms?

Protective CD4<sup>+</sup> T-helper cells. All T helper lymphocytes start out as Th0 cells, which after being activated by APC acquire the effector functions while differentiating into either T-helper type 1 (Th1) or Th2 effector cells based on the cytokines profile they secrete (Mosmann *et al.*, 1986; Sad and Mosmann, 1994). Th1 cells secrete IFN $\gamma$  with or without IL-2, while Th2 cells produce IL4, IL5, IL13, and IL10 (Purnama *et al.*, 2013; Perrigoue *et al.*, 2009; Wilson *et al.*, 2009; Zhu *et al.*, 2010). The polarization of T helper cells also reflected antagonistic effects of IFN- $\gamma$  and IL-4 on naïve T cell differentiation. IFN- $\gamma$  suppresses Th2 development that depends on the transcription factor GATA-3, and IL-4 blocks Th1 development that requires T-bet expression (Spellberg and Edwards, 2001). In broad strokes, Th1 cells are specialized to defend against intracellular microbial pathogens by inducing a strong cell mediated immune reaction (Spellberg and Edwards, 2001). The Th1-generated cytokine IFN- $\gamma$  stimulates phagocytosis (Szulc and Piasecki, 1988), oxidative burst (Johnston and Kitagawa, 1985), changes in intracellular iron and tryptophan metabolism, and intracellular killing of microbes (Dellacasagrande *et al.*, 1999; Maródi *et al.*, 2000). In contrast, Th2 cells are specialized to deal with extracellular pathogens, especially parasites, by facilitating B cell expansion, immunoglobulin production and recruitment of eosinophils (Lai and Mosmann, 1999; Lundgren *et al.*, 1989; Punnonen and de Vries, 1994).

Consistent with these general principles, protection against chlamydial infection is associated with Th1 immunity while Th2 responses are ineffective or exacerbate the infection (Gondek *et al.*, 2009). Investigation of the local cytokine response to chlamydial genital tract infection in mice demonstrated a predominant presence of IFN- $\gamma$ -secreting cells and very few cells secreting IL-4. Interestingly, highest levels of IFN- $\gamma$  secreting cells was observed during the first and the third week of infection (Cain and Rank, 1995). The initial peak of IFN- $\gamma$  secreting cells has been correlated with the presence of NK cells (Tseng and Rank, 1998) and the later peak with the influx of chlamydial- specific CD4<sup>+</sup> Th1 cells (Kelly and Rank, 1997; Kelly *et al.*, 2000). Moreover, adoptive transfer of an IFN- $\gamma$ -producing CD4<sup>+</sup> Th1 clone, but not an IL-4-producing CD4<sup>+</sup> Th2 clone, protected nude mice against Chlamydial genital tract infection (Hawkins *et al.*, 2002). Furthermore, a recent study demonstrated that chlamydial antigen-pulsed dendritic cells (DCs) derived from IL-10 knock-out mice induced a more vigorous Th1 response compared with that produced by stimulation with DCs from wild type mice (Igietseme *et al.*, 2000). This study also showed that IL-10 knockout mice cleared the genital chlamydial infection more rapidly and efficiently than the wild type mice. The findings suggest that a fast and vigorous Th1 response after an infection will rapidly arrest chlamydial replication, clear the infection, eliminate residual antigens and prevent the establishment of a latent infection. However, an inadequate or suboptimal Th1 response delays chlamydia clearance, leading to the establishment of a latent or persistent infection, which fuels a low grade chronic immune response and tissue damage (Igietseme *et al.*, 2000, 2003). It is obvious that the protective value of a T cell population is strongly dependent on their ability to efficiently migrate to infected tissue sites where they would perform their effector function. However,



it has been found that although significant numbers of CD4<sup>+</sup> Th2 cells are seen in the infected organs in IFN- $\gamma$  KO mice, these cells failed to localize to sites of chlamydial infection (Wang *et al.*, 1999). Thus, in addition to their inability to produce IFN- $\gamma$ , it is likely that the failure of Th2 cells to target infected cells is also a reason for their inability to control chlamydial infection.

Subsequent to the pioneering discovery of Th1/Th2 dichotomy, many other helper T cell subsets have been identified to include Treg (T regulatory, mucosal and peripheral tolerance) (Sakaguchi *et al.*, 1985), Th21 (Thf, follicular T cells) (Schaerli *et al.*, 2000), Th3 (mucosal tolerance) (Dicmann *et al.*, 2002), Th17 (neutrophil recruitment) (Infante-Duarte *et al.*, 2000; Langrish *et al.*, 2005; Park *et al.*, 2005), and Th22 cell (epithelial defense activation) (Nogales *et al.*, 2008, 2009). In addition, more recently there is an important category of Th1 T cells have been identified which are referred to as multifunctional Th1 T cell and capable of secreting IFN- $\gamma$ , TNF- $\alpha$  and IL-2 (Darrah *et al.*, 2007). Recently, chlamydial vaccine studies conducted in *C. muridarum* mouse model demonstrated that multi-functional CD4<sup>+</sup> T cells that co-secrete IFN- $\gamma$  and TNF- $\alpha$  were a better correlate of immunity against *C. muridarum* infection than CD4<sup>+</sup> T cells that secreted IFN- $\gamma$  alone (Yu *et al.*, 2010, 2011).

Th17 cells are a distinct class of CD4<sup>+</sup> helper T cells that secrete IL-17A, IL-17F, IL-21, and IL-22 (Harrington, *et al.*, 2005; Langrish *et al.*, 2005; Park *et al.*, 2005) and their differentiation is supported by several cytokines including transforming growth factor- $\beta$  (TGF- $\beta$ ), IL-1 $\beta$ , IL-6, IL-21, and IL-23 in mice and humans (Chung *et al.*, 2009; Sutton *et al.*, 2009; Yang *et al.*, 2008). Th17 cells play an important role in host defense against bacterial infections, generally extracellular bacteria, by recruiting and activating

neutrophils. However, they also have a major role in immunopathology, most prominently demonstrated for inflammatory autoimmune diseases (Waite and Skokos, 2011). A recent study conducted in a *C. muridarum* pulmonary mouse model showed that IL-17RA was consistently induced at higher level in the lungs of highly susceptible C3H/HeN mice than comparatively resistant C57/BL6 mice following infection suggesting its role in chlamydial immunopathology (Zhou *et al.*, 2009). In another study, it was demonstrated that IL-17 promotes Th1 induction and neutrophil infiltration in a murine *C. muridarum* genital infection model, however, its depletion did not interfere with resolution of the infection (Scurlock *et al.*, 2011). In contrast, a more recent study has shown that IL-22 plays an important role in host defense against murine *C. muridarum* respiratory tract infection through enhancing Th17 and Th1 immunity (Peng *et al.*, 2014). A recent study also investigated the role of Tregs in chlamydial immunity and found that they directly promote the induction of an IL-17/Th17 response during *C. muridarum* infection and thereby facilitate the development of *Chlamydia*-induced immunopathology (Moore-Connors *et al.*, 2013).

*CD4<sup>+</sup> Th1 effector mechanism of chlamydial clearance.* Evidence clearly suggests that the Th1 subset of CD4<sup>+</sup> T cell is essential for the protection against chlamydial infection. Resolution of the chlamydial infection by Th1 cells is mainly mediated by its cytokine IFN- $\gamma$ . Considerable *in vitro* and *in vivo* studies have demonstrated that production of IFN- $\gamma$  by *C. muridarum*-specific Th1 cells is essential for clearance of the infection from the genital tract (Morrison *et al.*, 2002). IFN- $\gamma$  can control chlamydial replication through multiple distinct mechanisms (Roan and Starnbach, 2008). It can upregulate the phagocytic potential of macrophages, thereby promoting the engulfment and

destruction of extracellular EBs (Zhong and de la Maza, 1988). IFN- $\gamma$  can also directly inhibit chlamydial growth within infected cells. It can induce the production of indoleamine-2, 3-dioxygenase (IDO) by the *Chlamydia*-infected cells (Beatty *et al.*, 1994). IDO catalyzes the catabolism of tryptophan, one of the essential amino acids that *Chlamydia* scavenges from the host cell, and thereby lead to the death of the organism through tryptophan starvation (Brunham and Rey-Ladino, 2005). In addition, IFN- $\gamma$  downregulates the transferrin receptor and thereby restricts chlamydial growth by intracellular iron depletion (Byrd and Horwitz, 1993; Freidank *et al.*, 2001). Finally and most importantly, IFN- $\gamma$  has been associated with activation of inducible nitric oxide synthase (iNOS) that catalyzes production of various antimicrobial reactive nitrogen intermediates which can kill intracellular *Chlamydia* organisms in infected cell lines (Chen *et al.*, 1996; Igietseme *et al.*, 1996). This mechanism has been found to be functional in both human and mouse epithelial cells.

The requirement for multifunctional Th1 in anti-chlamydial protective immunity is not well understood yet. It is possible that in combination with IFN- $\gamma$ , TNF- $\alpha$  (the effector cytokine of multifunctional CD4<sup>+</sup> T cells) boosts induction of iNOS expression via NF $\kappa$ B binding sites in the iNOS promoter and thereby synergizes to mediate killing of the pathogen (Drapier *et al.*, 1988). Although, IL-2 has no direct effector function, it strongly enhances the expansion of effector T cells. However, it is also probable that the multifunctional Th1 T cells possess a more robust degranulation phenotype than the Th1 T cells producing only IFN- $\gamma$  (Yu *et al.*, 2016). Consistent with the requirement for multiple cytokines, a recent *in vitro* study showed that treatment of murine epithelial cells with IFN- $\gamma$  alone was not sufficient to effectively terminate *C. muridarum* replication, but

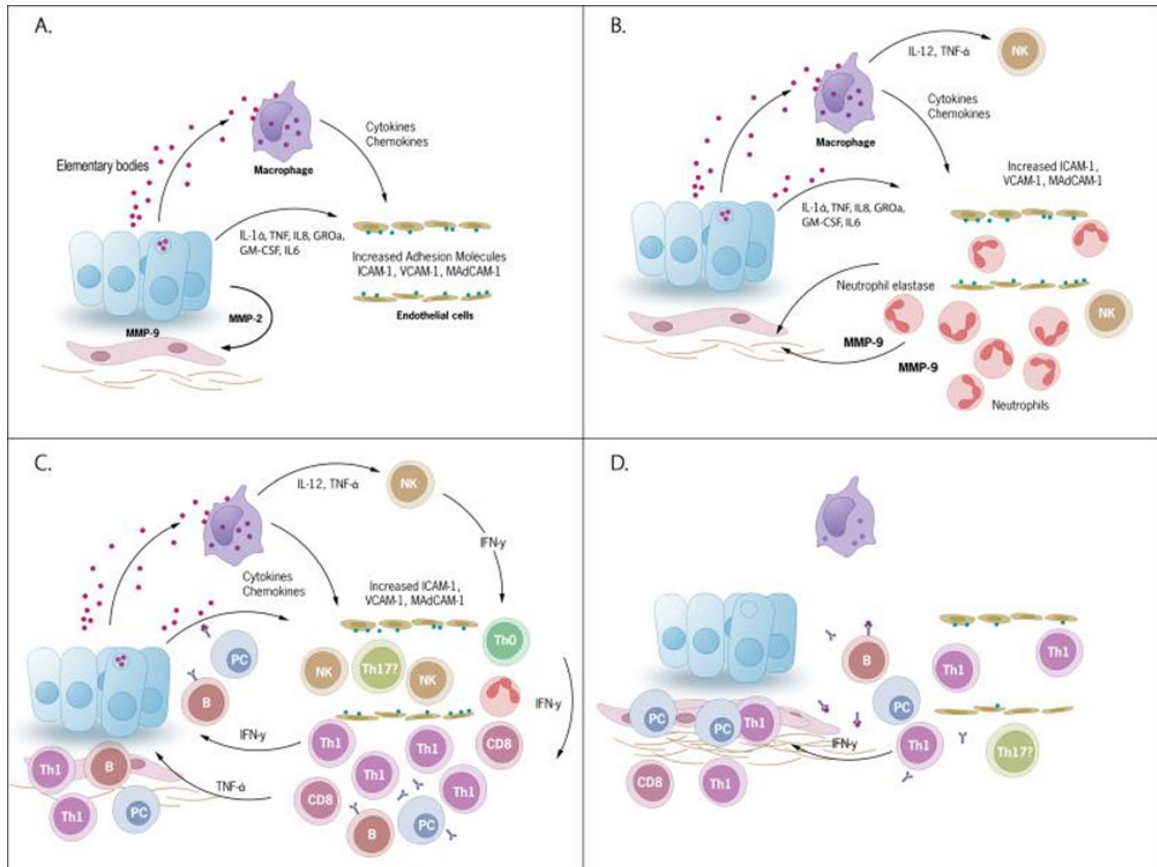
supernatants of activated T cells did so via an iNOS dependent mechanism (Johnson *et al.*, 2012).

CD8<sup>+</sup> T cells. The role and effector mechanism of *Chlamydia*-specific CD8<sup>+</sup>T cells are not well established. Multiple studies have demonstrated that MHC class I peptide presentation to CD8<sup>+</sup>T cells is not essential for clearance of infection with *Chlamydia* spp.:  $\beta$ 2-microglobulin (a component of MHC class I molecules) knockout mice resolved infection as efficiently as wild-type mice (Morrison *et al.*, 1995, 2000), and mice deficient in perforin or CD95 (also known as FAS) - which are crucial cytolytic effector molecules of CD8<sup>+</sup> T cells - also effectively cleared infection with *C. muridarum* (Perry *et al.*, 1999), suggesting that CD8<sup>+</sup> T cells are not essential for clearance of chlamydial infection. However, in an early study it has been demonstrated that *C. muridarum*-specific CD8<sup>+</sup>T cells efficiently lysed *C. muridarum*-infected cells when cells were transfected with the intercellular adhesion molecule 1 (ICAM1) (Beatty and Stephens, 1994). Thus, in some situations, CD8<sup>+</sup> T cells may be important for the elimination of cells infected with *Chlamydia* spp. Moreover, adoptively transferred *C. trachomatis* L2 serovar-specific CD8<sup>+</sup>T-cell lines protected mice against *C. trachomatis* challenge infection through a mechanism involving production of IFN- $\gamma$  (Starnbach *et al.*, 2003). Therefore, it appears to be that CD8<sup>+</sup>T cells might play supporting role in limiting infection with *Chlamydia* spp.

B-Cells. The significance of B cell and antibody-mediated immunity against *Chlamydia* infection is not fully understood (Li and McSorley, 2015). Although, *in vitro*, *C. trachomatis* specific antibodies can neutralize infection in tissue culture (Byrne *et al.*, 1993), high anti-*C. trachomatis* antibody titers do not correlate with resolution of infection

in humans, rather are more strongly correlated with increased severity of disease sequelae, such as tubal infertility in women (Punnonen *et al.*, 1979). Moreover, mice that lack B cells do not show a markedly altered course of primary genital infection with *C. muridarum* (Ramsey *et al.*, 1988). However, another study demonstrated that although B cell-deficient mice were capable of clearing *C. muridarum* primary infections with normal kinetics of bacterial shedding from the genital tract, knockout mice were more susceptible to reinfection than wild type control mice (Su *et al.*, 1997). Further studies showed that immune wild-type mice depleted of either circulating CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells by parenteral antibody treatment were able to effectively resolve a secondary *Chlamydia* infection (Morrison and Morrison, 2001). Notably, immune B cell-deficient mice (B cell knockout mice that had previously resolved a primary infection) were unable to clear secondary infection in the absence of CD4<sup>+</sup> - but not of CD8<sup>+</sup>-T cells, suggests that B cells and CD4<sup>+</sup> Th1 cells may function synergistically in providing immunity against chlamydial infection (Morrison *et al.*, 2000). However, adoptive transfer of immune serum into immune B cell knockout mice were able to reconstitute their ability to clear secondary infection in the absence of CD4<sup>+</sup> T cells (Morrison and Morrison, 2005). Surprisingly, when immune serum was passively transferred into naïve wild type mice it failed to provide protection from primary infection. Thus, although it appears that B cells and antibody have a role in the resolution secondary chlamydial infection, direct antibody-dependent neutralization or complement-mediated killing is unlikely to account for antibody-mediated protection since passive transfer of immune serum only protected antigen-experienced hosts rather than naïve mice.

Overall, these data show that *Chlamydia*-specific CD4<sup>+</sup>Th1 cells are required for control chlamydial infection, and CD8<sup>+</sup> T cells and B cells may further support the elimination of chlamydiae. An overview of the chlamydial immunity is shown in Fig. 1.3.



**Figure 1.3. Host immune response against chlamydial infection.** Infection of nonimmune host epithelial cells and resident tissue innate immune cells with chlamydiae results in production of proinflammatory cytokines and chemokines that lead to recruitment and activation of first innate and, later, adaptive immune cells to effect resolution of infection; subsets of these responses induce collateral genital tract tissue damage. A) Infection of reproductive tract epithelium results in production of interleukin (IL)–1, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-8, growth-related oncogene (GRO)– $\alpha$ , granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-6, which induce increased expression of endothelial adhesion molecules that aid in the attraction of immune cells. Resident tissue macrophages also contribute to early release of cytokines and chemokines. Infected epithelial cells release matrix metalloproteases (MMPs) that contribute to tissue proteolysis and remodeling. B) Neutrophils, natural killer (NK) cells, and monocytes are rapidly recruited into the

infected tissue site. Neutrophil release of MMPs and elastase contribute to tissue damage. C) NK cell production of interferon (IFN)- $\gamma$  drives CD4 T cells toward the Th1 (IFN-  $\gamma$  -producing) phenotype, and a mixture of CD4, CD8, B cells, and plasma cells (PCs) infiltrate the infected tissue. Antibodies released from PCs inactivate extracellular elementary bodies (EBs), and T cell production of IFN-  $\gamma$  inhibits intracellular chlamydial replication. Th17 cell involvement has not yet been determined. D) After infection has been resolved, inflammation abates, but chronic scarring may be the end result. (From Darville and Hiltke, 2010).

## 1.5. CHLAMYDIAL VACCINE

**Is a Vaccine Necessary?** In human medicine, considering the magnitude and near epidemic state of *C. trachomatis* and *C. pneumoniae* infections in some populations, the continued spread in global communities, and the economic stress on the healthcare system, several prevention and control strategies have been proposed and/or executed. These control measures include mass screening and treatment, mass antibiotic treatment of at-risk populations, health education programs on prevention methods, and the use of an efficacious vaccine as an immunoprophylaxis and preventive (Igiertseme and Black, 2013). However, data from the measures implemented so far have suggested that the vaccine option will likely represent the most reliable and cost effective means to achieve the greatest impact (Cohen and Brunham, 1999; Mahdi *et al.*, 2001) due to several reasons. First, the mass screening and treatment, or mass and targeted population treatment with antibiotics have not produced the desired long-term result to eliminate chlamydial ocular or genital infections (Brunham and Rekart, 2008; WHO, 2014). Secondly, although antibiotic therapy effectively cures chlamydial infection if detected early (Mahdi *et al.*, 2001), most of the chlamydial infections are asymptomatic and therefore treatment of symptomatic individuals alone is unlikely to be successful (Cohen and Brunham, 1999; Thein *et al.*, 2002). In addition, antibiotics are less effective when there is an established infection and pathology (Stagg, 1998). It has also been reported that a significant proportion of treated infections may lead to persistence (Bragina *et al.*, 2001; Byrne, 2001), casting doubt on the long-term value of certain chemotherapies. Likewise, most other prevention strategies have been found challenging to execute due to economic, convenience and acceptance issues. Furthermore, computer simulation modelling has



predicted that even the least efficacious chlamydial vaccination program would rapidly reduce the prevalence of genital infection (de la Maza and de la Maza, 1995). Thus, with epidemiologic data indicating persisting and sometimes increasing incidences of ocular and genital *C. trachomatis* infections in the human population worldwide, the urgency for an efficacious vaccine cannot be over emphasized. Unfortunately, until today no acceptable human chlamydial vaccine has been developed.

Antibiotics, mainly tetracyclines, are widely used to treat chlamydial infection in animals. However, most of the chlamydial infections are asymptomatic in nature and antibiotic treatment does not completely eliminate the disease (Owen *et al.*, 2003) and thus the carriers continually shed the organisms and infect others in the herd. Moreover, recently tetracycline-resistant *C. suis* strains have been isolated in both the USA (Lenart *et al.*, 2001) and Europe (Di Francesco *et al.*, 2008) which is somewhat alarming since *C. suis* and *C. trachomatis* are closely related species, and this is the antibiotic of choice for both animal and human chlamydial infections. Furthermore, treatment may require the daily use of oral antimicrobials for a long period, especially in cats against feline chlamydiosis, which often results in treatment failure as pet owners fail to adhere to such regimens (Owen *et al.*, 2003). Thus, although chlamydial infections could be effectively treated with antibiotics, their asymptomatic nature, possible development of antibiotic resistance strains, and the fact that antibiotic treatment does not clear the infection, means that infections are best managed through a combination of improved methods of detection and treatment, prevention, and control with vaccines. In sum, safe and effective anti-chlamydial vaccines are urgently needed in both human and veterinary medicine.

**Chlamydial Vaccine Development and Challenges.** Because of the asymptomatic nature of chlamydial infections, their diagnosis, treatment, and prevention of sequelae is a challenge. Chlamydial infections do not induce long-lasting immunity in the host, and protective immunity usually takes months or years to develop (Schachter and Stephens, 2008). Moreover, infection by a single serovar does not adequately protect against reinfection with other serovars (Schachter and Stephens, 2008). Furthermore, it is hypothesized that aggressive treatment may interfere with natural immunity (Brunham *et al.*, 2005). Thus, prevention and control of chlamydial infections rely on a safe and effective inactivated vaccine capable of inducing a better immune response than what occurs naturally (Taylor and Haggerty, 2011; Schachter and Stephens, 2008). However, the pathogenesis of *Chlamydia* has not yet been fully elucidated, and the role of host immunology is mostly unclear. Besides, lacking of a suitable animal model and difficulties to manipulate *Chlamydia* genetically, scientists in the chlamydial field have yet to develop a successful vaccine.

Early attempts to develop an effective vaccine for controlling both human and animal chlamydial infections started since 1950s with the use of inactivated or live, attenuated whole organism preparations (Longbottom and Livingstone, 2006). The common problems associated with these vaccines are the cost and the complexity of production, the requirement for cold storage, the presence of antigens which can induce autoimmunity or immunopathology, and the limited efficacy in neonates with high levels of maternal antibodies (van Drunen Littel-van den Hurk *et al.*, 2000). Therefore, over the last couple of decades, vaccine research has seen a gradual shift in philosophy away from the classical approach of using whole inactivated or attenuated organisms to subunit

vaccines. Subunit vaccines are safer to use as they cannot revert to a virulent form, and potential undesirable antigens, which can induce immunopathology or inflammatory damage, can be avoided (Olive *et al.*, 2001). In subunit vaccines, vaccine candidate antigens, or parts of antigens, may be represented as purified proteins, recombinant proteins or as synthetic proteins or peptides (Hess *et al.*, 2000). However, like inactivated vaccines, subunit vaccines are poor inducers of cell-mediated immunity (van Drunen Littel-Van Den Hurk, 2000), which is very important in the defense against chlamydial infections.

**Live attenuated vaccine.** The first vaccines that were used against *Chlamydiaceae* in humans were attenuated or modified living chlamydial organisms (Schautteet *et al.*, 2011). The potential usefulness of a live attenuated vaccine in the case of chlamydial infection would be due to the ability of such a vaccine to mimic the complex biphasic life cycle, thus ensuring the expression of antigens from both the EB and the RB. However, a potential pitfall with the use of such vaccines for protection against chlamydial disease is that antigens that could give rise to deleterious autoimmune or immunopathologic responses may also be produced (Brunham *et al.*, 2000). In fact, early vaccine trials demonstrated that individuals vaccinated with whole cells experienced exacerbated disease during subsequent infection (Grayston *et al.*, 1985). Therefore, whole-organism live vaccination is unlikely to be attempted in the near future especially in human, because there is a risk of immunopathology, the large-scale production of pure chlamydiae is extremely difficult (Stagg, 1998) and because of the possible spread of live *Chlamydiaceae* in the environment (Shewen, 1980).

A veterinary live attenuated *C. abortus* vaccine has been developed that is commercially available and is used against *Chlamydia*-induced abortion in sheep (Chalmers *et al.*, 1997; Rodolaki *et al.*, 1998). This vaccine was first developed in the early 1980s at the Institut National de la Recherche Agronomique, Nouzilly, France (Rodolakis and Bernard, 1984; Rodolakis and Souriau, 1983). It was produced by chemical mutagenesis, and consists of a temperature sensitive mutant strain that can grow at 35°C but not at 39.5°C, the body temperature of sheep, and therefore is diminished in its ability to grow *in vivo* and cause disease (Longbottom and Livingstone, 2006). However, the safety of using this vaccine is a matter of great concern, particularly since *C. abortus* can cause abortion and serious disease in pregnant women. Furthermore there is always a risk of the attenuated strain reverting to virulence, thus having the potential to cause disease and abortion in the vaccinated animal. In fact, using genetic markers, the attenuated *C. abortus* vaccine has been associated with abortions. In addition, the fact that the vaccine cannot be administered during pregnancy, or to animals being treated with antibiotics, severely limits its usefulness (Longbottom and Livingstone, 2006). Commercial live attenuated vaccines are also available against *C. felis* but these vaccines have not been thoroughly tested to ensure that they prevent infection or shedding of the organism into the environment (Masubuchi K *et al.*, 2010; Wills *et al.*, 1987).

**Inactivated Vaccine.** Due to the safety issues with live vaccines, early human chlamydial research had also attempted to use killed or inactivated vaccines. However, when such vaccines, derived by formalin inactivation of culture- or chick embryo-grown EBs, was delivered intramuscularly in alum or mineral oil adjuvant into children in

trachoma-endemic areas of Taiwan, East Africa, northern India and The Gambia in Africa, it produced mixed and some alarming results (Igietseme and Black, 2013). Depending on the trial, the results included a transient decline in trachoma in some vaccinated groups compared to placebo controls and more severe trachoma in some pre-vaccinated children compared to controls (Igietseme and Black, 2013). Similar to the outcome of attenuated live vaccines, the apparently disappointing outcome of these early vaccine trials was that the inactivated chlamydial organism vaccines contain components that induce both immunoprotective and immunopathogenic immune responses. Moreover, due to their inability to replicate, these inactivated organisms are poor inducers of cell-mediated immunity although they can induce an adequate level of humoral immunity (van Drunen Littel-Van Den Hurk, 2000). As a strong cell-mediated immunity is essential for clearance of chlamydial infections, inactivated or killed organisms seem to be less suitable for vaccine development against *Chlamydiaceae*.

Along with attenuated live vaccines, commercial inactivated vaccines are also available against *C. abortus* and *C. felis* (Longbottom and Livingstone, 2006). They are usually prepared from infected yolk sacs or cell cultures and incorporate whole organisms or fractions of them (Cello, 1967; Jones *et al.*, 1995; Tan *et al.*, 1990). Although using an inactivated vaccine in sheep is advantageous as it can be administered in pregnant ewes, care must be taken in handling and administering this vaccine, as it is adjuvanted with mineral oils, which have the potential to cause tissue necrosis. In cats, although inactivated vaccines are successful in reducing acute disease, again, like the live vaccines, they do not prevent shedding of the organism or re-infection (Wills *et al.*, 1987).

**Purified protein vaccine.** Since the early 1990s, chlamydial vaccine research has been focused largely on the use of the major outer membrane protein (MOMP) as a single antigen component. MOMP comprises approximately 60% of the total protein mass of the bacterial outer membrane (Brunham and Peeling, 1994). It has four variable domains, which are surface exposed and antigenically variable, hence responsible for serological differences between chlamydial strains (Stephens *et al.*, 1987). A predominant anti-MOMP response was observed from the serological analysis of convalescent sheep sera following natural or experimental infection with *C. abortus* and sera from humans infected with *C. trachomatis* (Anderson *et al.*, 1990; Miettinen *et al.*, 1990; Ward *et al.*, 1986). However, when the MOMP protein, eluted from SDS–PAGE gels, was orally administered to cynomolgus monkeys, it failed to induce protection against *C. trachomatis*-induced conjunctivitis (Taylor *et al.*, 1988). In contrast, protection was observed when a detergent-extracted chlamydial outer membrane complex (COMC) preparation, in which MOMP constitutes 90% or more of the protein content, was subcutaneously administered to pregnant ewes infected with *C. abortus* (Tan *et al.*, 1990). Since then, COMC preparations have been shown to induce variable levels of protection in guinea pig (Batteiger *et al.*, 1993) and mouse (Pal *et al.*, 1997) models of genital chlamydial infection, and in a mouse toxicity test for *C. felis* infection (Sandbulte *et al.*, 1996). Protection by native MOMP was observed in mice against a *C. muridarum* serovar after administration via immune stimulating complex (ISCOM) and adjuvantation with CpG oligodeoxynucleotide coupled to the nontoxic B subunit of cholera toxin (Cheng *et al.*, 2009; Igietseme and Murdin, 2000). More recently, a purified and refolded MOMP preparation induced protection in mice against upper genital tract challenge with *C. muridarum* (Pal *et al.*, 2001). However,

although successful protection has been acquired using refolded, purified MOMP preparations, the major disadvantages of these vaccines are that they are very expensive and there are problems to grow *Chlamydia* in bulk, which renders these kinds of vaccines commercially infeasible (Longbottom and Livingstone, 2006).

**Recombinant protein vaccine.** Due to hurdles associated with the purified MOMP and COMCs preparation, attention turned to the development of the cheaper and more cost-effective alternative of recombinant protein vaccines, particularly those based on MOMP (Longbottom and Livingstone, 2006). Studies conducted in sheep with *C. abortus* recombinant MOMP, expressed as insoluble inclusion bodies in a bacterial overexpression system, demonstrated some protection against infection, but the findings were not consistent (Herring *et al.*, 1998). Expression of *C. abortus* MOMP as overcoat protein on the surface of a plant virus was initially found promising in mouse studies, but ultimately proved unsuccessful and trials did not progress to sheep (Herring *et al.*, 1998, Longbottom and Livingstone, 2006). Different attempts were also made to induce protection against *C. trachomatis* infection by rMOMP vaccination. Mouse protection studies with rMOMP incorporated in lipid C induces partial protection of both the respiratory and genital mucosae against *C. muridarum* challenge (Ralli-Jain *et al.*, 2010). Similar studies also demonstrated that the degree of protection obtained with rMOMP was not as robust as that achieved with a native MOMP preparation (Sun *et al.*, 2009). Thus, the disappointing results achieved with recombinant MOMP vaccination could be due to a lack of native protein structure and conformationally intact protective epitopes (Longbottom, 2003), or, it may be that MOMP alone is insufficient and additional antigens are also required.

Moreover, this approach proved difficult as the expression of full-length recombinant MOMP (rMOMP) in prokaryotic expression systems is generally toxic (Schautteet *et al.*, 2011).

**Synthetic peptide vaccine.** Numerous *in silico* bioinformatics tools are now available to predict antigenic domains or epitopes. Thus, synthetic production of these epitopes makes it possible to produce synthetic peptides which correspond with the important immunogenic domains on the antigens. Several studies with chlamydial MOMP peptides and oligopeptide vaccines demonstrated variable results, with at best partial protection. Initial mouse model studies showed that intradermal injection of a peptide from a conserved region of the MOMP of *C. trachomatis*, conferred some protection against the development of salpingitis (Knight, *et al.*, 1995). In contrast to these findings, Caldwell's group at the Rocky Mountain Laboratory observed that parenteral immunization of mice with an alum-adsorbed synthetic oligopeptide of the *C. trachomatis* MOMP was ineffective in preventing chlamydial genital tract infection although mice produced high levels of antichlamydial neutralizing IgG serum antibodies (Su *et al.*, 1995).

*Potential of synthetic peptide vaccine against Chlamydia.* Synthetic peptide vaccine research in the chlamydial field is in its juvenile stage, and no striking successes have been demonstrated yet. However, considering the complexities and high cost related to other subunit vaccine approaches, the development of a peptide vaccine against chlamydial infection is very promising. The key advantages of peptide vaccines over traditional vaccines based on dead or live attenuated pathogens and recombinant vaccines are as follows (Skwarczynski and Toth, 2014):



- They are almost exclusively synthetically produced. Peptide antigen can be fully and precisely characterized as a chemical entity. This practically removes all the problems associated with the biological contamination of the antigens.
- Some pathogens are difficult to culture (e.g., *Chlamydia*, sporozoites for malaria vaccines), and a subunit-based vaccine (including peptide) might be the best solution in such cases.
- Recent developments in solid phase peptide synthesis using automatic synthesizers and application of microwave techniques have made peptide synthesis very simple, easily reproducible, fast, and cost-effective.
- Most of these vaccines are typically water-soluble, stable under storage conditions even at room temperature, and can be easily be freeze dried.
- Peptides can be customized to target very specific objectives. The current availability of bioinformatics tools make it possible to predict antigenic domains or epitopes *in silico* and chemically synthesize those peptide epitopes. The immune responses can be directed against naturally non-immunodominant epitopes. In addition, a peptide-based vaccine can be designed to target several strains, different phases of the life cycle, or even different pathogens in a multi-epitope approach.
- Because of the absence of the redundant proteins, peptide vaccines are less likely to induce allergic or autoimmune responses than any other vaccine.

However, it should be noted that the potential advantages that peptide vaccines have to offer are to some extent diminished by their inherent lack of immunogenicity, which so far has been reflected by the unimpressive outcome of clinical trial (Celis, 2002). The immune system in most species has usually evolved over time to combat life-threatening

infectious agents (and probably tumors), therefore vaccines consisting of aseptic, endotoxin-free peptides may be ignored and ineffective at inducing a protective immune response. Furthermore, peptides that are injected in aqueous solutions will probably be unsuccessful at stimulating T-cell responses, either due to their rapid biodegradation (e.g., by proteases) or, worse, because of the induction of T cell tolerance/anergy, which results from the antigenic stimulation of T-cells by non-professional APCs (Kyburz *et al.*, 1993; Toes *et al.*, 1996). Additionally, they are often not recognized equally by the whole outbred population, such as humans (Skwarczynski and Toth, 2014).

## 1.6. RESEARCH RATIONALE AND OBJECTIVE

The public health importance of chlamydial infections is evidenced by the vast socioeconomic burden of the genital, ocular and respiratory human diseases, as well as the economic losses in animal agriculture. With epidemiologic data indicating persisting and sometimes increasing incidence of chlamydial infections, the urgency for efficacious vaccines cannot be overemphasized. Unfortunately, acceptable chlamydial vaccines to date are not available, due to a number of challenges ranging from induction of appropriate immune response that would provide protection but not immunopathology, safety considerations through insufficient immunogenicity of vaccine candidates, lack of effective delivery systems, to the question of how to induce long-term immunity.

It is well established that IFN- $\gamma$  producing CD4<sup>+</sup> Th1 lymphocytes are indispensable for efficient and complete elimination of chlamydial infection (Perry *et al.*, 1997; Rottenberg *et al.*, 2000; Vuola *et al.*, 2000). Thus, an effective chlamydial vaccine design should consider the factors that trigger a CD4<sup>+</sup> Th1 immune response. Among various factors, antigen dose is one of the deciding factors in the induction of Th1 vs Th2 immune responses. A mathematical model predicted that a low antigen dose would favor Th1-dominant immune response whereas a high dose would induce a Th2 response (Bergmann and van Hemmen, 2001). This model is based on the differential sensitivity of these two T-helper cell types to activation-induced cell death (AICD). In this process, neighboring activated T cells undergo apoptosis after repeated ligation of the CD3/TCR complex that is mediated by mutual Fas-Fas-ligand (FasL) binding. Interestingly, FasL is mainly expressed on activated Th1 cells, thus making them very susceptible to AICD. Therefore, if the initial antigen concentration is very high, the continual antigen stimulation causes the

default Th1 cells to undergo AICD. In contrast, Th2 cells that randomly emerge a low frequency in the stimulated T cell population do not express FasL and are completely refractory to Fas-FasL mediated apoptosis. Therefore, Th2 cell will survive high antigen concentrations and overtake Th1 cells that will be eliminated by AICD.

Secondly, a vaccine should be safe and cost-effective. As mentioned earlier, chlamydial whole organism-based live and inactivated vaccines were not successful since they induced autoimmunity or immunopathology, excluding them from real-life application. Safe and cost-effective purified or recombinant protein subunit vaccines did not show promising results due to complexities in their development. Peptide vaccines would avoid these problems, but their effectiveness has been marginal. If the problem of ineffective vaccine response can be overcome, peptide vaccines should be very safe, can be directed to induce specific immunity, would be very easy to produce, and would be more cost-effective than any other vaccine.

In a peptide vaccine approach, multiple overlapping synthetic peptides (OSP) comprising whole proteins would be advantageous as compared to single peptides because 1) OSP vaccines would contain multiple epitopes that can bind to different MHC alleles, and hence the vaccine will generate antigen-specific immunity in vaccine recipients with different MHC backgrounds; 2) OSP vaccines could be designed rapidly against emerging strains of a pathogen or new pathogens because new sequences would serve as OSP templates, and neither epitopes nor vaccine MHC alleles need to be matched; 3) several vaccine candidate proteins of different chlamydial species have been identified, creating promising potential for human and animal OSP vaccines against chlamydial infection.

Finally, the selection of an appropriate adjuvant and delivery vehicle is essential for a subunit vaccine or an OSP peptide vaccine. Thus, in terms of chlamydial vaccine design one should select an adjuvant that triggers a Th1 response. Similarly, a delivery vehicle should be considered that will present the antigen and adjuvant simultaneously to the APCs and has controlled release properties. Biodegradable polymer microparticles, most commonly microspheres with microencapsulated peptide or protein antigens, have been studied for more than 20 years after early reports of the possibility to control the release of protein antigens over extended time periods for the purpose to eliminate booster vaccine doses. Primarily because of their safety, including everyday use in healthy people (including children) as resorbable sutures, and their use in several commercial controlled-release drug products, co-polymers of lactide and glycolide (PLG) have become the most widely studied polymer to help meet this goal. Moreover, PLG microspheres have several additional advantages, such as the ability to elicit cytotoxic T cell (CTL) responses, the potential for mucosal immunization, and DNA delivery in DNA vaccines.

We hypothesized that 20-mer overlapping synthetic peptide antigens derived from known vaccine candidate proteins of *C. abortus* should induce protection against *C. abortus* challenge when used (i) at very low dose of the peptide antigens and (ii) in a microparticulate delivery system. To this end, the overall purpose of this study was to develop a vaccine platform that can be utilized to design a safe and effective vaccine against different chlamydial species and other intracellular bacteria where a Th1 immune response is essential for clearance of infection.

The specific goals of this investigation were:

1. Synthesize 20-mer overlapping peptides from the best *C. abortus* vaccine candidate proteins;
2. Test whether low antigen (protein or peptide) can induce protection in a mouse model of respiratory *C. abortus* infection;
3. Identify the optimal adjuvant;
4. Identify and optimize a suitable delivery vehicle for this vaccine.

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## CHAPTER 2

### LOW-DOSE ANTIGEN VACCINATION

#### 2.1. INTRODUCTION

**Principle of peptide vaccines.** The vertebrate immune system is separated into innate and adaptive components, which cooperate to protect the host against microbial infection. The principal distinguishing factors between the two are the response time and the level of specificity. The innate response is initiated almost immediately and causes the migration of phagocytic cells, mainly leukocytes, macrophages and dendritic cells (DCs) to the site of infection (Murphy and Weaver, 2016; Akira, 2011). Upon encountering pathogens, these antigen presenting cells (APCs), particularly DCs, ingest a microbe or other antigens, and undergo directed activation and maturation. As the name APC implies, these cells present antigens by processing endogenous intracellular cytosolic antigens through proteasome-mediated degradation and exogenously derived antigens through the lysosomal degradation pathway. They present highly specific peptides (epitopes) produced by these proteolytic pathways on their surface, in combination with major histocompatibility complex (MHC) proteins. Endogenous peptides are presented in the antigen-binding cleft of MHC class I, and exogenous peptides on MHC class II. Cognate T cell receptors (TCR) can bind these peptide antigens only in the MHC context (MHC-restricted) because they need to simultaneously bind to non-antigen presenting regions of the MHC molecules. The combined TCR-MHC interaction provides the intracellular signal

that induces T cell maturation, specificity and subsequent clonal expansion (Leleux and Roy, 2013; Murphy and Weaver, 2016; Neeffjes *et al.*, 2011). Thus the final product that an APC presents to a T-cell is a short peptide. Moreover, T cells that identify peptide–MHC complexes derived from a parent protein after intracellular processing can also usually recognize the same peptides if they are directly loaded onto MHC molecules after being delivered exogenously to the cell (Mohan and Unanue, 2012). Thus, for antigen presentation it is well established that a given T cell will recognize cognate peptide–MHC complexes regardless of whether the peptide is generated internally from protein processing or delivered exogenously. These principles of antigen presentation generated the idea that peptides could substitute for whole proteins as antigens, and thus stimulated efforts for peptide vaccine development.

**Peptide length in a peptide vaccine.** Upon activation, maturation, and subsequent antigen presentation to T-cells, DCs initiate a cascade of events that are collectively known as the adaptive immune response (Cella *et al.*, 1997). It is important to note that the specific combination of peptide-loaded MHC complexes and costimulatory molecules expressed on the surface of an APC directs activation and functionalization of T lymphocytes. In general, MHC class I molecules bind CD8<sup>+</sup> naïve T-cells and trigger the differentiation of naïve T lymphocytes towards CD8<sup>+</sup> cytotoxic T lymphocytes (CTL). In contrast, MHC class II molecule bind naïve CD4<sup>+</sup> T-helper (Th) cells and induce their differentiation into one of several lineages of Th cells, including Th1, Th2, and Th17, as defined by their pattern of cytokine production and function (Zhu *et al.*, 2010). CTLs function to eliminate pathogen-infected cells and are being studied as a potential mechanism for cancer

eradication (von Andrian and Mackay, 2000). T helper cells are the master regulators of adaptive immunity by providing a wide range of direct and cytokine-mediated signals to immune and non-immune cells in their microenvironment. They have a wide variety of functions including facilitating B cell activation, which induces the humoral immune response, as well as initiating T-helper mediated cellular immune responses.

Therefore, targeting the appropriate MHC molecule is essential in design of a peptide vaccine, and the length of peptides or epitopes is an important consideration in this regard. In general, MHC class I molecules bind short peptides since they have a closed binding groove in which the N- and C-terminal ends of a peptide antigen are anchored into the pockets located at the ends of the peptide binding groove (Meydan, *et al.*, 2013; Natarajan *et al.*, 1999). While the majority of these peptides are about 9 residues in length, longer peptides can be accommodated through bulging of their central portion (Guo *et al.*, 1992; Speir *et al.*, 2001), resulting in binding peptides of length 8 to 15 amino acids (Schumacher *et al.*, 1991). In contrast, MHC class II molecules have an open binding groove, which allows greater flexibility in the length of bound peptides (Nelson and Fremont, 1999; Yassai *et al.*, 2002), which can vary from 8 to 30 amino acids in length (Liu and Gao, 2011; Rammensee, 1995).

**Adjuvants.** Although the innate immune response is specific in nature and does not retain any memory of a previous infection or antigenic stimulus, there are mechanisms at the APC level that can provide the initial direction of the adaptive immune response. The role of innate immune cells to drive the polarization of the adaptive immune response was first shown in mice: certain inbred mouse strains have a Th1 bias (like C57BL/6) and others

have a Th2 bias (like Balb/c). Studies demonstrated that the underlying reason for this bias is not in the T cell compartment, but rather in the innate immune system (Mills *et al.*, 2000). This was shown by using immunodeficient mice that do not possess an adaptive immune system, but still preserve the Th1/Th2 bias. The molecules that guide production of specific cytokine by APCs as well as promotes activation of APC and expression of surface proteins are termed pattern recognition receptors (PRRs). PRRs recognize various conserved constituents of microbial pathogens called pathogen associated molecular patterns (PAMPs) and, in turn, induce a specific array of inflammatory or non-inflammatory cytokines and thereby cause innate effector functions as well as initiate the development of acquired immunity to infecting pathogens. However, many injectable subunit vaccines do not possess PAMPs, or have PAMPs but not in a form that triggers PRRs to recruit innate immunity, and are therefore not effective in inducing protective immunity. For that reason such vaccines require adjuvants that substitute for pathogen-derived PAMPs and stimulate PRRs. Since synthetic peptide vaccines antigens are based on short sequences of amino acids that do not stimulate PRRs, adding an appropriate adjuvant is essential to elicit an effective and long-term immune response.

Adjuvants have been traditionally used to reinforce the magnitude of an adaptive response to a vaccine, measured by an antibody titer or by the ability to prevent infection. However, a second role for adjuvants has become increasingly important: guiding the type of adaptive response – cellular vs humoral - to produce the most effective forms of immunity for each specific pathogen (Coffman *et al.*, 2010). For polarization of helper T cells, there are striking differences in the type of response preferentially stimulated by different adjuvants (Table 2.1). Adjuvants such as MF59 and ISCOMs, as well as ligands

for Toll-like receptor 2 (TLR2) and TLR5, enhance T cell and antibody responses without altering the Th1/Th2 cell balance of the response to specific antigens (Coffman *et al.*, 2010). In contrast, more polarized Th1 cell responses are generated by adjuvants that incorporate agonists of TLR3, TLR4, TLR7-TLR8, and TLR9 ligands. Overall, selection of an appropriate adjuvant is influenced by the type of CD4<sup>+</sup> T cell response essential for protection.



**Table 2.1. Triggering of the Innate and Adaptive Components of the Immune System by Major Adjuvants.**

Adjuvant	Major Immunostimulatory Component(s)	Immune Stimulatory Mechanism	Principal Immune Responses Stimulated	Reviewed by
Alum	Aluminum salts	NLRP3 inflammasome (?) ↑Local cytokines and chemokines ↑Cell recruitment (eosinophils, monocytes, macrophages) ↑Ag presentation	Ab, Th2 Poor Th1 response	Awate <i>et al.</i> , 2013; Coffman <i>et al.</i> , 2010
MF59®	5% squalene emulsified in-water with 1% polyoxyethylenesorbitan monooleate and sorbitan trioleate	Tissue inflammation (no receptors defined)	Ab, Th1 + Th2	Awate <i>et al.</i> , 2013; Coffman <i>et al.</i> , 2010
Quil A, <i>Quillaja saponaria</i> 21 (QS-21)	Saponin (Quil A derived from the tree - <i>Quillaja saponaria</i> . QS-21 is purified product of Quil A)	↑ Cytokine (mainly IL-2 and INF-γ) ↑Ag presentation through MHC-I pathway	Th1, CD8 <sup>+</sup> T cells, Ab	de Paula Barbosa, 2014; Rajput <i>et al.</i> , 2007
Adjuvant System 03 (AS03)	Squalene-in-water emulsions	Spatio-temporal co-localization with Ag Transient ↑ cytokines locally and in lymph nodes draining the site of inoculation (dLNs) ↑Cell recruitment (granulocytes and monocytes) ↑Ag-loaded monocytes in dLNs	Ab, Immune memory	Awate <i>et al.</i> , 2013; Coffman <i>et al.</i> , 2010
Montanide ISA 71 VG	Water-in-oil (W/O) emulsion	Depot effect : produce ≤1µm droplet and thus ↑Ag presentation	Th1, Ab	SEPPIC, 2008
Incomplete Freund's adjuvant (IFA) (typically with Montanide formulations)	mineral or paraffin oil + surfactant	Mechanism undefined	Ab, Th1 + Th2	Coffman <i>et al.</i> , 2010
Complete Freund's Adjuvant (CFA)	IFA + peptidoglycan, trehalose dimycolate	NLR, inflammasome, Mincle, TLR?	Ab, Th1, Th17	Coffman <i>et al.</i> , 2010
Adjuvant System 04 (AS04)	Monophosphoryl lipid A (MPL) plus alum	TLR4 and inflammasome (?) ↑Local cytokines and chemokines ↑Cell recruitment (DCs and monocytes) ↑Ag-loaded DCs and monocytes in dLNs	Ab, Th1	Awate <i>et al.</i> , 2013; Coffman <i>et al.</i> , 2010

**Table 2.1. Continued.**

<b>Adjuvant</b>	<b>Major Immunostimulatory Component(s)</b>	<b>Immune Stimulatory Mechanism</b>	<b>Principal Immune Responses Stimulated</b>	<b>Reviewed by</b>
MPL and formulations (AS01, AS02)	MPL and purified Quil A saponin QS-21	TLR4	Ab, Th1	Coffman <i>et al.</i> , 2010
Polyinosinic-polycytidylic acid (poly-IC)	Synthetic derivatives of dsRNA	TLR3, MDA5	Ab, Th1, CD8+ T cells	Coffman <i>et al.</i> , 2010
Flagellin, flagellin-Ag fusion proteins	Flagellin from <i>S. typhimurium</i>	TLR5	Ab, Th1 + Th2	Coffman <i>et al.</i> , 2010
Imiquimods, Resiquimod,	Imidazoquinoline derivatives	TLR7, TLR8 or both	Ab, Th1, CD8+ T cells	Coffman <i>et al.</i> , 2010
CpG oligodeoxynucleotides and formulations (IC31, QB10)	Synthetic phosphorothioate-linked DNA oligonucleotides with optimized CpG motifs	TLR9	Ab, Th1, CD8+ T cells (when conjugated)	Coffman <i>et al.</i> , 2010
Juvimmune	CpG-containing plasmid packaged in cationic liposome	TLR9	Ab, Th1, CD8+ T cells	Coffman <i>et al.</i> , 2010
JVRS-100	CpG-containing non-coding plasmid packaged in cationic liposome-DNA complexes (CLDC)	TLR9; the CLDC are virus-sized particulates, with a mean diameter of ~120 nm, which facilitates trafficking to APC in dLNs.	Ab, Th1, CD8+ T cell	Chang <i>et al.</i> , 2009;
Trehalose-6,6'-dibehenate (TDB)	Synthetic analog of mycobacterial cord factor trehalose -6, 6-dimycolate (TDM)	TDB binds Mincle (macrophage inducible C-type lectin). Upon TDB recognition, Mincle interacts with FcR- $\gamma$ , resulting in CARD9-dependent NF- $\kappa$ B activation.	Th1, Th17	Althaus, 2009
Block copolymers (Poloxamers): Polygen, Pluronic L121	Hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) blocks arranged in A-B-A tri-block structure	In aqueous solutions self-assemble into micelles (10 nm to 100 nm in diameter). Thus they act as delivery vehicle and stabilize the native conformation of an antigen. The hydrophobic PPO interacts with PRR.	Ab, Th1, CD8+ T cells	Adams <i>et al.</i> , 2015; Batrakova & Kabanov, 2008

**Factors defining T-helper cell polarization – cytokine milieu and transcription factors.** After being activated by an APC in the periphery, a naive CD4<sup>+</sup> T cell differentiates into one of several major T helper (Th) cell subtypes to become a Th1, Th2, Th17, T follicular helper (Tfh), or peripherally derived induced regulatory T (iTreg) cell. The decision-making process is thought to be mainly directed by the available cytokines milieu during the activation process. Moreover, each Th subset is distinguished by specialized gene expression pattern, which is under the control of a lineage-defining transcription factor. The lineage-defining transcription factors are T-bet for Th1, GATA-binding protein 3 (GATA3) for the Th2, retinoic acid receptor-related orphan receptor- $\gamma$ t (ROR $\gamma$ t) for the Th17, B cell lymphoma-6 (BCL-6) for T fh cells, and forkhead box P3 (FOXP3) for Treg cells (Oestreich and Weinmann, 2012). CD4<sup>+</sup> Th cell subsets are characterized by the signature cytokines that they secrete, their distinct homing properties, and their specialized effector functions, which make them best equipped to target a particular class of pathogens.

Th1 cells. The development of Th1-cell begins with the secretion of cytokines IL-12 and type 1 interferons (IFN- $\alpha$  and IFN- $\beta$ ) by macrophages and DCs upon activation by intracellular pathogens (Farrar *et al.*, 2002). IL-12 triggers the secretion of IFN- $\gamma$  by these same cells and from natural killer (NK) cells. IFN- $\gamma$  acts in an autocrine manner to generate a positive feedback loop, producing further IL-12, and also acts as an inhibitor of the Th2 pathway by preventing Th2 cell proliferation (Kaiko *et al.*, 2007; Lafaille, 1998; Murphy *et al.*, 2000). Bindings of IFN- $\gamma$  to naïve Th cells leads to the Janus Kinase 1 and 2 (JAK1 and JAK2; tyrosine kinase proteins)-mediated activation of the transcription factor signal transducer and activator of transcription 1 (STAT1), which then induces the expression of

T-bet (a member of the TATAAA box family of transcription factors). T-bet is the master regulator of Th1 cell differentiation and its selective expression initiates the remodeling of the IFN- $\gamma$  gene locus, the production of IFN- $\gamma$ , expression of the IL-12 receptor and stabilization of its own expression through the autocrine activity of IFN- $\gamma$  (Lazarevic *et al.*, 2013; Mullen *et al.*, 2001). Once the IL-12 receptor is expressed, the IL-12 cytokine can bind its receptor and further augment the differentiation of Th1 cells. IL-12 signaling activates the STAT3, STAT4 and nuclear factor- $\kappa$ B transcription factors, and thus promote the synthesis of cytokines associated with the Th1 phenotype (Afkarian *et al.*, 2002; Kaiko *et al.*, 2007). T-bet also prevents the differentiation of the Th2 lineage by sequestration of GATA3 (GATA binding protein 3) away from Th2 cell-specific genes (primarily the *Il4-Il5-Il13* locus) and by binding a T-bet-Runt-related transcription factor 3 (T-bet-RUNX3) complex to the *Il4* silencer. T-bet therefore suppresses the expression of Th2 cell-specific cytokine genes in developing Th1 cells (Hwang *et al.*, 2005; Djuretic *et al.*, 2007; Kanhere, *et al.*, 2012).

Th2 cells. The development of Th2 effector cells primarily involves the action of IL-4, IL-6, IL-10 and IL-11 (Kaiko *et al.*, 2007). IL-4 induces the STAT6 production in naive T cells, which in turn leads to expression of the zinc finger transcription factor GATA-3 (Kaplan *et al.*, 1996; Ouyang *et al.*, 1998). GATA-3 enhances promoter activity and reverses chromatin structure-based suppression of regions associated with controlling Th2 cytokine gene expression and thus directs in the release of cytokines characteristic of the Th2 phenotype (IL-4, -5, -9, -10 and -13), and also inhibits the expression of the IL-12 receptor and therefore Th1 development (Farrar *et al.*, 2002). IL-6 is released during the early stages of a Th2 immune response and induces the Th2 phenotype through the up-

regulation of IL-4 and inhibition of STAT1 phosphorylation, thereby preventing IFN- $\gamma$  gene expression (Detournay *et al.*, 2005; Dodge *et al.*, 2003). IL-10 appears to inhibit IL-12 synthesis, and thus the Th1 pathway (Koch *et al.*, 1996).

Th17 cells. Th17 cells produce IL-17, IL-17F, IL-22 and TNF- $\alpha$ , which, in turn, act on fibroblasts, endothelial and epithelial cells, and macrophages, and trigger the release of inflammatory mediators and chemokines (Kaiko *et al.*, 2007). The resultant environment recruits neutrophils and creates a general state of tissue inflammation (Nakae *et al.*, 2003; Iwakura and Ishigam, 2006; Romagnani, 2006). The development of Th17 depends on the action of TGF- $\beta$ , IL-6, and IL-21 (Korn *et al.*, 2009). IL-6 acts on naïve T cells and induces the downstream expression of IL-21, which causes an autocrine loop that results in self-induced expression. The regulatory cytokine TGF- $\beta$  then acts in synergy with IL-21 to induce the expression of ROR $\gamma$ t through a STAT3-dependent mechanism (Korn *et al.*, 2007; Zhou *et al.*, 2007). The expression of ROR $\gamma$ t induces transcription of IL-17 and IL-17F encoding genes (Ivanov *et al.*, 2006).

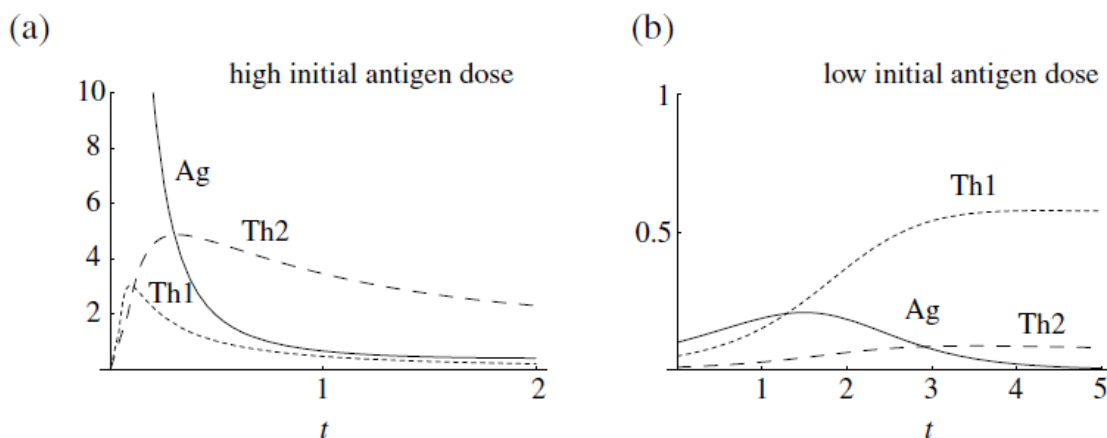
**Influence of antigen dose on T-helper cell polarization.** Besides the cytokine milieu, many other factors influence the selective differentiation of CD4<sup>+</sup> T cells, including the specificity and avidity of antigen recognition by the T cell receptor, the expression of the costimulatory molecules, and the dose of the antigen applied to stimulate T lymphocytes (Constant and Bottomly, 1997; Cho *et al.*, 2000; Xu *et al.*, 2010). Although there is disagreement on whether Th1- or Th2-type responses are elicited by high versus low doses of antigen, ample evidence suggest that low antigen doses favor a Th1 immune response, and higher doses favor antibody production (Constant and Bottomly, 1997;

Power *et al.*, 1998) . In fact, Salvin in the 1950s, examined how the dose of antigen (purified diphtheria toxoid or ovalbumin) administered affected the class of immunity expressed at different times following immunization. When guinea pigs were intradermally administered with different doses of diphtheria toxoid, low doses generate an exclusive cell-mediated, DTH response; medium doses more rapidly generated a cell-mediated response that evolves, with time, into a humoral mode; the administration of even larger doses results in more rapid responses, sometimes resulting in a barely detectable cell-mediated phase (Bretscher, 2014; Salvin, 1958). Similar findings were observed for diverse routes of antigen administration, for diverse antigens such as xenogeneic red blood cells (Lagrange *et al.*, 1974), the protozoan parasite *Leishmania major* (Menon and Bretscher, 1998), for mycobacteria given to adult (Power *et al.*, 1998) or neonatal mice (Kiros *et al.*, 2010), and in different species of immunized animals (Buddle *et al.*, 1995).

The question of why low doses of antigens trigger Th1 differentiation is difficult to resolve, although a potential explanation is provided by the differential susceptibility of Th1 and Th2 cells to activation induced cell death (AICD) at high doses of antigen (Constant and Bottomly, 1997). AICD is the process by which cells undergo apoptosis in a controlled manner through the interaction of a death factor and its receptor, the Fas (CD95)-Fas ligand (FasL/CD95L) interaction (Maher *et al.*, 2002). It is considered the primary mechanism for deleting mature CD4<sup>+</sup> T cells in the periphery and it plays a crucial role during adaptive immune responses by ensuring that a defined number of specialized T cells remain in the organism (Baumann *et al.*, 2002; Krammer *et al.*, 2007). However, it has been demonstrate that Th1 cells express high levels of FasL, whereas its expression in Th2 cells is very low level (Ramsdell *et al.*, 1994). Therefore, Th1 clones are more

susceptible than Th2 clones to AICD triggered by high doses of antigens, and, in fact, it has been found that stimulation of recently derived CD4<sup>+</sup> T cells induces apoptosis more readily and more quickly in the Th1 than the Th2 subset (Constant and Bottomly, 1997). In 2001, Bergmann *et al.* published an interesting approach modeling T helper cell immunity with a regulatory feedback loop on the basis of the differential sensitivity of T helper cells to Fas-Fas ligand-mediated AICD. Their model suggests that if antigen levels are high or the default Th1 response to a pathogen is not successful in eliminating the pathogen, the sustained antigenic stimulus would drive Th1 cells into activation-induced apoptosis and Th2 cells would overtake the population of activated pathogen-specific T cells (Fig. 2.1).

Thus, the only way to maintain a default Th1 immune response is either by very low antigen concentration to begin with, or by rapid antigen removal with a successful Th1 response against a pathogen such as *Chlamydia*.



**Figure 2.1.** Time plots showing antigen (Ag) dose dependence. (a) The Th1 bias promotes Th1 but high antigen doses (initial antigen dose = 50) induce a rapid shift to Th2 dominance. (b) At low initial antigen levels (initial antigen dose = 0.1) Th1 eliminates the antigen and the initial Th1 bias is maintained. Reproduced from Bergmann *et al.*, 2001.

Similar to other intracellular pathogens, T-lymphocytes play a key role in a protective host response to *Chlamydia* infection (Morrison *et al.*, 1995; Rank, 2006). In particular, IFN- $\gamma$  producing Th1 helper lymphocytes are indispensable for efficient and complete elimination of chlamydial infection (Perry *et al.*, 1997; Rottenberg *et al.*, 2000; Vuola *et al.*, 2000), and ablation of Th1 cells or effector functions results in increased chlamydial disease and failure to eliminate chlamydiae (Cotter *et al.*, 1997; Lu *et al.*, 2000; Morrison *et al.*, 1995; Wang *et al.*, 1999). Th1 cells restrict chlamydial replication via Th1-type effector cytokines, most prominently IFN- $\gamma$ , contributing to a delayed type hypersensitivity (DTH) response (Perry *et al.*, 1997; Rottenberg *et al.*, 2000; Wang *et al.*, 2008). Such protective DTH responses are characterized by tissue infiltration of CD4<sup>+</sup> T cells and macrophages and release of proinflammatory Th1 cytokines such as IL-1, IL-2, IL-12, IFN- $\gamma$ , and TNF- $\alpha$ . Therefore, factors favoring a Th1-dominant immune response, such as Th1-promoting adjuvant and low antigen dose, should be considered while developing a vaccine against chlamydial infection.



## **2.2. HYPOTHESIS**

We hypothesize that low dosage of potential protective protein or peptide antigens of *Chlamydia* spp. will induce a protective Th1 response against *Chlamydia* challenge, if delivered with a safe and efficient adjuvant.

## **2.3. OBJECTIVES**

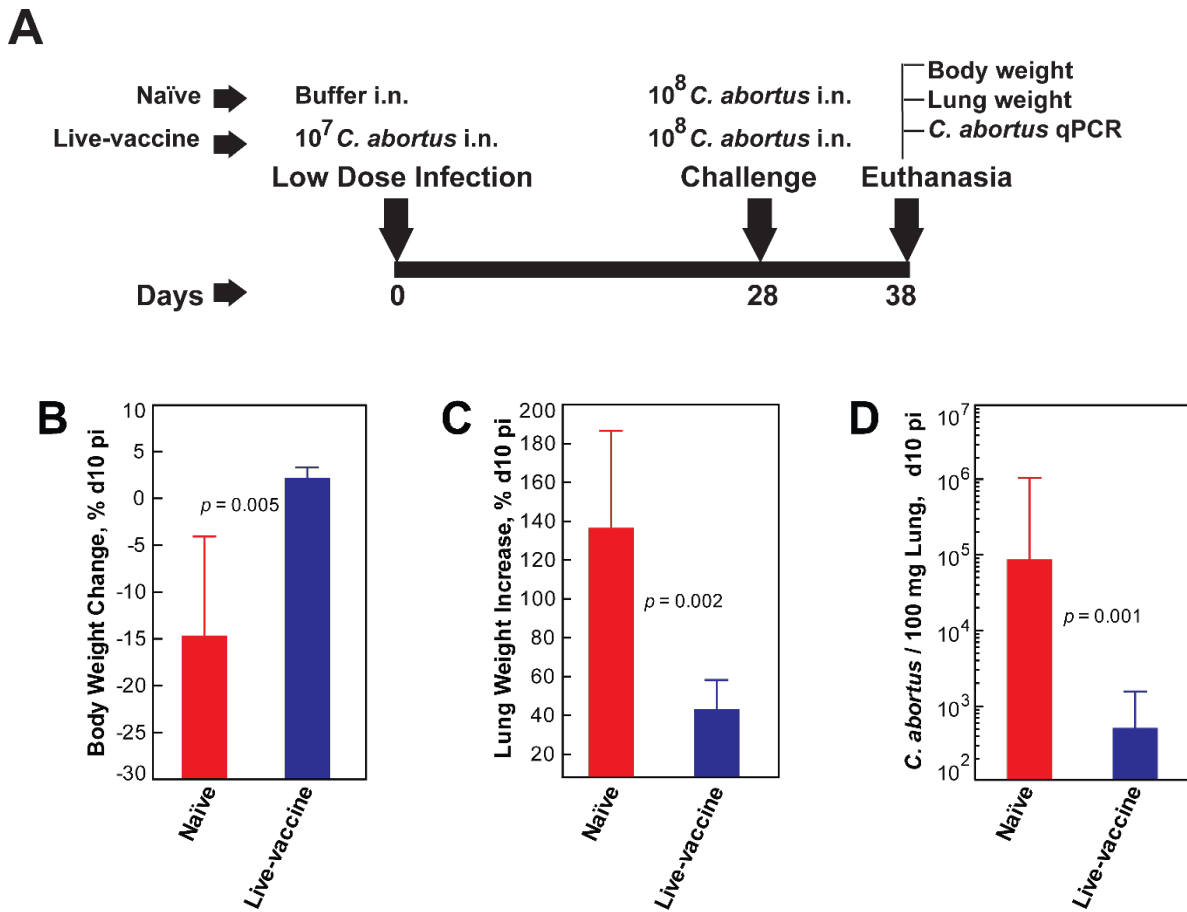
The aim of this experiment was to examine in a murine model of *C. abortus* respiratory disease the effect of

- 1) varying high to low dose vaccine antigen administration on the protection efficacy against chlamydial challenge, and
- 2) to identify a suitable adjuvant for an effective Th1 vaccine formulation.

## 2.4. RESULTS

**A/J mouse model of chlamydial respiratory disease.** In this experiment, we first examined the A/J mouse strain as chlamydial lung challenge model by contrasting the disease outcome between naïve and previously infected mice 10 days after challenge inoculation. As shown in Fig. 2.2A, one group of mice received a low dose of *C. abortus* inoculation four weeks before the high dose challenge. This low dose mimics natural subclinical chlamydial infection and induces a protective immune response that lasts approximately 10 weeks. Therefore, this low dose is comparable to an attenuated live-vaccine and hence is termed the live-vaccine group. The disease outcome was determined by analyzing the following parameters: i) the changes in body weight; ii) severity of lung inflammation as determined by lung weight; and iii) the chlamydial burden in the lungs as detected by real-time PCR.

As expected, naïve mice had a significantly lower body weight 10 days after challenge inoculation than live-vaccine group (Fig. 2.2 B,  $p=0.005$ ). In fact, the live-vaccine group gained 2.09% body weight during the challenge infection, whereas the naïve group heavily lost 14.72% weight. Similarly, naïve mice had highly inflamed lungs with a mean lung weight increase of 136.18% over healthy adult female A/J mice. Conversely, the live-vaccine group had a highly significantly lower local inflammatory response than naïve mice ( $p=0.002$ ) with a mean lung weight increase of 42.77%. Finally, significantly greater numbers of 79,433 chlamydial genomes were detected per 100 mg lung of naïve mice than the 468 genomes of the live-vaccine group ( $p=0.001$ ). Overall, there was a sharp contrast between naïve and live vaccine mice: live-vaccine mice remain completely healthy following *C. abortus* challenge whereas naïve mice become very sick.



**Figure 2.2. Chlamydial respiratory disease in A/J mice.** Disease severity (body weight loss and lung weight increase) and chlamydial lung load after intranasal inoculation of mice with  $10^8$  genomes of *C. abortus* elementary bodies. (A) Schematic presentation of experimental protocol. Each mouse in the live vaccine group received  $10^7$  *C. abortus* EBs intranasally (i.n.) in 20  $\mu$ l SPG buffer on day 0, and four weeks later every mouse in both naïve and live-vaccine groups was challenged i.n. with  $10^8$  *C. abortus*. Ten days after challenge, all mice were euthanized, body weight and lung weight were measured, and *C. abortus* burdens in lungs were determined by qPCR. (B) Percentage of mean body weight change in naïve and live-vaccine mice after 10 days of challenge. Whereas live vaccine group gained body weight (positive mean body weight change), the naïve group significantly lost weight (negative mean body weight change). (C) Percentage of lung weight increase over the average lung weight of healthy adult female A/J mice 10 days after challenge, indicative of lung inflammation. The naïve mice had significantly higher lung weights than the live vaccine group. (D) Lung chlamydial burden in naïve and live-vaccine mice 10 days after challenge. Naïve lungs were heavily loaded with chlamydiae, whereas the live vaccine had significantly lower levels. Data were analyzed by Student's t-test (n=15 mice / group).

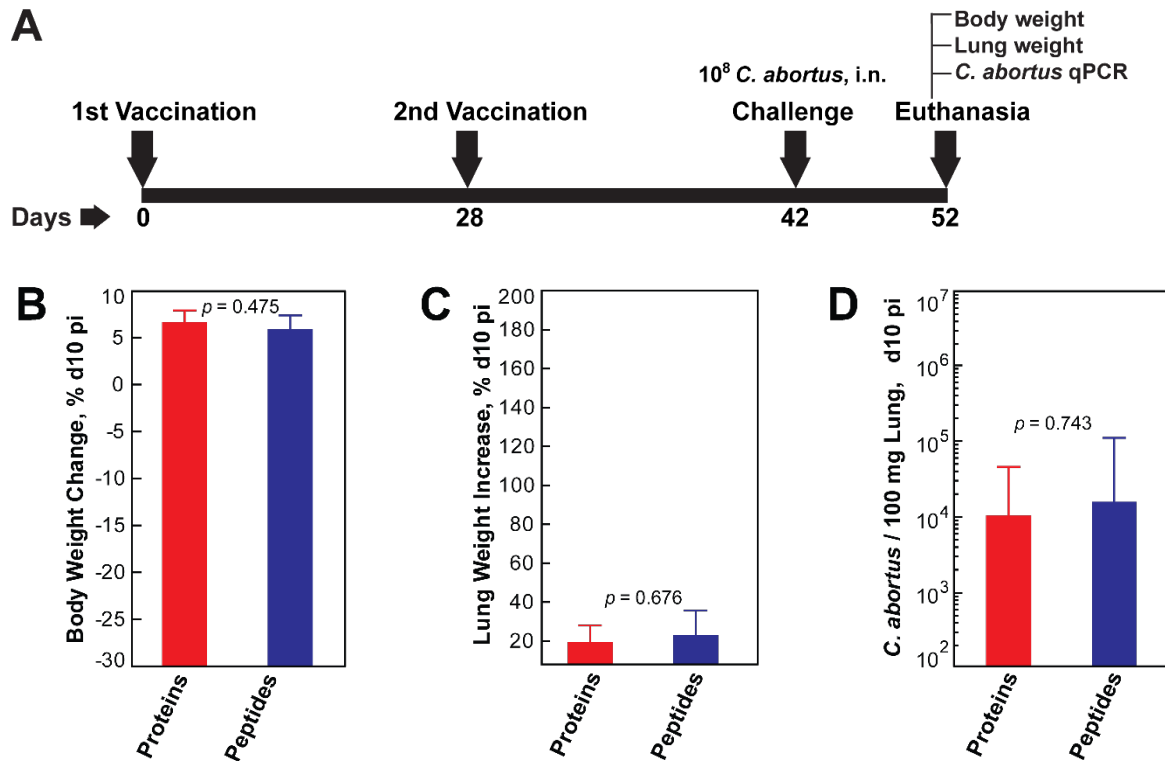
**Twenty femtoMole-dosed *C. abortus* vaccine candidate recombinant proteins or their equivalent-dosed synthetic peptide antigens induce immune protection.** In an initial approach towards development of a subunit *C. abortus* vaccine, we examined if, and what dose, vaccine antigen doses lower than those customarily used in vaccines are able to induce immune protection from chlamydial infection. For testing in an A/J mouse model of *C. abortus* respiratory disease (Fig. 2.2), we vaccinated the mice two time in a four-week interval (Fig. 2.3A). As the vaccine protein antigens, the mice received 20 femtoMoles (~1 ng) of each of three recombinant *C. abortus* vaccine candidate proteins - DnaX2, GatA, and GatC. Alternatively, they received 10 femtoMoles of each of 20-mer peptides overlapping by 10 amino acids and comprising each protein. Since these peptides were overlapping by 10 amino acids, every sequence of 10 or less amino acids of these proteins was available for antigen presentation by the T cell receptor. The 10 fM dosage of each peptide was matched to the 20 fM protein dosage because these overlapping peptides represented each protein molecule twice. We used 1 ng of vaccine antigen assuming that this dose would be at least 1,000-fold lower than the lowest dose (1µg) researchers usually use in mice for chlamydial vaccine research. As a vaccine adjuvant, Polygen, a Th1 promoting immune stimulator (Andrianarivo *et al.*, 1999), was included in the vaccine formulation.

As shown in Fig 2.3B, 10 days after challenge inoculation both the protein and peptide vaccine mice had a similar body-weight gains of 6.77% and 6.00%; respectively (Fig. 2.2B), and there was no significant difference between these vaccines ( $p=0.475$ ), but significantly higher weight gains than the protected live-vaccine control in the A/J model ( $p=0.00005$  and  $p=0.0007$ ; Fig. 2.2B). Similarly, protein-vaccinated mice showed a lung

weight increase of 19.00%, and peptide-vaccinated mice showed 22.47% (Fig 2.3C), without significant differences between the two groups ( $p=0.676$ ). Importantly, these lung weight increases of the protein vaccine group were significantly lower than the 42.77% of the protected live-vaccine control in the A/J model ( $p=0.03$ ; Fig. 2.2C) and barely failed to reach significance for the peptide vaccine group ( $p=0.08$ ).

While body weight change and lung weight increase data indicated complete protection, mice in both vaccine groups showed intermediate chlamydial lung burdens, ranging midway between live-vaccine and naïve control mice in the A/J model. Protein-vaccinated mice had 9,885 *C. abortus* genomes per 100 mg lung, and peptide-vaccinated ones 15,136 ( $p = 0.743$ , Fig. 2.3D). These chlamydial burdens were lower, but not significantly different, than the 79,433 genomes per 100 mg lung of naïve mice ( $p = 0.231$  and  $p = 0.359$ ; Fig. 2.2D), but significantly higher than the 468 genomes of live-vaccine mice ( $p = 0.004$ ,  $p = 0.011$  Fig. 2.2D).

Overall, these data clearly indicate that the low vaccine antigen dose can induce a substantial protective immune response against chlamydial challenge infection. It is also evident that the overlapping synthetic peptide antigens are as protective as recombinant proteins in this vaccination approach. However, considering the relatively high chlamydial loads in vaccine groups, we assume that there is still potential to improve the low antigen dose immunization approach. Therefore, we hypothesize that further lowering the antigen dose should induce more potent Th1 responses that will clear chlamydiae more efficiently. Thus we performed a dose titration analysis in our subsequent experiment, using only synthetic peptide antigens with the aim to develop a synthetic peptide vaccine.



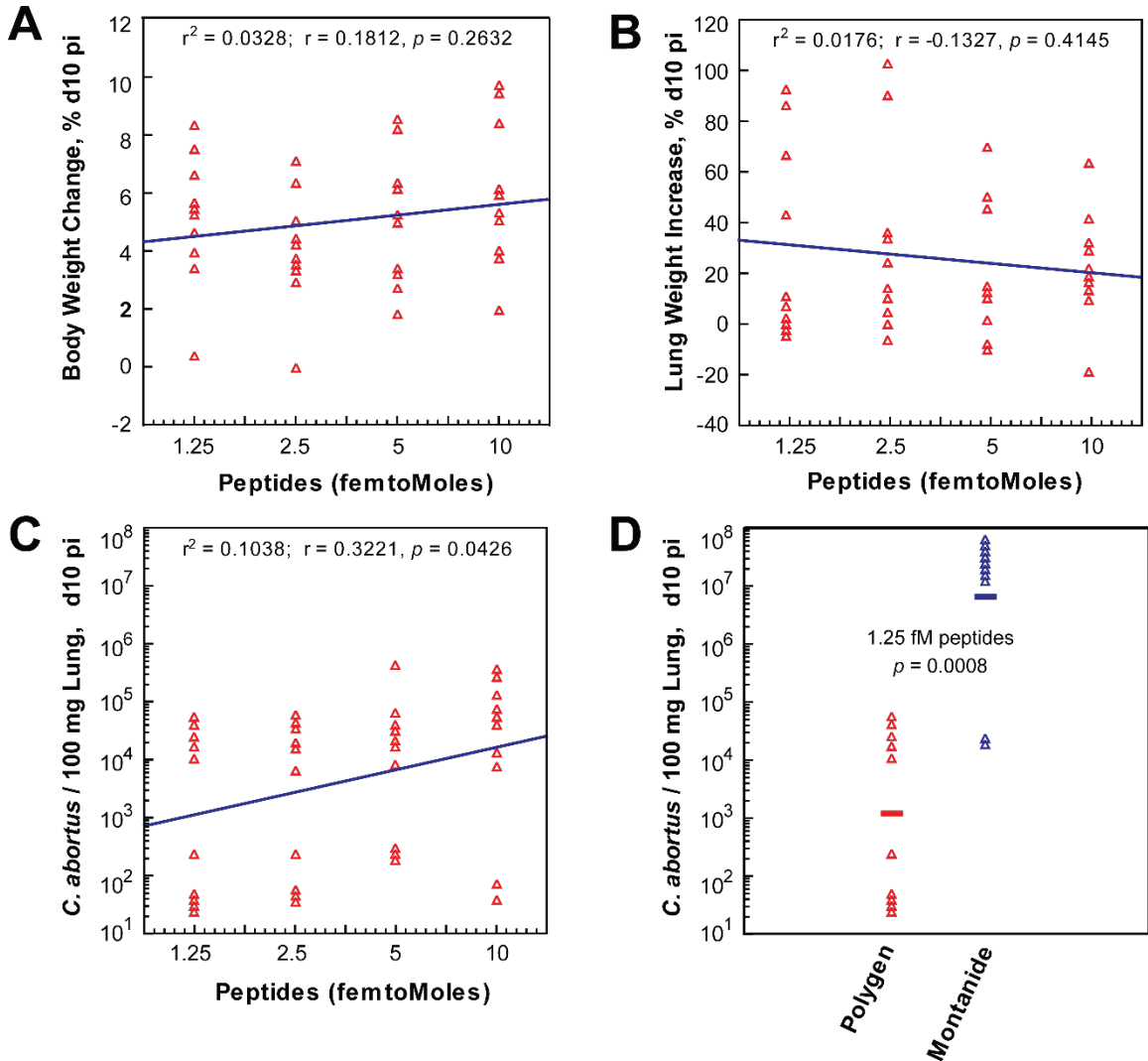
**Figure 2.3. Comparison between low dose proteins and peptides vaccine antigens for protection against *C. abortus* in A/J mice.** (A) Schematic representation of the experimental protocol. Two groups of mice were vaccinated twice at four weeks intervals with vaccines containing protein or peptide antigens. The protein antigens were comprised of *C. abortus* DnaX2, GatA, and GatC recombinant proteins, and the peptides antigens were composed of 102 synthetic peptides which were 20 amino acids long and overlapped each other by 10 amino acids, representing the entire sequence of each of the three proteins. Mice received 200  $\mu$ l of vaccine subcutaneously containing 20 femtoMoles ( $\sim$ 1ng) of each protein or 10 femtoMoles of each 20-mer peptide mixed with 2% Polygen in PBS. Four weeks after the 2<sup>nd</sup> vaccination, all mice were challenged intranasally with 10<sup>8</sup> *C. abortus* organisms. Ten days after challenge, all mice were euthanized, body weight and lung weight were measured, and *C. abortus* burdens in lungs were determined by qPCR. (B) Percentage of body weight change 10 days after challenge. Mice in both of groups had similar and positive body weight changes (gained weight). (C) Lung weight increase, indicative of lung inflammation. Mice in both protein and peptides groups had minimal lung weight increases. (D) Lung chlamydial burdens 10 days after challenge. Lung chlamydial loads did not differ significantly between protein and peptide antigen vaccines. Data were analyzed by Student's t-test (n=10 mice/group).

**Dose titration of peptide antigens.** In this dose titration experiment we examined whether lowering the peptide antigen dose per mouse from 10 femtoMoles to 1.25 femtoMoles would promote better chlamydial clearance. Similar to the previous experiment, the A/J mice were vaccinated twice 4 weeks apart with either 10-, 5-, 2.5-, or 1.25-femtoMoles per vaccine dose of each peptide antigen along with 2% Polygen adjuvant.

As shown in Fig. 2.4A, mice in all four vaccine groups gained weight during challenge infection at similar levels, with mean positive body weight changes of 5.11%, 4.10%, 5.08%, and 5.99%, for the 1.25-, 2.5-, 5-, and 10-femtoMoles group, respectively. Linear regression analysis showed no correlation in the body weight change pattern among the groups ( $r=0.1812$ ,  $p=0.2632$ ; Fig. 2.4A). Similarly, the mean lung weight increases were also very low in all four groups (30.27%, 30.13%, 17.12%, and 22.47%), and was not correlated with peptide dose ( $r=-0.1327$ ,  $p=0.4145$ ; Fig. 2.4B). Interestingly, the mean chlamydial burden was progressively lower in the mice as the peptide dose was reduced: 15,136 genomes per 100 mg lung for 10 fM, 8,318 for 5 fM, 2,239 for 2.5 fM, and 1,202 for 1.25 fM of peptides. Linear regression model analysis of the data clearly revealed a positive correlation for the chlamydial burden with peptide dose ( $r=0.3221$  and  $p=0.0426$ ; Fig. 2.4C), with the corollary that chlamydial elimination is significantly better at 1.25 fM than at 10 fM each peptide per mouse vaccine dose. These findings clearly established that ultralow doses of vaccine antigens, 1.25 fM or even lower peptide doses, are essential to induce a robust and protective immune response against chlamydial infection.

Although a significant level of chlamydial clearance was found in mice that received 1.25 fM peptides per vaccine dose, a clear bimodal response was also observed in

regard to chlamydial burden (Fig. 2.4C). Whereas four mice had marginal numbers of chlamydial lung genomes ( $\leq 100$  organisms/100 mg lung), five mice clustered at substantially higher chlamydial burdens ( $>10,000$



**Figure 2.4. Dose titration of synthetic peptides vaccine antigens to induce protection against *C. abortus* in A/J mice.** The A/J mice were vaccinated with either 1.25, 2.5, 5, or 10 femtoMoles of peptides along with 2% Polygen adjuvant (Fig. A-C) or with only 1.25 femtoMoles of peptides along with Montanide ISA 71 VG adjuvant (D), in a dual immunization approach as depicted in Fig. 2.3A. (A) Percentage of body weight change in vaccinated mice after 10 days of challenge. Mice in all four vaccine groups had similar and positive body weight changes (gained weight) and there was no dependence on peptide dose. (B) Percentage of lung weight increase over the average



lung weight of healthy adult female A/J mice 10 days after challenge. Most of the mice in all groups showed minimal or no lung weight increase and no dependence on peptide dose. (C) Lung chlamydial burdens 10 days after challenge. Chlamydial burdens were significantly lower as the peptide antigen doses decreased. (D) Lung chlamydial burden in mice that received 1.25 fM peptide vaccines with either Polygen or Montanide ISA 71 VG adjuvants. Low dose vaccination with the Polygen adjuvant cleared chlamydiae highly significant better than vaccination with w/o emulsion adjuvant Montanide ISA 71 VG. The horizontal lines in D indicates mean.

genomes/100 mg lung; Fig. 2.4D). We attributed this to unequal vaccine antigen exposure due to the difficulty to target such low amounts of dissolved peptides to antigen presenting cells. The block-copolymer adjuvant Polygen entraps the antigen in micelle in aqueous solution and thus also acts as a delivery vehicle (Table 2.1). But it cannot fully function as an adjuvant with particulate depot formation properties that slowly releases the antigen. We assumed that an adjuvant with such depot formation and hence particulate delivery properties would deliver and release antigens to APCs more consistently, and would stimulate immunity more uniformly, resulting in robust protection. Therefore, we included in this experiment also a group of mice that received 1.25 fM peptides with Montanide ISA 71 VG, a water in oil (W/O) emulsion Th1 adjuvant that produces  $\leq 1$   $\mu\text{m}$  droplets of PBS-dissolved peptide antigens and a mineral oil delivery vehicle. The A/J mice were vaccinated twice at 4 weeks interval with this vaccine. As shown in Fig. 2.4D, delivering the 1.25 femtoMoles peptides with Montanide ISA 71 VG yielded very uniform chlamydial loads in 8 out of 10 mice. However, all of the mice had a very high chlamydial loads of 6,165,950 *C. abortus* genomes per 100 mg lung, highly significantly higher than those that received the same amount of antigen with the Polygen adjuvant ( $p=0.00001$ ; Fig. 2.4D). This result suggest that a particulate delivery vehicle is essential to induce a uniform immune response, but that Montanide ISA 71 VG is not an appropriate adjuvant for this immunization approach.

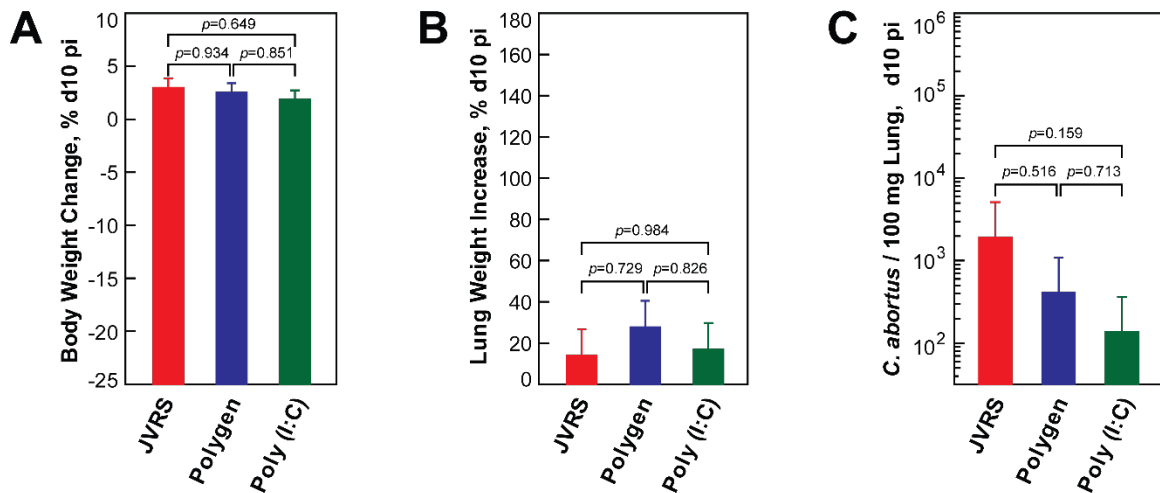
**Selection of an Optimal Adjuvant.** With the Polygen adjuvant we achieved significant protection against chlamydial infection by the low dose peptide immunization approach. However, we sought more robust and reproducible protection that resulted in

homogeneous immune protection of all vaccinated animals without bimodal response distribution. Therefore we also examined various other known Th1 adjuvants. To this end, we evaluated the immunostimulatory effect of three different Th1 adjuvants with low-dose immunization.

**Th1 adjuvants - Polygen, Poly (I:C), and JVRS-100™ showed similar and protective response in low dose vaccination against *C. abortus*.** In this experiment, we tested two known Th1 promoting adjuvant – JVRS and Poly (I:C) along with Polygen in the dual immunization approach with 1.25 fM peptide antigens. We included JVRS because this adjuvant has particulate delivery properties by way of virus-like enclosure of antigen in liposomes and a TLR9-stimulating non-coding plasmid with CpG sequences (Table 2.1), and has been reported to induce strong cellular immune responses (Chang *et al.*, 2009). Poly (I:C) resembles double-stranded RNA and consists of chains of hundreds of inosinic acids molecules complementary, but also mismatched, to chains of cytidylic acid molecules. It is not known to form specific particulate aggregates when mixed with antigens, but does promote a strong Th1 response via the TLR3 and MDA-5 (Melanoma Differentiation-Associated protein-5 (MDA5) signaling pathways (Martins *et al.*, 2015). Thus, this experiment allowed us to compare whether a micelle delivery like that of Polygen, or a virus-like particulate delivery like that of JVRS-100, or a strong Th1-promoting adjuvant with unknown delivery mechanism like poly(I:C) would make a difference in protection in this low-dose peptide immunization approach.

As shown in Fig. 2.5, 10 days after *C. abortus* challenge inoculation the mice in all three adjuvant groups had similar body weight increases as well as similar, low lung weight

increases, both indicative of protection with no significant differences among the three different adjuvant groups. In fact, mice in all groups gained body weights 5.58%, 5.11%, and 4.38% for JVRS, Polygen, and Poly (I:C), respectively (Fig. 2.5A). Similarly, the lung weight increase was minimal in all groups with 15%, 30% and 19%, respectively (Fig. 2.5B). Furthermore, very low and similar levels of chlamydial lung burden was observed in all three adjuvant groups. Although, JVRS-vaccinated mice had relatively higher chlamydial loads (5,381 genomes/100 mg lung) than the Polygen (1202 genomes) and Poly (I:C) (407 genomes) adjuvants groups, there was no significant difference between the groups (Fig. 2.5C).



**Figure 2.5. Comparison of Th1-promoting adjuvants in 1.25 fM low-dose *C. abortus* peptide vaccinations.** The A/J mice were vaccinated with twice with 1.25 fM peptides along with either JVRS-100, Polygen, or Poly (I:C) adjuvant (Fig. 2.3A). (A) Percentage of mean body weight change 10 days after *C. abortus* challenge. Mice in all vaccine groups had similar and positive body weight changes (gained weight) which was comparable to that observed in live-vaccine protected control mice (Fig 2.2B). (B) Percentage of lung weight increases over the average lung weight of healthy adult female A/J mice 10 days after challenge, indicative of lung inflammation. Mice in all vaccine groups had similarly minimal lung weight increase. (C) Lung chlamydial burdens. All

vaccine mice had similarly low chlamydial lung burdens. Data were analyzed by one way ANOVA, and the  $p$  value is determined by Tukey HSD test (n=10 mice/group).

## 2.5. DISCUSSION

Studies in different animal species with a variety of antigens have demonstrated that the dose of vaccine antigen is pivotal in determining the type of immunity induced. These studies indicate that low antigen doses initiate a cell-mediated response, and higher doses trigger antibody production (Hernandez-Pando *et al.*, 1997; Howard, 1986; Lagrange *et al.*, 1974; Parish, 1972; Power *et al.*, 1998; Salvin, 1958; Wortis *et al.*, 1966). We show here that vaccination of A/J mice with femtomole doses of *C. abortus* vaccine candidate recombinant proteins – DnaX2, GatA, and GatC, as well as their derived overlapping synthetic peptide antigens mediate significant protection following a respiratory chlamydial challenge, as determined by changes in body weight, lung weight and number of *Chlamydia* genomes determined in their lungs. To our knowledge, this is the first time that a vaccine formulated with such a low dose of vaccine antigens has been shown to be protective against a chlamydial infection.

To determine the protective efficacy of our low dose vaccine formulations, we chose the well-established murine model of chlamydial respiratory disease (Finco *et al.*, 2011; Huang *et al.*, 1999; Sun *et al.*, 2009; Williams *et al.*, 1981; Yu *et al.*, 2009). We used A/J mice because these mice allow maximum readout amplitude between disease protection versus susceptibility in the chlamydial lung disease model. Our challenge model study has clearly demonstrated that naïve A/J mice develop severe lung disease when intranasally challenged with a high *C. abortus* inoculum. However, if the mice have been previously infected with a lower dose, they develop protective immunity and show a high level of protection when challenged with a high dose of chlamydial EBs. This is in contrast to high inflammatory responder mouse strains (e.g., C57BL/6J, C3H/HeJ) that develop

enhanced disease after secondary challenge (Huang *et al.*, 2002; Min-Oo, 2008). The results of the three parameters that we used to determine protection, body and lung weights and number of chlamydiae in the lungs, showed good correlation in our model analysis experiment. When the mice developed disease (naïve mice) they lost body weight, the lung were inflamed and heavier, and high chlamydial burdens were detected in the lungs. Conversely, the protected mice (live-vaccine) gain body weight, showed minimal lung inflammation with little lung weight increase, and only small numbers of chlamydiae were detected in the lung.

In our initial vaccine experiment we first examined whether protein or peptide antigens make a difference in this low dose vaccination approach. The findings clearly demonstrated that vaccination with both protein or overlapping synthetic peptide antigens at 1 ng of per vaccine dose equally and substantially protect mice against chlamydial challenge. To our knowledge, this is an antigen dose that is orders of magnitude lower than those typically used for immunization. In fact, usual antigen dose that are used in chlamydial MOMP protein based vaccine development research in mice are 10 to 100 µg per vaccine dose (Badamchi-Zadeh *et al.*, 2016; Farris *et al.*, 2010; Tifrea *et al.*, 2014; Tifrea *et al.*, 2013; O'Meara *et al.*, 2013; Tu *et al.*, 2014; Zhang *et al.*, 2008) with few instances 3 or 7 µg (Tifrea *et al.*, 2014). However, in a recent influenza vaccine study, 40 ng of H1N1 influenza viral antigen was used in a direct deep pulmonary bronchoscope immunization approach in sheep and demonstrated the induction of a detectable serum antibody response (Wee *et al.*, 2008). Since a mouse of 20 g body weight weighs 1,500 times less than a young sheep of 30 kg, it requires a 240 times lower antigen dose when calculated by allometric scaling of the ratio of body weights by the power of 0.75 (West *et*

*al.*, 2002). Thus, the extremely low antigen dose in sheep suggests that a comparable mouse dose could actually be lower than the 1 ng dose that we used.

In our experiments, animals both in protein and peptide vaccine groups gained weight and showed minimal lung inflammation similar to the protected live vaccine groups. Also, chlamydial lung burdens were approximately 10-fold lower than those of naïve mice, but these differences were not significant. However, we observed improved chlamydial elimination at further lowered antigen dose in our subsequent dose titration experiment with *C. abortus* peptide antigens in the murine model (Fig. 2.4). Besides the importance of low antigen dose, the findings of this experiment also clearly demonstrated that we can use overlapping peptides as an alternative to whole recombinant proteins in a subunit vaccine. These finding has potential clinical implications since a fully protective chlamydial vaccine is not yet available, and a peptide vaccine will be highly preferable over a recombinant protein vaccine because of simpler and cheaper manufacturing.

Our most striking finding is the dose titration analysis of the peptide antigens. We performed an experiment with a log<sub>2</sub> peptide dilution series from 10 to 1.25 fM of each *C. abortus* peptide antigen per vaccine dose in a dual immunization approach. Although the complete peptide dose range resulted in protection from disease, interestingly the reduction of peptide dose per vaccine progressively associated with enhanced chlamydial elimination. While the vaccine containing 10 fM peptides failed to significantly clear the chlamydiae as compared to the naïve control, the 1.25 fM peptides vaccine did so highly significantly. Thus, maximal protection can be achieved by an ultralow dose of peptide antigens. Our 10-, 5-, 2.5-, and 1.25-fM of per vaccine peptide dose is equivalent to 1, 0.5, 0.25, and 0.125 ng of each 20-mer vaccine peptide.



Since protection against *Chlamydia* is only possible with a Th1 response, these findings clearly indicate that such a low dose of vaccine antigen is essential to induce a protective anti-chlamydial Th1 response. In fact, in the 1950s Salvin (Salvin, 1958) performed a similar dose titration experiment by immunization in guinea pigs. He used 30 ng diphtheria toxoid antigen as the lowest dose and observed only a DTH response with this dose, but antibody and immediate hypersensitivity responses at higher doses. Subsequently, Parish and Liew (1972) conducted a similar dose-dependent experiment in rats with bacterial flagellin and demonstrated that daily antigen administration as low as 10 fg/rat for 28 days induced a significant DTH, whereas only the larger doses of antigen (100 ng – 100 µg/rat for 28 days) produced detectable antibody. Studies with intracellular pathogens such as *Leishmania major* parasites (Menon *et al.*, 1998) and mycobacteria (Kiros *et al.*, 2010; Power *et al.*, 1988) showed that infection with low numbers elicited a potent and exclusive Th1 responses, without the production of detectable antibody, whereas infection with higher numbers generated a mixed response still dominated by Th1 cells, but containing also IL-4-producing CD4 Th2 cells, and production of IgG2a antibodies. These early experimental findings strongly suggest that the anti-chlamydial protection in our ultralow antigen dose immunization approach is mediated by a Th1-dominated DTH response.

Finally, we have demonstrated that low dose immunization approach is possible with different Th1 biased adjuvant such as Polygen, JVRS-100, Poly (I:C), and possibly many others. However, the findings also indicated that an appropriate adjuvant is mandatory to elicit a correct immune response. Therefore, we observed a fully unprotective

immune responses when we used Montanide ISA 71 VG as adjuvant with lowest antigen dose that induced significant protection with adjuvants that are mentioned.

In conclusion, this study indicates that 1) overlapping synthetic peptides of the protective vaccine candidate proteins mediate protection as efficiently as the corresponding whole proteins; 2) further reduced doses of the peptides as compared to the 1 ng protein dose mediate better immunoprotection by enhancing chlamydial elimination; and 3) different adjuvants mediate the induction of protective immunity against *C. abortus* in low antigen-dose vaccination.

**Consequences for clinical vaccine development:** The present data clearly demonstrate that overlapping 20-mer peptide antigens at amounts as low as 1fM in total combined with an appropriate Th1 biased adjuvant mediate essentially 100% protection as compared to live-vaccine controls. However, one of the challenges of such ultralow antigen dose immunization is to produce consistent and uniform protection. In fact, we observed a strong bimodal response, particularly for chlamydial elimination, even with the lowest antigen dose that showed a significant reduction of mean chlamydial burden as compared to the naïve control. This suggests that in some animals the Th1 immune response was not potent enough to effectively eliminate the infection. We assume, this might be due to suboptimal antigen targeting and presentation in this solution-based immunization approach. It is highly probable that most of the low-concentrated peptide antigens are further diluted such that they never associate with the MHC-II of antigen presenting cells and hence cannot induce an immune response. It is obvious that such a bimodal response is not desirable in developing a potent vaccine for clinical use. We

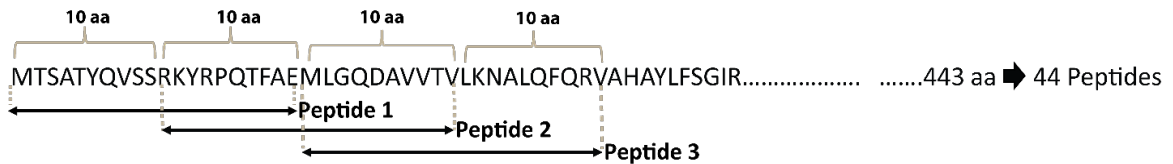
assume that particulate delivery of antigens can overcome this difficulty. In fact, we observed uniform, albeit increased, chlamydial burdens when we used Montanide ISA 72 VG, an adjuvant with microparticulate delivery properties (Fig. 2.4D). This finding suggests that a uniform and robust immune response is possible using an appropriate delivery vehicle. Micro- and nano-particle delivery and controlled release by antigen encapsulation in biodegradable poly-lactide-co-glycolide (PLG) polymers is now widely used in vaccine research. Such delivery systems not only potentiate the immune response of subunit vaccines but also reduce the number of immunization required. Therefore, in our subsequent vaccine studies we will attempt to combine the low antigen-dose immunization approach with a biodegradable polymer-based microparticulate delivery system.

## 2.6. MATERIALS METHODS

**Proteins and Peptides.** DNA polymerase III subunits gamma and tau (DnaX2), aspartyl/glutamyl-tRNA amidotransferase subunit A (GatA), and aspartyl/glutamyl-tRNA amidotransferase subunit C (GatC) of *Chlamydia* spp., which had been identified previously by Expression Library Immunization technique as the best vaccine candidate proteins, were used as protein antigens in this experiment (Stemke-Hale *et al.*, 2005). Large-scale protein production as recombinant antigen followed the protocol described by Li *et al.* (2010). Briefly, sequence-confirmed DnaX2, GatA, GatC genes were cloned into pEXP5-NT (Invitrogen, Carlsbad, CA), and expressed in *E.coli* strain BL21( $\lambda$ )DE3. IPTG-induced cells were harvested after 3–4 h by centrifugation and the resulting cell pellet lysed by resuspension in PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, and protease inhibitors (Roche Applied Sciences, Indianapolis, IN). Cell walls were permeabilized with 10 mg of lysozyme and 3 freeze/thaw cycles. The viscous lysate was cleared by incubation with DNase I and MgCl<sub>2</sub>, followed by centrifugation at 27,000×g for 10 min at 4°C, and the supernatant containing the soluble material was transferred to a fresh tube. The insoluble material, remaining in the pellet of the cleared lysate, was washed 4 times in PBS containing 1% Triton X-100 and 0.5 M guanidine followed by 3 washes with PBS. Cells were collected between washes by centrifugation at 3000×g for 5 min at room temperature. After the final PBS wash, the inclusion bodies were re-suspended in PBS. To solubilize the inclusion bodies, the pellets were re-suspended in PBS containing 8 M urea and 10% glycerol. Insoluble material was removed by centrifugation at 14,000×g for 5 min at room temperature, and the soluble

protein was collected in the supernatant and dialyzed against PBS, and quantified by NanoOrange protein quantification fluorescent assay (Molecular Probes, Eugene, OR).

Twenty amino acid long peptide antigens of *C. abortus* vaccine candidate proteins were commercially synthesized by Thinkpeptides<sup>®</sup>, Inc., Bradenton, FL. *C. abortus* DnaX2 (CAB0327) comprised 44 peptides, *C. abortus* GatA (CAB0286) 49 peptides, and *C. abortus* GatC (CAB0285) 9 peptides. Each of the peptides were 20 amino acids (aa) in length, with 10 aa overlaps between sequential peptides and spanning the entire consensus sequences of the 3 *C. abortus* proteins. For example DnaX2 has a molecular weight of 49,183 Daltons and is composed of 443 amino acids. DnaX2 was broken up into 44 peptides according to the approach described above and schematically shown in Fig. 2.6. To prevent potential *in vitro* polymerization, all peptides were synthesized with N-terminal and C-terminal amide and used as crude preparation with >70% purity.



**Figure 2.6.** Graphic illustration of first 3 peptides (20-mers, overlapping by 10 amino acids). Shown is the partial sequence of DNA polymerase III subunit gamma/tau (DnaX2) [*Chlamydia abortus* S26/3].

**Adjuvants.** The adjuvants used in this experiment were: Polygen (MVP Laboratories, INC., Omaha, NE, USA), poly(I:C) (Sigma Chem. Co., St. Louis, MO, USA), JVRS-100 (Juvaris, Burlingame, CA) and Montanide™ ISA 71 VG (Seppic Inc., Fairfield, NJ, USA).

***Chlamydia abortus.*** *C. abortus* strain B577 (ATCC V-656) was grown in Buffalo Green Monkey Kidney monolayer cell cultures, purified by differential centrifugation, and quantified as previously published (Li *et al.*, 2005). Purified infectious EBs were suspended in sucrose-phosphate-glutamate (SPG) buffer, stored in aliquots at  $-80^{\circ}\text{C}$ , and their infectivity was confirmed in female A/J mice.

**Preparation of Protein Vaccines.** The recombinant *C. abortus* proteins DnaX2, GatA, and GatC were dissolved in PBS at a concentration of 670  $\mu\text{g/ml}$ , 668  $\mu\text{g/ml}$ , and 495  $\mu\text{g/ml}$ , respectively and stored at  $-80^{\circ}\text{C}$ . Required volumes of proteins were collected, combined and further diluted in HBSS and mixed together with 2% Polygen to obtain 20 femtoMoles of each protein per 200  $\mu\text{l}$  mouse vaccine dose. This dose corresponded to  $\sim 1$  ng of DNAX2 and GatA, and  $\sim 0.25$  ng of GatC.

**Peptide Vaccines.** The peptides were collected in a deep-well 96-well polypropylene plate, and each peptide was dissolved in  $\sim 400$   $\mu\text{l}$  of dimethyl-sulfoxide (DMSO; Amresco, OH, USA) to create a  $10^{-6}$  M solution of each peptide, calculated from MW and mg yield of each peptide. For preparation of the  $10^{-8}$  M vaccine stock of each protein, 5  $\mu\text{l}$  of each peptide of a protein were pooled and the solution filled up to 500  $\mu\text{l}$

with DMSO. The plates were stored at  $-80^{\circ}\text{C}$ . Required volumes of combined peptides for each protein were further diluted in HBSS and mixed together with either 2% (v/v) Polygen, or 50  $\mu\text{g}$  poly (I:C), or 8  $\mu\text{g}$  JVRS-100, or Montanide ISA 71 VG (at 30% aqueous peptide solution) to obtain 10 fM, 5fM, 2.5 fM, or 1.25 fM of each of combined peptide of each protein in 200  $\mu\text{l}$  of per mouse vaccine dose. Since the average molecular weight of a 20-mer peptide is 2,200 Daltons, the average amount of the 10 femtoMoles of each peptide corresponds to 22 picograms (pg).

**Animal and Immunization.** Inbred female A/J mice were sourced from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age. Udel “shoebox” type cages with spun fiber filter tops were maintained in static air or ventilated cage racks. Five to ten animals were housed per cage in a temperature-controlled room on a 12-hour light/dark cycle, with ad libitum access to water and standard rodent chow. All animal experiments were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Each group was consisted of either 15 (controls) or 10 mice. Mice received the vaccines twice under light isoflurane inhalation anesthesia by subcutaneous injection of 200  $\mu\text{l}$  protein or peptide vaccine between the shoulder blades in a 4-week interval at 6 weeks and 10 weeks of age.

**Positive and Negative Controls.** Naïve, mock-vaccinated mice served as controls for a complete lack of protective immunity against *C. abortus*. These mice developed maximum disease and eliminated chlamydiae less efficiently than immunoprotected mice. Mice that received a low intranasal dose of  $10^6$  *C. abortus* bacteria (genomes) 4 weeks

before the high-dose challenge infection served as controls for protective immunity (live-vaccine controls).

**Intranasal *C. abortus* Challenge and Monitoring.** Mouse intranasal inoculation was performed as previously described (Huang *et al.*, 1999), and optimal doses for live immunization and challenge inocula were determined in preliminary experiments. All mice were challenged 4 weeks after the second vaccination under light isoflurane anesthesia intranasally with  $10^8$  *C. abortus* elementary bodies suspended in 20  $\mu$ l sucrose-phosphate-glutamate buffer. All animals were weighed during challenge infection and every second subsequent day until euthanasia on day 10 post challenge. Mice were monitored every day and death, if any, was recorded. Ten days after challenge, mice were sacrificed by CO<sub>2</sub> inhalation and weighed. Lungs were collected, weighed, snap frozen in liquid nitrogen, and stored at -80°C until further processing. Percent lung weight increase was based on naïve lung weights of 138.4 mg for adult female A/J mice. For mice that died before sacrificing on day 10, body weight losses, lung weight increases and chlamydial lung loads of the mouse in any group were taken as the highest of each of these parameters prior to death for the day 10 values.

**Mouse Lung Nucleic Acid Extraction.** Mouse lungs were homogenized in guanidinium isothiocyanate Triton X-100-based RNA/DNA stabilization reagent by shaking with a BeadRaptor device to create a 10% (wt/vol) tissue suspension. This suspension was used for total nucleic acid extraction by the High Pure® PCR template



preparation kit (Roche Applied Science, Indianapolis, IN) (DeGraves *et al.*, 2003; Wang *et al.*, 2004).

**Analysis of *C. abortus* Lung Loads by Quantitative PCR (qPCR).** The PCR primers and probes were custom synthesized by Operon, Alameda, CA. The *C. abortus* genomes copy number per lung was determined by *Chlamydia* genus-specific 23S rRNA FRET (fluorescence resonance energy transfer) qPCR (DeGraves *et al.*, 2003).

**Data Analysis.** All analyses were performed with the Statistica 7.1 software package (StatSoft, Tulsa, OK). Data of *C. abortus* genome copies were logarithmically transformed. Results were analyzed by Student's t-test, linear regression, and one-way ANOVA with Tukey's honest significant differences test for correction of the *p* value in multiple comparisons. *P* values  $\leq 0.05$  were considered significant.

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## CHAPTER 3

### MICROPARTICULATE DELIVERY FOR THE LOW DOSE ANTIGEN VACCINE PLATFORM

#### 3.1. INTRODUCTION

**What is Particulate Delivery?** Antigen presenting cells (APC) have evolved to engulf microorganisms. It is therefore possible that particles with dimensions that are similar to pathogens, ranging from viruses (20 – 100 nm) to bacteria and even cells (0.5 – 10 µm) act as adjuvant by direct targeting of the antigen to these cells (Bachmann and Jennings, 2010). The term ‘particulate delivery system’ denotes any strategy addressed to endow an antigen with dimensions of a microbe (Espuelas *et al.*, 2005).

**Advantages of Particulate Delivery of Antigens.** There are three important steps of immune mechanisms that are essential for a vaccine to be efficacious: targeting, activation and transfection/antigen presentation. A particulate delivery system facilitates the immune system to perform these mechanisms more efficiently than soluble antigens, and hence facilitates induction of a robust and stable immune response.

Before an antigen is up taken by an APC, it is subject to extensive dilution and vulnerable to its surrounding environment containing numerous enzymes that can easily degrade the antigen such that it completely loses its immunomodulating abilities. Therefore, the first benefit of particulate antigen delivery is the protection it provides the

Ag from dilution and premature degradation in biological environments (O'Hagan, 1989; Slütter, 2010). Once the antigen is present and stable within a host, it is very important that it is found and taken up by an APC. Herein lies another advantage of particle delivery, the tight control of active and passive targeting to APCs, and the enhanced uptake of the antigens by APCs (Tacke *et al.*, 2011). Regardless of the route of delivery, soluble antigens and adjuvants rarely reach the appropriate APCs; hence the resulting immune response is not potent enough to provide long term protection. Particles mimic the size and structure of a pathogen with charged, hydrophobic or receptor-interacting properties. Therefore, in contrast to small protein or peptide antigens in solution, particles are more efficiently taken up by APCs and induce robust and long lasting immune responses (Ahsan *et al.*, 2002; Bachmann and Jennings, 2010). Studies have suggested that macrophages present the antigens 100- to 1000-fold more efficiently to the MHC class I and II pathways when antigens are incorporated into degradable particles than when antigens are free in solution (Raychaudhuri and Rock, 1998). Additionally, particle-based antigen carriers may serve as a depot for controlled release of antigen and other molecules, thereby prolonging the availability of antigens to APCs. It has been reported that release of antigens over a long period may enhance the level as well as the quality of immune responses (Rice-Ficht *et al.*, 2010; Thomasin *et al.*, 1996). Furthermore, particles also facilitate the endosomal release of antigens after uptake, which is essential for antigen presentation and cross-presentation (Mui *et al.*, 2001; Hubbell *et al.*, 2009). Finally, particulate delivery system allow the co-delivery of antigen and adjuvants to the same cell. This enhances the probability that the desired response will be observed due to the discrete heightened

response on an individual cell basis (Leleux and Roy, 2012; Mallapragada and Narasimhan, 2008).

Particulate delivery systems also possess other desirable properties: they are typically safe, stable, and therefore the induced effects are highly reproducible. Besides, they can be administered by several routes which offer the possibility of developing both mucosal and systemic immune responses. Moreover, it has become increasingly evident that in order to elicit a Th1-dominant immune response, the antigen should be delivered to DC in a particulate form (Gamvrellis *et al.*, 2004; Waeckrle-Men and Groettrup, 2005; Couvreur and Vauthier, 2006). Collectively, these data re-emphasize the critical importance of particle vaccine delivery for a peptide-based subunit vaccine against chlamydial infection. They also clarify that a number of critical choices in the selection of vaccine carrier, adjuvant, and production methodology must be made to achieve optimal results.

**Different Types of Particulate Delivery Vehicles.** Based on their lipidic or polymeric composition, particulate delivery vehicles can be classified in two major groups (Singh and O'Hagan, 2002): lipid-based particles, such as liposomes, ISCOMs, and virosomes; and non-lipidic particles, e.g. emulsions, microparticles, and nanoparticles. Liposomes are small (30 nm - 10  $\mu$ m) artificial spherical vesicles that can be created from cholesterol and natural nontoxic phospholipids (Akbarzadeh *et al.*, 2013; Espuelas, 2005; Kersten and Crommelin, 2003). They are composed of one or more phospholipid bilayers enclosing an aqueous phase, where the polar head groups are oriented in the pathway of the interior and exterior aqueous phases. (Akbarzadeh *et al.*, 2013; Kersten and Crommelin,

2003). They are extensively used as carriers for numerous molecules in cosmetic and pharmaceutical industries.

Virosomes (50 nm - 10  $\mu$ m) are virus-like particles, composed of reconstituted viral envelopes including membrane lipids and viral spike glycoproteins, but devoid of viral genetic material (Akbarzadeh *et al.*, 2013; Huckriede *et al.*, 2005). They are generated from virus by a detergent solubilization and reconstitution procedure (Stegmann *et al.*, 1987; Bron *et al.*, 1993). Since they possess viral envelope glycoproteins, which stimulate humoral responses in their native conformation, they are highly effective as vaccine antigens and adjuvants (Huckriede *et al.*, 2005). Virosomes were first prepared by Almeida *et al.* (1975) by inserting purified influenza spike proteins into preformed liposomes. Subsequently a range of viral envelopes have been reconstituted, including those of Sendai virus (Bagai *et al.*, 1993; Uchida *et al.*, 1979), Semliki Forest virus (Helenius *et al.*, 1977 and 1981), vesicular stomatitis virus (Metsikkö *et al.*, 1986; Petri *et al.*, 1979) and Sindbis virus (Scheule, 1986).

Immunostimulating complexes (ISCOMs) are micellar assemblies that are formed by cholesterol, lipid, immunogen, and Quil A saponins from the bark of the tree *Quillaia saponaria* Molina (Pearse and Drane, 2005; Sjölander *et al.*, 1998). Iscom Matrix (also called ISCOMATRIX™) is an empty carrier, similar to ISCOM but without immunogen or protein (Sjölander *et al.*, 1998). Typically, both ISCOMs and Iscom Matrix exist as spherical, hollow, rigid, cage-like particles of about 40 nm in diameter with a strong negative charge (Sjölander *et al.*, 1998). However, they can also form rings and aggregates with properties essentially identical to the 40 nm particle.

Emulsions are fine dispersions of one liquid in another one (water-in-oil or oil-in-water) stabilized with emulsifiers. Some of the commonly used adjuvants, such as, Syntex adjuvant formulation (SAF), Freund's adjuvants, MF59, belong to this category (Espuelas, 2005). Initially they included adjuvants derived from mycobacterial muramyl dipeptide (MDP) or MDP analogues. However, their toxicity was unacceptable for human or veterinary use. MF59 is a squalene-in-water emulsion with nonionic surfactants – polysorbate 80 and sorbitan trioleate 85 – as emulsifiers, and is produced by a microfluidization technique that yields droplets of around 160 nm (Shah *et al.*, 2014; Podda *et al.*, 2006). It was originally developed as a vehicle for an MDP derivative – lapidated muramyl tripeptide (MPT-PE) (Wintsch *et al.*, 1991). However, studies demonstrated that MF59 emulsion alone was well tolerated and had immunogenicity comparable to the formulation containing MTP-PE, when administered with a recombinant envelop antigen from human immunodeficiency virus (Kahn *et al.*, 1994; Keffer *et al.*, 1996). After testing in several human clinical trials, it was successfully introduced onto the market in Europe in conjunction with an influenza vaccine.

In recent decades, polymers have been extensively used as biomaterials due to their favorable properties such as good biocompatibility, easy design and preparation, structural diversity and interesting bio-mimetic character (Bennet and Sanghyo Kim, 2014). Particularly in the field of drug delivery, polymers have played a significant role as they can deliver therapeutic agents directly into the intended site of action, with superior efficacy and release properties. There are various biological applications have been reported for nano-scale (10 to 1000 nm) to micro-scale (1 to 1000  $\mu\text{m}$ ) sized polymeric particles, such as site-targeted, controlled, and enhanced bioavailability of hydrophobic

drugs (Bennet and Sanghyo Kim, 2014; Kreuter, 1996; Panyam,*et al.*, 2003; Soppimath *et al.*, 2001). Moreover, polymeric particles proved their effectiveness in stabilizing and protecting the drug molecules such as proteins, peptides, or DNA molecules from various environmental degradation hazards (Kawashima, 2001; Kreuter, 1996; Panyam, *et al.*, 2003; Soppimath *et al.*, 2001; Cui *et al.*, 2002; Cohen *et al.*, 200). The potential of polymeric particles as vaccine delivery systems has also been widely recognized (Akagi, 2012; Rice-Ficht, *et al.*, 2010; Lin *et al.*, 2015; Yue and Ma, 2015).

**Potential of Synthetic Biodegradable Polymer-based Particles as Vaccine Delivery Vehicles.** In preparation of polymeric vaccine microparticles, biodegradable polymers are considered superior to non-degradable polymers as the latter may require additional removal procedures. They are natural or synthetic in origin and are degraded *in vivo*, either enzymatically or non-enzymatically, or both, to yield biocompatible, toxicologically safe by-products which are further eliminated from the body by normal metabolic pathways (Tian *et al.*, 2012). One of the most important features of biodegradable polymers is their unique degradation kinetics for which they are gaining exponential interest in the field of controlled delivery of active pharmaceutical ingredients (API: drugs, vaccine antigens, adjuvant, *etc.*). Such controlled API delivery is achieved by incorporating the API into the biodegradable polymeric particle, which allows the continual release of the agent as the polymer degrades (Edlund and Albertsson, 2002; Engineer *et al.*, 2011). The release kinetics of API from controlled delivery systems is governed by diffusion and/or erosion mechanisms (Ramtoola *et al.*, 1992; Parikh *et al.*, 1993). In non-erodible polymers, diffusion of the API results in elution kinetics that causes

an initial burst release of surface-embedded API followed by diffusion from the interior. In contrast, for biodegradable polymers both phenomena - diffusion and degradation - contribute to the API elution response. Therefore, release kinetics of API can be tailored precisely using biodegradable polymers (Engineer *et al.*, 2011). Because of reliability and reproducibility, synthetic biodegradable polymers (Table 3.1) are the best choice for antigen encapsulation in single-dose vaccine development (Lin *et al.*, 2015; Mao *et al.*, 2012).

Poly (glycolic acid) (PGA), poly (lactic acid) (PLA), and their copolymers are the most widely used synthetic biodegradable polymers in medicine. Among the co-polyesters investigated, extensive research has been performed in synthesizing a full range of poly (lactide-co-glycolide) (PLG) polymers. Different ratios of PLGs have been commercially developed and are being investigated for a wide range of biomedical applications (Azimi *et al.*, 2014; Nair and Laurencin, 2007). The major popularity of these biocompatible copolymers can be attributed in part due to their approval by the United States Food and Drug Administration (FDA) and the European Medicine Agency (EMA) in various drug delivery system in humans, resulting in their generally recognized as safe status (GRAS) (Chevalier *et al.*, 2015; Danhier *et al.*, 2012; Pillai and Sharma, 2001). They possess also good process ability which enables fabrication of a variety of structures and forms, their controllable degradation rates, and possibilities for sustained drug delivery (Azimi *et al.*, 2014; Makadia and Siegel, 2011). In the body, the PLG polymers undergo hydrolysis of their ester linkages in the presence of water to break down into the original monomers - lactic and glycolic acids (Gadad *et al.*, 2012). Lactic acid enters the tricarboxylic acid (TCA) cycle and is metabolized and subsequently eliminated from the body as carbon

dioxide and water. Glycolic acid is either excreted unchanged in the kidney or it enters the TCA cycle and is eventually eliminated as carbon dioxide and water (Gadad *et al.*, 2012; Makadia and Siegel, 2011). The potential of PL, PG, and PLG as vaccine delivery has been successfully tested with multiple antigens for generating both humoral as well as cellular responses (Johansen *et al.*, 2000; O'Hagan and Singh, 2003).



**Table 3.1. List of biodegradable polymers commonly used in drug delivery.**

Name of the Polymer	Chemical Structure
Poly(lactide) (PL)	$\left[ \begin{array}{c} \text{CH}_3 \quad \text{O} \\   \quad    \\ \text{CH} - \text{C} - \text{O} \end{array} \right]_n$
Poly(glycolide) (PG)	$\left[ \begin{array}{c} \text{O} \\    \\ \text{CH}_2 - \text{C} - \text{O} \end{array} \right]_n$
Poly(D,L-lactide-co-glycolide) (PLG)	$\left[ \begin{array}{c} \text{O} \\    \\ \text{O} - \text{CH}_2 - \text{C} \end{array} \right]_m \left[ \begin{array}{c} \text{CH}_3 \quad \text{O} \\   \quad    \\ \text{O} - \text{CH} - \text{C} \end{array} \right]_n$
Methoxypoly(ethylene glycol)-block-PLG (mPEG-PLG)	$\text{H}_3\text{C} \left[ \text{O} - \text{CH}_2 - \text{CH}_2 \right]_m \text{O} \left[ \begin{array}{c} \text{O} \\    \\ \text{C} - \text{CH} - \text{O} \\   \\ \text{CH}_3 \end{array} \right]_x \left[ \begin{array}{c} \text{O} \\    \\ \text{C} - \text{CH}_2 - \text{O} \end{array} \right]_y \text{H}$
Polycarbonate	$\left[ \text{O} - \text{C}_6\text{H}_4 - \text{C} \begin{array}{c} \text{CH}_3 \\   \\ \text{CH}_3 \end{array} - \text{C}_6\text{H}_4 - \text{O} - \text{C} \begin{array}{c} \text{O} \\    \end{array} \right]_n$
Polyanhydrides	$\left[ \begin{array}{c} \text{O} \quad \text{O} \\    \quad    \\ \text{C} \quad \text{C} \\   \quad   \\ \text{R} \quad \text{O} \end{array} \right]_n$
Poly(caprolactone) (PCL)	$\left[ \text{O} - \left( \text{CH}_2 \right)_5 - \text{C} \begin{array}{c} \text{O} \\    \end{array} \right]_n$
Poly(ortho esters) IV	$\left[ \begin{array}{c} \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \\   \quad   \quad   \quad   \\ \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \\   \quad   \quad   \quad   \\ \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \end{array} \right]_n \left( \begin{array}{c} \text{CH}_3 \quad \text{O} \\   \quad    \\ \text{CH} - \text{C} - \text{O} \end{array} \right)_{n=1-6} \text{R}' - \text{O} - \begin{array}{c} \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \\   \quad   \quad   \quad   \\ \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \\   \quad   \quad   \quad   \\ \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \end{array} - \text{O} - \text{R} \right]_n$
Poly(phosphoesters) (PPE)	$\left[ \begin{array}{c} \text{O} \\    \\ \text{P} - \text{O} - \text{R} - \text{O} \\   \\ \text{OR}' \end{array} \right]_n$
Polyhydroxybutyrate	$\left[ \text{O} - \begin{array}{c} \text{CH}_3 \\   \\ \text{CH} \end{array} - \text{CH}_2 - \text{C} \begin{array}{c} \text{O} \\    \end{array} \right]_n$

**Size of the Polymeric Particles.** Based on the size, polymeric particulate delivery can be classified into nano- and micro-particles. However, the dividing line between nanoparticles and microparticles is not well defined and many investigators have used the terms ‘nanoparticles’ and ‘microparticles’ interchangeably in the literature to describe various particles that have been used as vaccine adjuvants (Jung *et al.*, 2001; Sharp *et al.*, 2008; Tabata *et al.*, 1996). Although, the United States patent office has the class definition for nanotechnology using the scale 1–100 nm or slightly larger, some sources considering 1000 nm particles to be nanoparticles (Quintanar-Guerrero *et al.*, 1998; Wendorf *et al.*, 2008). Thus, theoretically, nanoparticles are solid particles ranging in size from 1 to 1000 nm while microparticles are particles in the size range of 1 to 1000  $\mu\text{m}$  (Kreuter, 1996; Oyewumi *et al.*, 2010).

*Nanoparticle versus microparticle effect on the immune response.* Available data from studies evaluating the effect of particle size on the immune response offer conflicting outcomes, depending on the specific system evaluated. After mucosal administration there are findings that show higher antibody responses for nanoparticles compared to microparticles (Jung *et al.*, 2001; Nagamoto *et al.*, 2004), however, the reverse has also been demonstrated (Gutierrez *et al.*, 2002), and yet in another study nanoparticles and microparticles were comparable (Vila *et al.*, 2004). The studies with systemic administration also have inconsistent findings. It has been demonstrated that nanoparticles and microparticles are comparable (Katare *et al.*, 2003; Nagamoto *et al.*, 2004; Wendorf *et al.*, 2008), that microparticles are preferable to nanoparticles (Gutierrez *et al.*, 2002; Katare *et al.*, 2005; Katare *et al.*, 2006), and that nanoparticles are preferable to microparticles (Nixon *et al.*, 1996; Fifi *et al.*, 2004). The type of immune response with nanoparticles

compared to microparticles may also be different, in one study it was shown that PLG microparticles favored Th1 type responses, while nanoparticles triggered more Th2 type cells (Conway *et al.*, 2001). However, the numerous differences in other parameters related to microparticles, such as polymer type, uniformity in size, antigen encapsulation efficiency, preparation techniques, etc., may also account for the lack of consistency in the literature. In our low dose immunization approach we were interested to use an optimal sized synthetic polymeric microparticulate delivery system to induce Th1 response against *Chlamydia*.

*Optimal size of microparticles for vaccine delivery.* Size is considered to be one of the crucial parameter affecting the immunogenicity of microparticles, since smaller particles (<10  $\mu\text{m}$ ) has been found significantly more immunogenic than larger ones (Eldridge *et al.*, 1991; O'Hagan *et al.*, 1993). When PLG particles of 1–10  $\mu\text{m}$  diameter (mean of 3.5  $\mu\text{m}$ ) were compared to 10–110  $\mu\text{m}$  particles (mean of 54.5  $\mu\text{m}$ ) with encapsulated staphylococcal enterotoxin B, the generation of serum IgG antitoxin response was more rapid and substantially more vigorous with the smaller particles (Eldridge *et al.*, 1991). Similarly, with ovalbumin (OVA) entrapped in PL particles, an increased serum anti-OVA antibody titer was observed with particles <5  $\mu\text{m}$  compared to particles with mean sizes larger than 5  $\mu\text{m}$  (Nakaoka *et al.*, 1996). The effect of particle size on immunogenicity is likely to be a consequence of enhanced uptake of smaller-sized particles into lymphatics and greater uptake into APC. An earlier study demonstrated that only microspheres <5  $\mu\text{m}$  were transported to the spleen after oral administration in mice (Tabata *et al.*, 1996). Recently, it has been demonstrated that macrophages effectively engulf microparticles, especially in the 2 – 3  $\mu\text{m}$  range, the curvature of which corresponds

with that of the macrophage's membrane ruffles (Champion *et al.*, 2008; Pacheco *et al.*, 2013). Thus, microparticles with a mean size of less than 5 microns is essential for an optimal immune response.

**Synthesis of Polymeric Microparticles – Factors to be considered.** A complicated array of factors including type of polymers and preparation technique directly influence the morphology of microparticles, and subsequently their *in vivo* degradation kinetics, antigen release, and immune response that they elicit. Therefore, a detailed understanding of these parameters is essential while developing such a microparticulate vaccine delivery vehicle.

*Physio-chemical properties of polymers.* The release of antigens from the microparticulate biodegradable delivery vehicle depends primarily on the degradation and erosion kinetics of the polymers used in preparation of the particles, but also on the intrinsic diffusion of the API in and from the polymer. All parameters driving release, i.e. degradation, erosion of, and diffusion from, the polymers rely mostly on their inherent physiochemical properties such as molecular weight, crystallinity, glass transition, and copolymer composition (Kamaly *et al.*, 2016; Kim and Pack, 2006). Therefore, selection of appropriate polymer is critical for the production of a targeted microparticulate vaccine delivery system.

*Polymer degradation and erosion mechanism.* All biodegradable polymers possess hydrolysable bonds that make them prone to degradation by chemical-mediated or enzyme-catalyzed hydrolysis. Enzymatic degradation is not known to occur in polymers of lactide/glycolide family (Pitt *et al.*, 1981; Therin *et al.*, 1992). The hydrolytic bond

cleavage generates acidic degradation products that can dissolve in an aqueous environment (Griffith, 2000; Merkli *et al.*, 1998). Thus, whereas degradation is a chemical event, erosion encompasses physical phenomena, such as dissolution and diffusion (Engineer *et al.*, 2011). However, polymer erosion is a more complex phenomenon than degradation, since it depends on many other processes, such as degradation, swelling, dissolution and diffusion of oligomers and monomers, and morphological changes (Engineer *et al.*, 2011).

Based on the erosion mechanism, polymers can be broadly classified into two types: bulk eroding and surface eroding (Burkersroda *et al.*, 2002; Kumar *et al.*, 2002; Tamada and Langer, 1993). Bulk-eroding polymers, such as PLG, PG, PL, PCL, allow permeation of water into the polymer matrix and degrade throughout the particle's matrix. Particles consisting of bulk-eroding polymer are often characterized by four stages of the polymer erosion process (Engineer *et al.*, 201; O'Donnell and McGinity, 1997 ): the first stage of water diffusion is followed by the second stage, in which oligomers with acidic end-groups autocatalyze the hydrolysis reaction. At the beginning of the third stage, when a critical reduced molecular weight is reached, oligomers start to diffuse out from the polymer. Consequently, water molecules diffuse into the void created by the removal of the oligomers, which in turn accelerates oligomer diffusion. Marked decrease in polymer mass and a sharp increase in the API release rate occur during the third stage as the API diffuses from the porous regions. In the fourth stage, the polymeric matrix become highly porous, and degradation proceeds homogeneously and more slowly until the complete breakdown of the biodegradable particle (Proikakis and Mamouzelos, 2006; Agrawal *et al.*, 1992).

The release of the API is in turn driven by the polymer erosion kinetics. Initially, a “burst” diffusion of the surface-embedded active pharmaceutical ingredient (API) occurs. As much as 50% of the total API load (O’Donnell and McGinity, 1997) is released within the first few hours after suspension of biodegradable microparticles in aqueous medium. This is followed by a slow continuous release that is controlled by the enhanced diffusion enabled by polymer matrix erosion. Finally, sometimes in a third phase the remaining API is quickly released as a result of severe degradation and complete breakdown of the polymer matrix into soluble oligomers (Kim and Pack, 2006).

In contrast, surface-eroding polymers, such as poly (ortho) esters and polyanhydrides, are composed of relatively hydrophobic monomers linked by labile bonds. Thus they can resist water penetration into the polymer bulk, while degrading quickly into oligomers and monomers at the polymer/water interface via hydrolysis (Saltzman, 2001). For polymeric particles comprised of surface-eroding polymers, embedded APIs are released primarily at the surface as the polymer breaks down around it. Erosion of such polymers usually proceeds at a constant velocity (Göpferich and Langer, 1993). If the drug of interest is homogeneously dispersed throughout a microsphere, the largest rate of release will occur at the beginning. As time proceeds, the surface area of the sphere and the release rate decrease asymptotically.

*Polymer molecular weight.* The physical properties of polymers, such as viscosity in solution, solubility, crystallinity, mechanical strength, and degradation rate, are associated with its molecular weight (MW) (Kamaly *et al.*, 2016). Therefore, the MW of degradable polymers has a notable impact on the antigen release profile from polymeric nano or micro particles and can influence the biological properties of polymeric vaccine

delivery systems, such as elimination, phagocytosis, and other biological activities (Knopp *et al.*, 2015; Valencia *et al.*, 2013; Kim and Fassihi, 2000). Usually, low-MW polymers degrade more rapidly and therefore release APIs more quickly, while high-MW polymers have slower degradation and antigen release rates (Kamaly *et al.*, 2016; Kim and Fassihi, 1997; Green *et al.*, 2009). Moreover, high-MW polymers have a low elastic modulus, generating a relatively non-deformable matrix that limits the amount of pore-forming channels. In contrast, low-MW polymers have a high elastic modulus, and the matrix is more deformable that results in expansion of pores due to osmotic pressure (Kamaly *et al.*, 2016). For example, it has been demonstrated that low-MW 50:50 hydrophilic PLG (MW 8.6 kD; Resomer® RG502H) encapsulating leuprolide acetate resulted in porous microspheres that burst-released ~50% of the peptide within 3 days, followed by continuous slow release of the remaining peptide over the next 30 days (Ravivarapu *et al.*, 2000). In contrast, the higher-molecular-weight formulation (MW 28.3 kD; Resomer® RG503H) resulted in dense microspheres and produced only ~5% burst release followed by gradually accelerating erosion/degradation release of the remaining peptide over 50 days.

It should be noted that commercially available polymers are usually characterized in terms of intrinsic viscosity (IV), a measure of a polymer's ability to increase the viscosity of a solvent, which is directly related to their molecular weight ( $M$ ) (Burn, 2001; Lu *et al.*, 2013; Makadia and Siegel, 2011). In short, IV is a widely used, simple viscometric method for measuring a polymer's molecular weight  $M$ , and based on the flow time of a polymer solution through a narrow capillary relative to the flow time of the pure solvent through

the capillary, and is expressed in deciliters per gram (dL/g) (Burn, 2001; Charlier *et al.*, 2014).

Polymer crystallinity. The terms crystalline and amorphous are used to indicate the ordered and unordered polymer regions, respectively (Odian, 1991). Polymer crystallinity refers to the degree of crystalline regions within a polymer sample in relation to amorphous regions and is an important concept in drug or vaccine antigen delivery, since only amorphous regions are permeable and therefore accessible to water molecules (Kamaly *et al.*, 2016; Ordian, 1991). The mechanical strength, swelling, hydrolytic, and biodegradation rates of polymers depend on the degree of crystallinity, which in turn is governed by the nature of the monomers. A high degree of crystallinity leads to relatively slower antigen release states, particularly for low MW polymers with high porosity (Kamaly *et al.*, 2016). For effective design of degradable polymers in vaccine delivery applications, a balance between amorphous and crystalline states is necessary. Therefore, polymeric particles are usually synthesized from copolymers that possess both hydrophobic and hydrophilic segments, which make physical properties such as antigen-release rates more predictable (Kaushal *et al.*, 2004).

Due to the asymmetric  $\alpha$ -carbon, poly lactide (PL) can be described as possessing D- or L- stereochemical centers, giving rise to two enantiomeric forms PDL and PLL, with PLG being generally described as poly(D,L-lactic-co-glycolide) with an equal distribution D- and L-lactic acid. PLL is highly crystalline and PDL is fully amorphous owing to disordered polymer chains. In contrast, PG is highly crystalline as it lacks asymmetric methyl groups on the side chain (Kamaly *et al.*, 2016). In the case of PLG (copolymerization of PL and PG), the degree of crystallinity and amorphousness depends



on the ratio of the monomers; e.g., a 50:50 ratio of lactide to glycolide results in an amorphous polymer, with increases in the lactic acid content leading to a more crystalline polymer (Müller *et al.*, 2014). Studies have shown that the rate of drug release is higher in polyesters with a low degree of crystallinity because of higher macromolecular chain mobility (Karavelidis *et al.*, 2011; Zilberman, 2005).

Polymer glass transition. Another important factor that influences polymer physicochemical properties and is related to crystallinity is the glass transition temperature (T<sub>g</sub>). The type of polymer and temperature dictate whether the amorphous region is in a “glasslike” or “rubberlike” state, and this depends on the polymer’s T<sub>g</sub>. In short, T<sub>g</sub> is the temperature range where a thermosetting polymer changes from a hard, rigid or “glassy” state to a more pliable, compliant or “rubbery” state. T<sub>g</sub> can be determined by differential scanning calorimetry (Allcock and Lampe, 1981). Below the T<sub>g</sub>, the polymer exists in a glassy state and thus it has limited mobility and low diffusion rates. In contrast, above the T<sub>g</sub>, the polymer exists in a rubbery state, which enables higher mass transfer rates of water and antigens or drug molecules throughout the matrix (Kamaly *et al.*, 2016; Liechty *et al.*, 2010). The T<sub>g</sub> of the PLG copolymers are reported to be above the physiological temperature of 37°C and therefore are glassy in nature (Makadia and Siegel, 2011). It has been also reported that the T<sub>g</sub> of PLG polymers decreases with a reduction of lactide content and with a lower molecular weight (Passerini and Craig, 2001).

Co-monomer ratio. The ratios of co-monomer in many copolymers can also affect release rates. Most often, increasing the content of the more rapidly degrading or more soluble monomer enhance the release rate (Kim and Pack, 2006; Lin *et al.*, 2000; Spenlehaue *et al.*, 1989). For example, increasing the glycolic acid portion of PLG, which

makes the polymer more hydrophilic, can lead to faster degradation rates, and thus to faster release of API (Lu *et al.*, 2000; Park, 1995). This also suggest that solubility of the monomer is a critical factor in the rate of antigen or drug release from polymeric particles.

**Technologies to Synthesize Microparticles.** Microencapsulation of vaccine antigens in synthetic biodegradable polymers is commonly achieved by several approaches: i) emulsion-solvent evaporation which includes a water-in-oil-in-water (w/o/w) double emulsion method, and a single o/w emulsion method (Chang and Gupta, 1996; Jiang and Schwendeman, 2008); ii) phase separation or coacervation (Johansen *et al.*, 1999 and 2000); and iii) spray drying (Johansen *et al.*, 1999; Makadia and Siegel, 2011; Murillo *et al.*, 2002). Problems encountered with emulsion-solvent evaporation and coacervation formulation processes include the use of high shearing forces, risk of significant degradation of vaccine antigens due to the long exposure to the interfaces existing between the water and oil phases, and the necessity of lyophilization to obtain a stable powder (Baras *et al.*, 2000; Desai *et al.*, 2013; Giteau *et al.*, 2008; Zhu *et al.*, 2000). During the last decades a number of efforts have been made to overcome these problems, such as co-incorporation of stabilizing excipients with the biopharmaceuticals in the inner water phase to minimize the interfacial effects (Jiang and Schwendeman, 2008; Katare *et al.*, 2006). However, these traditional techniques are still facing a number of challenges, particularly associated with industrial large-scale production (Desai *et al.*, 2013).

Compared with those conventional laboratory-scale techniques, emulsion-solvent evaporation and coacervation, spray drying is a well-established industrial processing technology and is a fast, single step process with a potential for operation in a continuous

mode. It is a widely used technical method to produce fine particles, coarse powders, agglomerates or granulates in various industries, including the pharmaceutical industry (Yiang, 2011; Vehring, 2008). It has been commonly used to manufacture sophisticated and functionalized microparticles for delivery of API (Tsapis *et al.*, 2002; Wan *et al.*, 2013; White *et al.*, 2005). All spray-dryers include a nozzle for spraying by compressed gas that atomizes the feed fluid, the desiccation chamber (spraying cylinder), a fan and heater for the drying gas (air, nitrogen), the cyclone (for the separation between the product and the drying gas flow), and a final vessel which collects the spray-dried product (Baras *et al.*, 2000; Okuyama *et al.*, 2006; Patel *et al.*, 2009). This technique is attractive for the preparation of vaccine microparticles and it appears to come close to the properties desired: simple, reproducible, rapid and easy to scale-up (Bodmeier and Chen, 1988). Another advantage of the spray drying technique is its ability to control the particle size and the morphology of the dried powder by varying the process parameters and the formulation factors (Haggag and Faheem, 2015).

*Spray drying procedure.* Spray drying consists of spraying an emulsion of polymer and API through the nozzle of a spray dryer apparatus. The mechanism of spray-drying involves five fundamental sequential stages: 1) Feedstock suspension/solution; 2) atomization of the feedstock by a spray nozzle; 3) spray – heated drying gas (air) contact; 4) drying of the sprayed droplets; 5) collection of the solid product (spray-dried microparticles) (Baras *et al.*, 2000; Broadhead *et al.*, 1992; Patel *et al.*, 2009). Generally, the API is dissolved or dispersed in the polymer solution, for which volatile solvents (e.g., dichloromethane and acetone) are usually preferred. In order to generate droplets, the feed solution or suspension can be atomized by rotary disks, two-fluid nozzles, or ultrasonic

nebulizers, depending on the droplet size required (Okuyama *et al.*, 2006). The droplets are sprayed into a drying chamber into which gas heated to a temperature above the vaporization temperature of the solvent streams; the solvent evaporates very quickly upon contact with the drying gas, leaving solid microparticles behind (Baras *et al.*, 2000). The powder of microparticles can be collected using a cyclone, filter bag or electric field precipitator (Okuyama *et al.*, 2006). The size of the atomized droplets depends on the viscosity and surface tension of the liquid, the pressure drop across the nozzle, and the spray velocity (Zuidam and Shimoni, 2010). The morphological features of spray-dried microparticles, like size and shape, are controlled by both spray the drying process and formulation parameters such as inlet and outlet temperatures, spray rate of feedstock, spraying gas concentration, nature of organic solvent, and API-polymer ratio of the polymeric feed solution (Baras *et al.*, 2000; Giunchedi and Conte, 1995; Patel *et al.*, 2011). Therefore, optimization of these parameters is essential to produce vaccine microparticles with the desired physical characteristics.

*Stability of polymer and API in spray drying.* Although there is always a concern for stability of peptide and protein molecules in spray drying, many reports have shown that thermal denaturation of peptides and proteins is usually not observed during the spray drying process (Maltesen *et al.*, 2008; White *et al.*, 2005; Yang *et al.*, 2007), and the atomization shear stress does not appear to influence stability (Maa and Hsu, 1996; Maa and Prestrelski, 2000). During the process, the spray temperature is usually not very high and, more importantly, the residence time of the droplet/particles is very short, and thus after spraying the inherent properties of the particles do not change appreciably. For example, when water is used as the dispersing medium, around 150°C of an operating

temperature is sufficient for completely drying of the droplets within a few seconds (Okuyama *et al.*, 2006). Moreover, although the air temperature for drying gas can be relatively high, the actual temperature of the evaporating droplets is significantly lower, owing to the cooling effect of the evaporating solvent (Haggag and Faheem, 2015; Okuyama *et al.*, 2006). During spraying, the evaporated moisture forms a skin around the droplets, which absorbs most of the heat. Therefore, the mean temperature of the droplet remains 15–20°C lower than the temperature of the surrounding environment (Broadhead *et al.*, 1995). To date, there is no vaccine produced by spray drying available in the market. However, numerous studies have shown that spray drying is a promising method for stabilizing vaccines.

#### **Co-delivery of an Immune Potentiator by Polymeric Microparticle Delivery:**

The particulate delivery system (e.g. polymeric micro/nano particle) mainly functions as an adjuvant by delivering antigen into APC and improve / regulate antigen presentation by APC (Foged, 2011). Although microparticles by themselves show excellent adjuvant activity (Moretti and Blander, 2014), sometimes such a delivery system alone is not sufficient enough to induce a desired immune response, which is particularly true for subunit vaccines (Zhang *et al.*, 2015). However, another type of adjuvant, termed immunopotentiator, activates innate immunity directly (e.g. cytokines) or through stimulation of pattern recognition receptors (PRRs) (bacterial components such as peptidoglycan, LPS) (Coffman *et al.*, 2010; Foged, 2011; Kornbluth *et al.*, 2006; Mohan *et al.*, 2013; Sahdev *et al.*, 2014). To achieve better efficacy in accelerating development of a vaccine-induced immune response in a subunit vaccine, an immunopotentiator is

usually incorporated into polymeric microparticles. Such incorporation can not only overcome the drawbacks associated with the use of a free immunopotentiator, such as high dose requirements and unwanted side effects, but also enhance its efficacy by co-targeting antigen and adjuvant to the same APC (Bramwell and Perrie, 2006). Therefore, the combination of an antigen, a delivery system and an immunopotentiator constitutes a paradigm for the development of subunit vaccines (Guy, 2007). In the present study, we investigated the immune-stimulating ability of a polymeric particulate vehicle encapsulating different adjuvants, without antigen, as biological response modifiers (BRM) that tested the reversal of the susceptibility of C3H/HeJ mice to *Chlamydia abortus* respiratory challenge.

**What is Biological Response Modifier?** Biotherapy, often termed as biological therapy or immunotherapy, aims at supporting and helping in the treatment of diseases without chemical drugs and invasive therapies, by restoring the body's natural immune system (Bodey, 2002). It is also used to minimize certain side-effects that may be caused by some treatments against cancer, autoimmune diseases, or other diseases. The substances that are used in biotherapies are called biological response modifiers (BRMs). Thus, BRMs, a term first coined in 1982, are referred to as biologics, which connotes an agent and treatment approach whose perceived action involves the modification of an individual's own biological response (Boyle, 2010; Oldham, 1983; Rusthoven, 1993). They modulate the immune system by stimulating or replacing the function of one or more of the system's components, and hence the term BRM is often used synonymously with the terms immunomodulator and immunostimulant (Ritts, 1990). BRMs used in

biotherapy are usually classified into two groups (Kuroki *et al.*, 2012; Smit, 2009). The first group includes specific BRMs, such as vaccines and monoclonal antibodies that provide an antigen-specific immune response, and may exhibit a direct antitumor effect. The second group consists of non-specific BRMs, such as cytokines and adjuvants, which augment or stimulate the immune system without antigenic specificity. Some examples of non-specific BRMs are recombinant form of interferons, interleukins, colony stimulating factors, or traditional Chinese medicine (Rusthoven, 1993). BRM are extensively used in the treatment of cancer and also against certain other diseases, e.g., rheumatoid arthritis and Crohn's disease (Kuroki *et al.*, 2012). Since entrapping a molecular adjuvant in particulate delivery has the potential to enhance the immunomodulating effect of a particulate vaccine matrix, we sought to identify the adjuvant that would modulate the response to chlamydial infection towards enhanced protection. By identifying a potential vaccine adjuvant this way, we assumed that such innate adjuvant/matrix protection would also induce appropriate immunity against specific antigens later incorporated into this vaccine carrier platform.

**Why C3H/HeJ Mice?** C3H/HeJ mice possess a missense mutation in the TLR4 gene which results a single amino acid change in the cytoplasmic portion of TLR4, hindering signal transduction and leading to a phenotype similar to that of TLR4 knockout mice (Hoshino *et al.*, 1999; Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). TLR4 is activated by gram-negative bacterial lipopolysaccharide (LPS), and its ligation is essential in activating DCs toward the initiation of Th1-type or Th17 responses (Wynn, 2005). Conversely, absence of TLR4 stimulation during antigen presentation causes

disproportionate expansion of regulatory T lymphocytes (Treg) and subsequent suppression of immunity (Pasare and Medzhitov, 2003 and 2004). Therefore, C3H/HeJ mice are highly susceptible to Gram-negative bacterial infections including *Escherichia coli* and *Salmonella* spp. for which LPS is the principal trigger of the innate immune response (Nowicki *et al.*, 1999; O'Brien *et al.*, 1980). Similarly, C3H/HeJ mice are also highly susceptible to *C. trachomatis* infection (Morrison *et al.*, 2010).



### 3.2. HYPOTHESIS

We hypothesize that

- (i) *Chlamydia abortus* respiratory challenge inoculation of naïve C3H/HeJ mice will result in severe, potentially lethal disease, making such inoculation an appropriate model for evaluating protective biological response modifier effects;
- (ii) microparticles composed of biodegradable poly-lactide-co-glycolide (PLG) polymers and an adjuvant can be synthesized by spray drying technology; and
- (iii) microparticles with PLG polymer/adjuvant combination that results in optimally protective BRM effects can be identified which then can serve as a carrier platform for a low-dose peptide vaccine against *C. abortus*.

### 3.3. OBJECTIVES

The aim of this investigation was to synthesize by spray drying a PLG-based microparticle delivery platform that incorporated an adjuvant, and to examine its immunopotentiating effect against the *C. abortus* respiratory infection in C3H/HeJ mice.

The specific objectives were as follows:

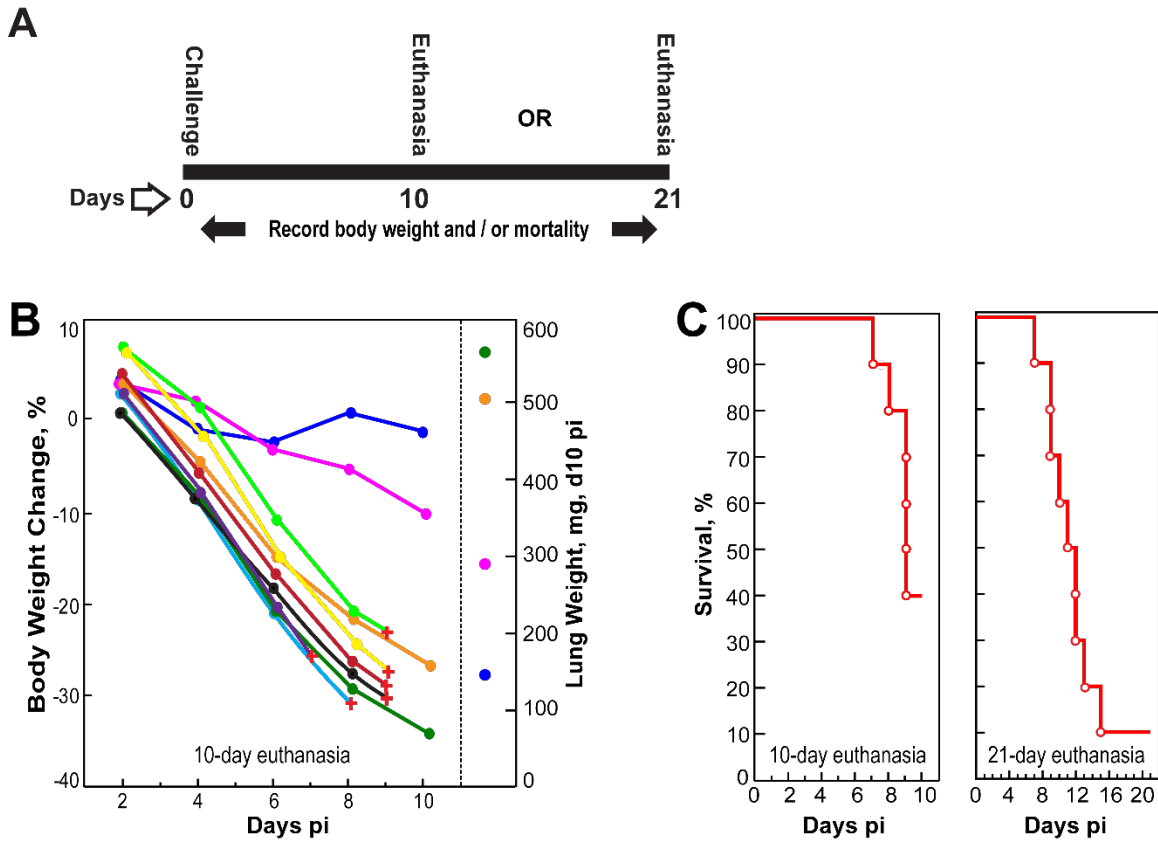
1. Establish a C3H/HeJ/*C. abortus* challenge model for testing of BRM effects of the particulate vaccine carrier.
2. Optimize spray drying parameters and synthesis of microparticles based on a suitable PLG polymer.
3. Identify an optimal dose ratio of polymer versus adjuvant for optimal BRM microparticles.
4. Analyze suitable microparticles compositions for morphology, degradation and adjuvant release kinetics, and *in vivo* uptake by APC.
5. Examine the BRM effect of optimized microparticles in the C3H/HeJ/*C. abortus* challenge model.

The optimal BRM microparticle formulation would then be used as delivery platform for the low-dose peptide antigen immunization approach.

### 3.4 RESULTS

**C3H/HeJ mice as the BRM model of *C. abortus* respiratory disease.** In this experiment, we examined the outcome of the *C. abortus* lung challenge model in the C3H/HeJ mouse strain in comparison to the A/J mouse model with the lung disease readout on day 10 post inoculation. In first experiment, the naïve C3H/HeJ mice were intranasally challenged with  $1 \times 10^8$  of *C. abortus* elementary bodies, and the mice were observed for disease (body weight) and mortality until 10 days after challenge inoculation when the surviving mice were euthanized (Fig. 3.1A). As shown in Fig. 3.1B, four days after challenge inoculation six out of 10 mice had lost substantial body weight, and these mice died by day 9 of challenge inoculation (Fig. 3.1B and 3C-left panel). Moreover, among the four mice that survived until day 10 post challenge, two mice were very sick with an approximately 30% of body weight loss on day 10 of challenge inoculation (orange and blue circles, Fig. 3.1B, left panel) and very high lung weight (505 and 566 mg, respectively, Fig. 3.1B right panel) indicative of highly inflamed lungs. The pattern of body weight loss and high lung weights, which were similar to those of dead mice, indicated that these two mice would have succumbed to the chlamydial infection within one or two days. The remaining two surviving mice showed little signs of body weight loss or lung disease, as indicated by lung weight less than 300 mg. These findings also indicated that the *C. abortus* challenge inoculation was highly lethal to C3H/HeJ mice, and the bi-modal outcome prompted us to convert the experimental readout into survival rates. In a separate experiment, using the same *C. abortus* challenge dose, we monitored the mice for three weeks, and 90% of the mice died by 15 days after challenge, starting on day 8 (Fig. 3.1C-right panel). Therefore, unlike A/J mice, C3H/HeJ mice are highly susceptible to challenge

inoculation with  $1 \times 10^8$  *C. abortus* organisms. When tested with a 10-fold lower intranasal challenge dose of  $1 \times 10^7$  *C. abortus* organisms, typically used for A/J live-vaccine



**Figure 3.1. Chlamydial respiratory disease in C3H/HeJ mice.** Disease severity (body weight loss and lung weight increase) and mortality after intranasal inoculation of mice with  $10^8$  *C. abortus* elementary bodies. (A) Schematic representation of the experimental protocol. Six week-old female naïve C3H/HeJ mice were challenged i.n. with  $10^8$  *C. abortus*. Mice were observed daily after challenge inoculation, and body weight and mortality, or mortality only was recorded in two separate experiments of 10 or 21 day duration. (B) Left Panel - Body weight change of each mouse over the course of the challenge infection. Red crosses indicate the death of the mouse. Right panel - Lung weight of the mice that survived until day 10. (C) Survival analysis. Left panel - The survival curve corresponds to the dead mice as shown in the left side of panel B. Right panel - Survival curve of mice in the 21-day experiment. Data were analyzed by repeated measure Anova (body weight), scatter plot (Lung weight), and Kaplan-Meier survival analysis (n=10 mice/group).

controls, C3H/HeJ mice showed the same 10% survival rate, but the time of death was delayed by 3-5 days (data not show). This observation established that immune protection by the low-dose live vaccine approach was impossible to achieve with this mouse strain. Overall, these observations indicated that a BRM effect of microparticles in C3H/HeJ mice could only be analyzed by observing whether microparticle treatment can reduce the mortality in a three weeks' time frame after challenge inoculation.

**Production of microparticles by spray drying.** Typically, for spray drying multiple parameters must be quantitatively determined to achieve consistent production of microparticles of the desired properties. Key parameters that are determined by the material to be spray dried are the use of the appropriate solvent, inlet temperature of drying gas as well as outlet temperature of the spray-dried particles. Dichloromethane (DCM) is the ideal solvent for spray drying of poly-lactide-co-glycolide polymers because of its high solvation capacity for DL-PL and DL-PLG biopolymers (Youan, 2004) and the low boiling point of 40°C. It is also considered one of the least toxic of the halogenated solvents (Blanco *et al.*, 2005). Optimal temperatures of 55-60°C for inlet and 35-38°C for outlet are defined by boiling point of the DCM solvent and glass transition and melting temperatures of DL-PL and DL-PLG (Arpagaus and Schafroth, 2007).

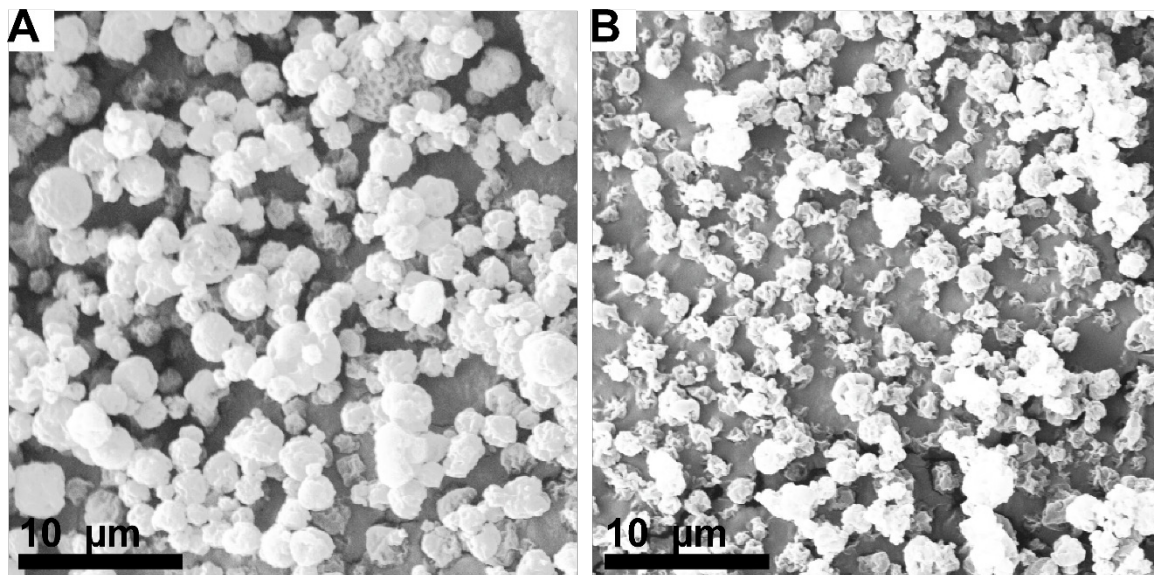
We used DCM as solvent, and 55-60°C inlet- and 35-38°C outlet temperature. Under these restrictions, the key parameters that determine microparticle size are (i) solids concentration in spray drying feed solution, (ii) flow rate of the feed solution (spray flow), (iii) flow rate of nitrogen gas used for atomization of the feed solution by the spray dryer nozzle, and (iv) flow rate of the drying gas (air). In preceding experiments, and following standard procedures, we determined that a 600 L/hr nitrogen atomizing gas flow and

maximum drying air (aspirator) flow rates ( $\sim 45 \text{ m}^3/\text{hr}$ ) were optimal. For effective phagocytosis by APC, a diameter of the microparticles of less than 5 microns, optimally 1-3  $\mu\text{m}$ , is essential (Champion *et al.*, 2008; Pacheco *et al.*, 2013). For that reason we aimed to develop spray drying conditions that produced particles of this size, which is at the lower size limit that can be achieved with two-fluid nozzle spray drying (Arpagaus and Schafroth, 2007).

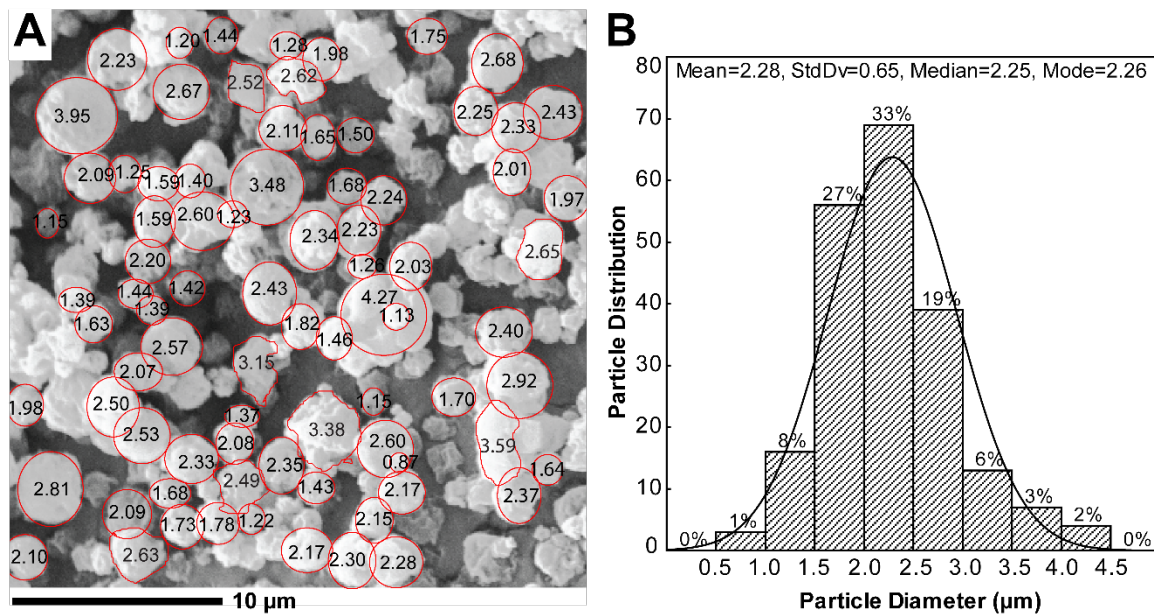
*Effect of pump rate of the feed solution.* For determination of an optimal set of spray drying parameters, we first performed a preliminary determination of the flow rate for a 1% feed solution of PLG-PEG in DCM. At an inlet temperature of  $58^\circ\text{C}$ , outlet temperature  $36^\circ\text{C}$ , aspirator rate 100%, and gas spray flow  $600 \text{ L/hr}$ , approximately 1 to 3  $\mu\text{m}$  spherical microparticles were produced at a spray flow rate of  $3.2 \text{ ml/min}$  (Fig. 3.2A). However, when the spray-rate of feed was increased to  $8 \text{ ml/min}$ , the same feed solution produced irregular small deformed particles with mostly open surfaces (Fig. 3.2B). Therefore, we considered a  $3.2 \text{ ml/min}$  feed solution pump rate for a 1% polymer solution, under constant remaining parameters, as standard for our experiments.

*Microparticle size and distribution.* The size of the microparticles is an important criterion since it directly influences the rate of phagocytosis of the particles by APCs, and an optimal diameter in the range of 1-3  $\mu\text{m}$  is essential. We measured the size of the spray dried particles by analyzing the SEM image of the particles by the ImageJ software version 1.51 (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). ImageJ is widely used to determine the micro- and nano-particle's size (Larson, *et al.*, 2013; Liu *et al.*, 2010; Xie and Smith, 2010; McCall and Sirianni, 2013; Baldelli *et al.*, 2016; Carver and Yang, 2016; Sameni *et al.*, 2008).

In case of non-aggregating individually isolated particles, one can analyze the particle size using automated tools of the ImageJ software. However, the SEM images of our spray dried microparticles demonstrated extensive aggregation of the particles (Fig. 3.2A). Therefore, we manually marked individual particles in the SEM image, and the ImageJ software then automatically determined the diameter, and aggregated and analyzed these data. As shown in Fig. 3.3A, the size of the spray dried DL-PLG-PEG microparticles, synthesized with a 1% feed solution containing only the polymer, was measured with ImageJ. The statistical analysis of the data derived from ImageJ, revealed that the mean diameter of these DL-PLG-PEG microparticles is 2.28  $\mu\text{m}$ , ranging from 0.5 to 4  $\mu\text{m}$  (Fig. 3.3B). It is also evident that 80% of the particles are between 1.5 to 3  $\mu\text{m}$ , the optimal range as we expected. It also important to note that the mean (2.28), median (2.25), and mode (2.26) of the data are essentially equal, indicating a symmetrical distribution of the particle size (Fig. 3.3B).



**Figure 3.2.** Scanning electron micrographs of spray-dried microparticles synthesized at different spray-rates. 50:50 DL-PLG-PEG (Table 3.4) microparticles produced from 1% feed solution sprayed at (A) 3.2 ml/min, (B) at 8 ml/min.



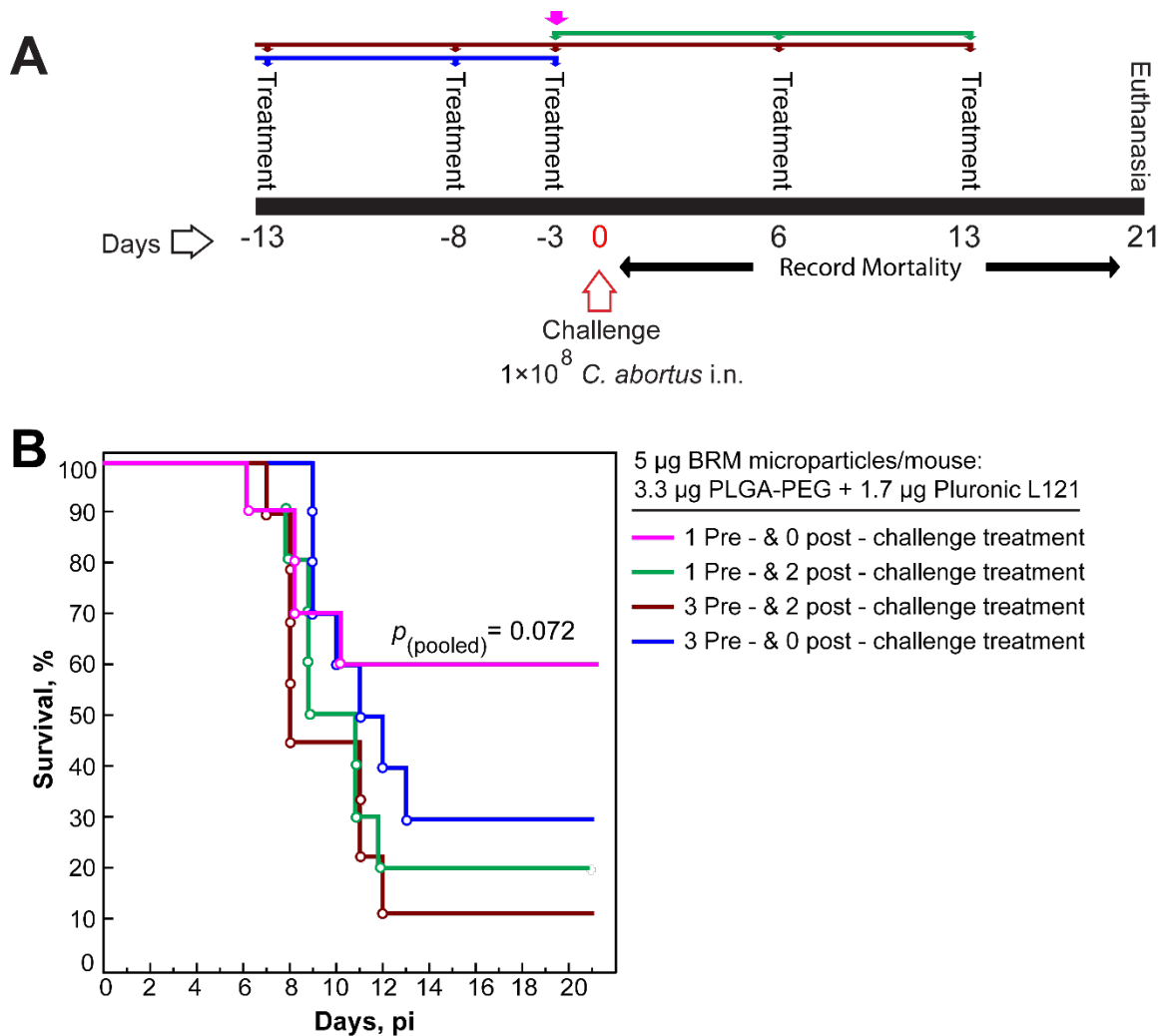
**Figure 3.3.** Size determination of spray dried microparticles. (A) Randomly distributed DL-PLG-PEG particles in the SEM micrograph were manually marked, and the ImageJ software then automatically determined the particle diameter. (B) Statistical analysis of the diameter of randomly marked particles (N=207).



### **Evaluation of Adjuvanted Microparticles as Biological Response Modifiers.**

After defining spray-drying parameters for synthesis of the desired 1.5-3  $\mu\text{m}$  microparticles, we investigated the immunomodulating effect of such microparticles when an appropriate adjuvant was incorporated into these particles. Among the previously successfully used adjuvants, JVRS-100 represented a separate liposome platform that cannot be adapted to spray-drying, and Poly (I:C) is insoluble in DCM. Only the active ingredient of the Polygen adjuvant, a poloxamer co-polymer, was soluble in DCM and therefore amenable to incorporation in spray-dried DL-PLG microbeads. For these reasons, we selected Pluronic L121, a highly hydrophobic poloxamer with proven potent adjuvant properties (Adams *et al.*, 2015; Andrianarivo *et al.*, 1999; Hunter and Bennett, 1984 and 1986; Hunter *et al.*, 1981 and 1990; Kabanov, 2008; Zigterman *et al.*, 1987), for inclusion in the microbeads. We designed two experiments asking two questions – (i) when and how frequently would the BRM microparticles have to be administered to achieve maximum immunopotentiating effect; (ii) what would be the ideal doses of polymer and adjuvant that would induce the best protective response.

*Optimal time and frequency of BRM administration.* Initially, we prepared microparticles using DL-PLG-PEG (Table 3.3) and Pluronic L121 at 2:1 ratio, dissolved in DCM at the concentration of 1% total solids. As an initial approach, we used 5  $\mu\text{g}$  of total polymer and adjuvant (3.3  $\mu\text{g}$  DL-PLG-PEG and 1.7  $\mu\text{g}$  of Pluronic L121 per BRM dose per animal). The microparticles were synthesized utilizing the optimal spray drying parameters as aforementioned.



**Figure 3.4. Determination of optimal time and frequency of BRM microparticle administration in C3H/HeJ mice.** (A) Schematic representation of the experimental protocol. Six week-old female naïve C3H/HeJ mice received i.p. injections of 5  $\mu$ g BRM microparticles comprised of DL-PLG-PEG and Pluronic L121 at a 2:1 ratio at one or multiple time points before or after challenge inoculation. Mice were challenged i.n. with  $10^8$  *C. abortus* and observed daily until termination on day 21 day after challenge. (B) Survival analysis of mice after 4 different BRM microparticle administration regimens. Data were analyzed by Kaplan-Meier survival analysis,  $p$  value was determined by Cox's F test ( $n = 10$  mice/group).

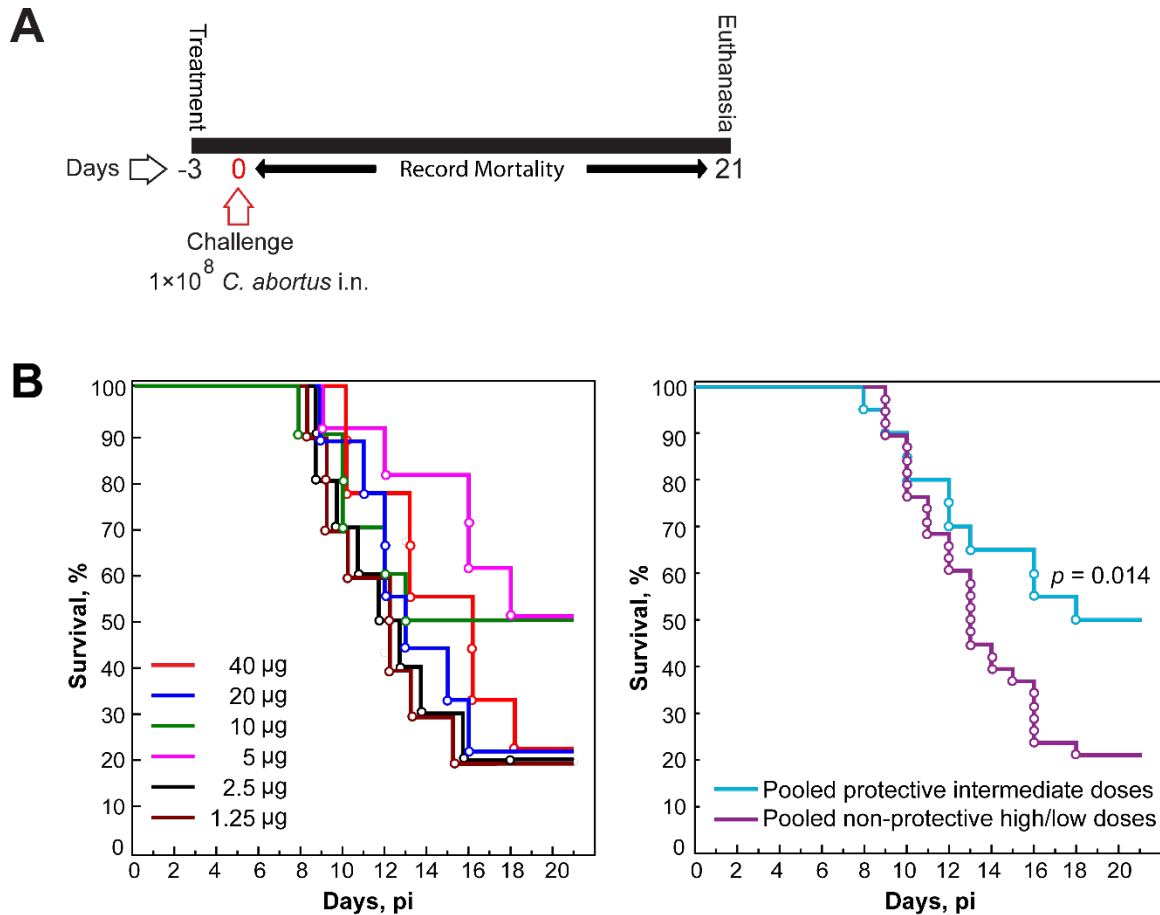
To evaluate the immunopotentiating effect, the C3H/HeJ mice were intraperitoneally injected with the amount of microparticles containing 5  $\mu\text{g}$  of total polymer and adjuvant concentration first mixed with 25  $\mu\text{g}$  Lactose Microfine excipient, and then dissolved in 200  $\mu\text{L}$  PBS containing 0.1% Kolliphor HS 15. These injections were administered on four different time points relative to the challenge inoculation (Fig. 3.4A): i) a single injection 3 days before challenge; ii) one injection 3 days before and two injections 6 and 13 days after challenge; iii) three injections 13, 8, and 3 days before and two injections 6 and 13 days after challenge; and iv) three injections 13, 8, and 3 days before challenge. All mice were challenged intranasally with  $1 \times 10^8$  *C. abortus* organisms and monitored for three weeks, and mortality was recorded. As shown in Figure 3.3B, the single treatment 3 days before challenge inoculation significantly reduced the mortality of C3H/HeJ mice and resulted in 60% survival (Fig. 3.4B). In contrast, neither three treatments nor five treatments showed any protection. The three treatments at 3 days before- and 6 and 13 days after challenge inoculation resulted in 30% survival, and at 13, 8, and 3 days before challenge inoculation reduced survival to 20% (Fig. 3.4B). Similarly, five treatments at 13, 8, and 3 days before- and 6 and 13 days after challenge inoculation resulted in 10% survival (Fig. 3.4B). Survival after single treatment was significantly higher than after five treatments ( $p=0.025$ ; Cox's F test) and after three treatments ( $p=0.166$  and  $0.069$ , respectively). Comparison of the 60% survival after single treatment to the 20% survival of the pooled multiple treatments almost reached significance at  $p=0.05$  ( $p=0.072$ ). Moreover, the single treatment also resulted in higher survival than the 10% survival of the naïve control ( $p=0.057$ ; Fig. 3.1C). Thus, the findings indicate that Pluronic L121-containing PLG-PEG microparticles modulate the innate immune response in *tlr4*<sup>-/-</sup>

C3H/HeJ mice and protected against lethal *C abortus* challenge. However, the findings also demonstrate that overstimulation of the innate immune system was counterproductive and most likely induced an uncontrolled excessive inflammatory response. This resulted in death rates similar to that of naïve challenged mice that succumb to excessive chlamydial replication due to a non-responsive innate immune system. Therefore, a discrete optimum exists for the BRM use of DL-PLG-PEG-Pluronic L121 microbeads, and a single treatment three days before challenge inoculation induces a protective response in C3H/HeJ mice.

Optimal BRM dose. After determining the optimal time and frequency of treatment required to induce a maximal BRM effect, we investigated the BRM microparticle dose requirements that evoked maximum protection. Following the composition evaluated for timing of administration, we used a 2:1 ratio of DL-PLG polymer to Pluronic L121 adjuvant. However, to maximize phagocytosis while potentially extending adjuvant release, we substituted 50:50 DL-PLG (RG502H) as carrier for the more hydrophilic DL-PLG-PEG. We performed a dose titration experiment with a two-fold logarithmic dilution series of total BRM solids (RG502H plus Pluronic L121) per mouse, from 40 µg to 1.25 µg. The spray dried microparticles were prepared with the standard procedures as mentioned earlier.

To evaluate the immunopotentiating effect, the C3H/HeJ mice were intraperitoneally injected 3 days before challenge inoculation with spray dried microparticles containing either 40, 20, 10, 5, 2.5, or 1.25 µg of total polymer and adjuvant (2:1 ratio). The BRM microparticles were first mixed with the 5-fold amount of Lactose Microfine excipient, and then suspended in 200 µL PBS containing 0.1% Kolliphor HS 15 (Fig. 3.5A). All mice were challenged with  $1 \times 10^8$  *C. abortus* organisms and monitored for three weeks. As shown in

Fig. 3.5B, neither of the high doses of 40 and 20  $\mu\text{g}$  nor the low doses of 2.5 and 1.25 $\mu\text{g}$  total of polymer and adjuvant was able to reduce mortality, with only ~20% survival. In contrast, both intermediate doses of 10 and 5  $\mu\text{g}$  resulted in 50% survival (Fig. 3.5B). Comparison of the protective 50% survival of the pooled intermediate doses (5 and 10  $\mu\text{g}$ ) to that of the non-protective 20% survival in the pooled high (40 and 20  $\mu\text{g}$ ) and low (2.5 and 1.25  $\mu\text{g}$ ) doses revealed significant differences of combined ( $p=0.014$ ; Fig. 3.5C) or of separate pooled high and low doses ( $p=0.057$  and  $p=0.021$ , respectively). These findings clearly demonstrated that similar to the multiple treatments with low doses of microparticles (Fig. 3.4B), a single treatment with high doses of BRM microparticles (RG502H + Pluronic L121) is counterproductive due to the overstimulation of the innate immune response. Conversely, very low doses of BRM microparticles most likely are not sufficient to trigger an optimal stimulation to the innate immune response and therefore cannot elicit a protective response in naïve C3H/HeJ mice. Therefore, for optimal protection a narrow dose range exists at which the amount of BRM micobeads is sufficient for reproducible protection yet does not overstimulate pathological inflammation.



**Figure 3.5. Dose-dependent immunopotentiating effect of BRM microparticles in C3H/HeJ mice.** (A) Schematic representation of the experimental protocol. Six week-old female naïve C3H/HeJ mice received via i.p. injection different doses of RG502H:Pluronic L121 = 2:1 microparticles. After three days mice were challenged i.n. with  $10^8$  *C. abortus* and then observed daily until 21 days post challenge. (B) Survival analysis of mice after receiving 1.25 – 40  $\mu\text{g}$  of BRM microparticles. (C) Survival analysis of mice pooled by 5 & 10  $\mu\text{g}$  doses versus mice pooled by 40, 20, 2.5, and 1.25  $\mu\text{g}$  doses. Data were analyzed by Kaplan-Meier survival analysis [n= 9 (40 and 20  $\mu\text{g}$ ) or 10 (10 – 1.25  $\mu\text{g}$ ) mice/group].

Given the identical survival rate at 5 and 10 g BRM dose, albeit at different survival kinetics, we decided on the future use of 10  $\mu\text{g}$  doses of BRM as carrier for vaccine trials. We reasoned that at the 10  $\mu\text{g}$  dose innate stimulation was sufficiently strong to cause rapid death in mice susceptible to overstimulation, while fully protecting the lower inflammatory responders. In contrast, at 5  $\mu\text{g}$ , mice succumbed more slowly, but at the same final survival rate. Thus we reasoned that the 5  $\mu\text{g}$  dosage presented a higher risk for understimulation than the 10  $\mu\text{g}$  dose for overstimulation.

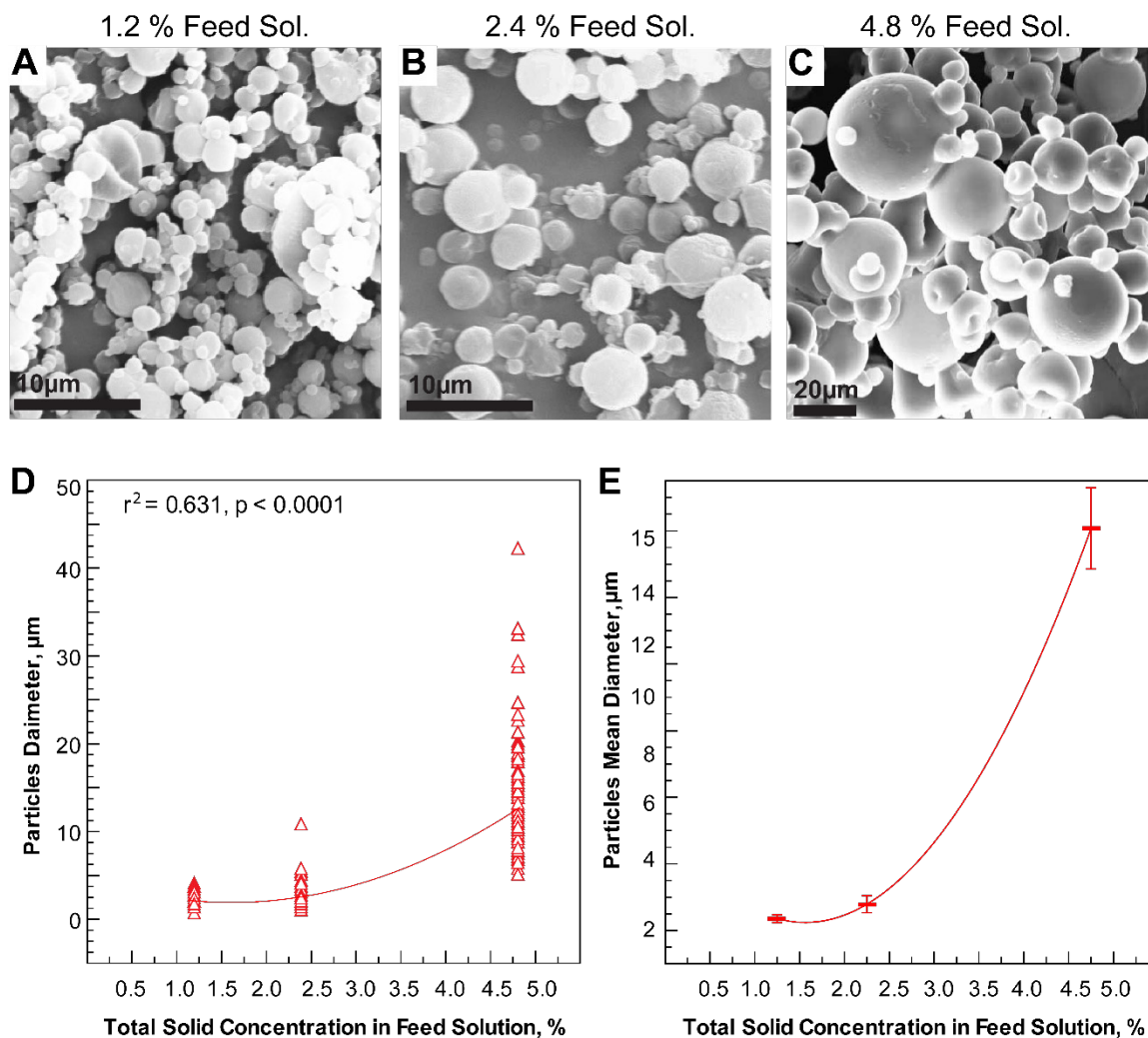
**Synthesis of optimal microparticles.** After defining initial spray-drying conditions for production of suitable BRM microparticles that mediated significant protection, we re-analyzed spray drying production parameters in order to fine-tune robust synthesis conditions of optimally sized BRM, and future vaccine, microparticles. For ease of calculation, we changed the polymer to adjuvant ratio to 6.5 $\times$  polymer and 3.5 $\times$  adjuvant. In the subsequent experiment, we studied the morphological features and functional properties of microparticles in depth by *in vitro* and *in vivo* experiments.

*Effect of the concentration of feed solution on size and shape of microparticles.* In our previous experiments, we found that the feed solution containing 1% of total solid resulted in the synthesis of 2.28  $\mu\text{m}$  spherical microparticles by spray drying. However, the surface of these microspheres was rough and irregular (Fig. 3.2A), which is undesirable for ideal BRM/vaccine microparticles. A low surface roughness is essential for extended release of API. Irregularities will result in an increase in surface area, creating an accelerated diffusive release of API (Dawes *et al.*, 2009). Previously, we had observed that the concentration of feed solution had the greatest influence on size and shape of the

microparticles. Therefore, we performed a concentration titration experiment with a two-fold logarithmic dilution series from 1.2% to 4.8% of total solids comprising DL-PLG-PEG and Pluronic L121 at 6.5:3.5 ratio in the feed solution.

Spray drying of all feed solutions containing either 1.2%, 2.4%, or 4.8% of DL-PLG-PEG and Pluronic L121 resulted in spherical microparticles with smooth surfaces as observed in their SEM micrographs (Fig. 3.6A-C). The SEM micrographs also demonstrated that microparticles became sequentially larger as the feed concentration of solids increased (Fig. 3.6A-C). Quadratic polynomial regression analysis revealed a positive correlation for the diameter of the particles with the concentration of solids in the feed solution ( $r=0.794$ ;  $p<0.0001$ ; Fig. 3.6D), with the mean diameter of 2.36, 2.79, and 14.07  $\mu\text{m}$  for the 1.2, 2.4, and 4.8% feed solutions, respectively (Fig. 3.6E). It is apparent from the data that a 1.2% feed solution is the lowest concentration that should be used to synthesize spray-dried DL-PLG-PEG-Pluronic L121 microparticles with optimal size and shape. Although, 2.4% feed solution produced DL-PLG-PEG microparticles with 2.79  $\mu\text{m}$  in diameter – which is at the upper limit for an optimal size (1 to 3  $\mu\text{m}$ ), and hence risks the production of substantial numbers of particle with more than 3  $\mu\text{m}$ . Analysis of the particle mean diameter provides a theoretical mean diameter of 2.5  $\mu\text{m}$  for a 2% feed solution (Fig. 3.6E). Therefore, a feed solution with the concentration in between 1.2 to 2% would be the best choice to synthesize microparticles with optimal size and shape while using PLG-PEG as polymer and with Buchi 190 mini spray dryer.



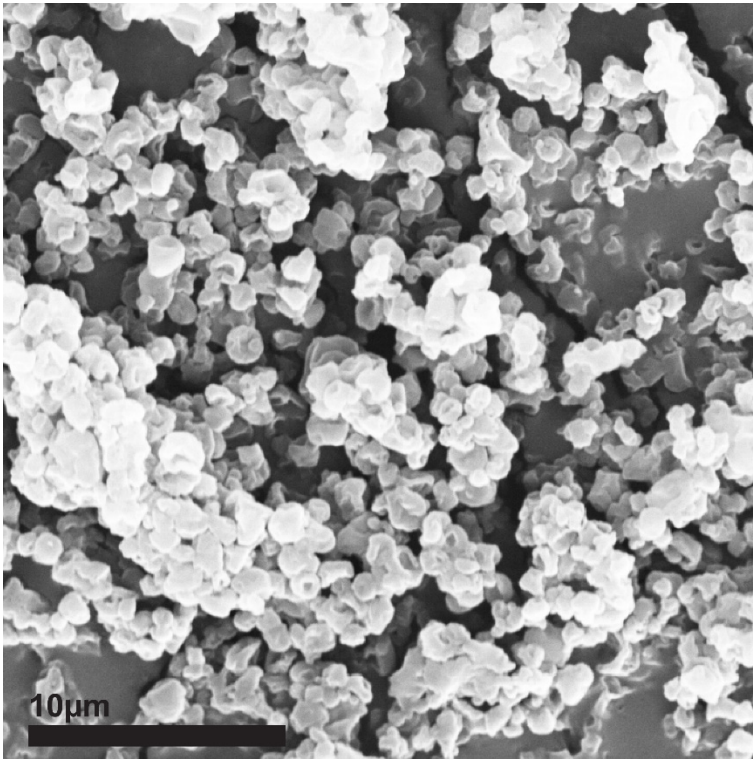


**Figure 3.6. SEM images and size analysis of spray-dried DL-PLG-PEG-Pluronic L121 microparticles.** DL-PLG-PEG and Pluronic L121 were dissolved in DCM at a 6.5:3.5 ratio at a final w/v solid concentration of 1.2% (A), 2.4% (B), or 4.8% (C). (D) Polynomial quadratic regression between the diameter of the particles, as determined by ImageJ software from SEM images, and the percentage of total solids used in the feed solution. Each triangle represents one particle ( $n = 100$  of randomly marked microparticles in the SEM micrograph of each of 1.2, 2.4, and 4.8% concentration) (E) Linear regression analysis with polynomial fit of the mean diameter of the microparticles at 1.2, 2.4, and 4.8% feed solution concentration as shown in D.

*Effects of polymers type and molecular weight on microparticles size and shape.*

As mentioned earlier, the type of polymer and its molecular weight significantly affect the degradation kinetics of the spray dried microparticles (Kamaly *et al.*, 2016) and therefore the release of vaccine antigen and subsequent immune response. For this reason, we tested spray drying to optimal particle size of six different PLG polymers that later would be analyzed for kinetics of degradation and antigen release, and mechanisms of antigen release.

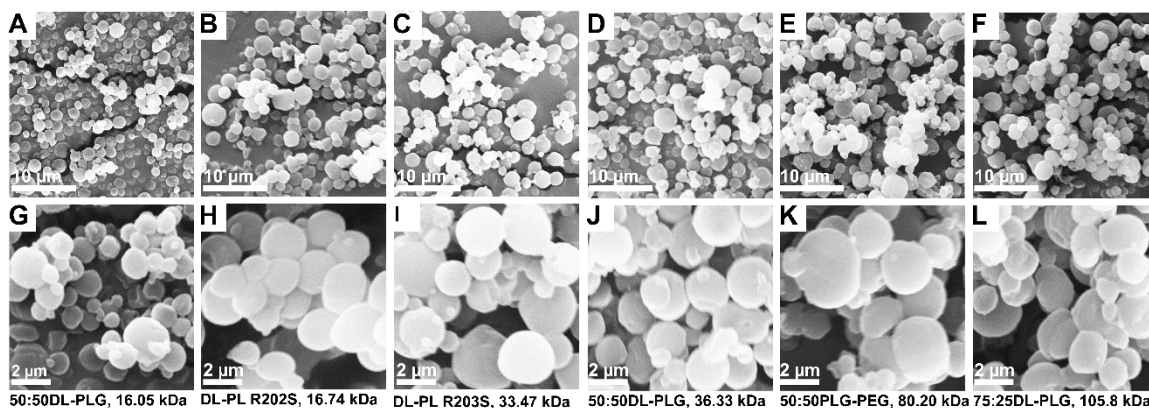
In our previous experiment, we used PLG-PEG of 80.2 kDa molecular weight as polymer which is comprised of 50:50 DLG and contains 5 wt% poly-ethylene-glycol (PEG) of 5 kDa MW. In our next experiment, we examined whether different DL-Poly-lactide (DL-PL) or DL-Poly-lactide-co-glycolide (DL-PLG) polymers of different molecular weight and composition could produce equally sized microparticles. In an initial attempt we used DL-PL (R202S) with 16.74 kDa molecular weight and synthesized microparticles with 1.2% feed solution. As shown in Fig. 3.7, at the 1.2% feed solution, R202S with five-fold lower molecular weight than DL-PLG-PEG produced small microparticles (1.21  $\mu\text{m}$  mean diameter) that were not fully spherical (Fig. 3.7). We anticipated that a higher concentrated 2% feed solution would produce larger and optimally shaped microparticles. Therefore, as next step we determined the shape and size of microparticles produced from six different PLG and PL polymers (Tables 3.2 and 3.4) by use of a 2% feed solution.



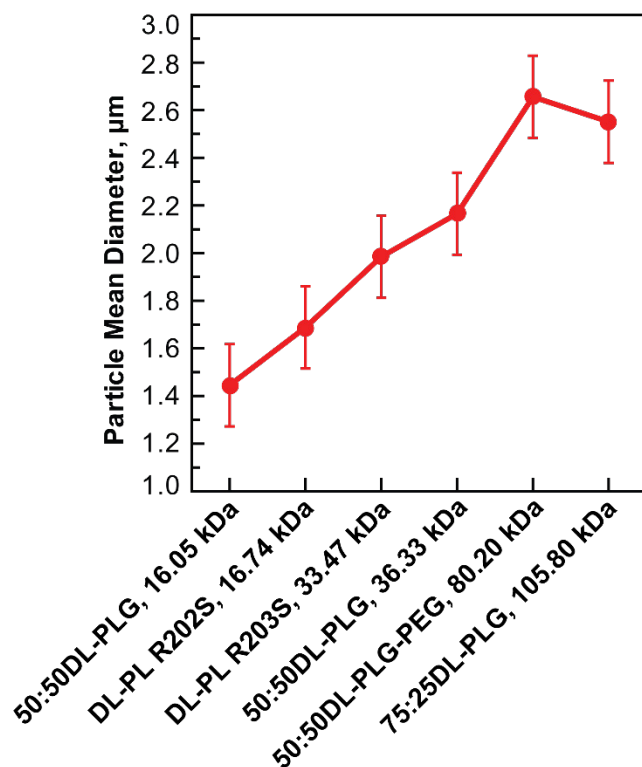
**Figure 3.7.** SEM micrograph of the spray-dried DL-PL (R202S) and Pluronic L121 microparticles synthesized by use of a 1.2% DCM feed solution. DL-poly-lactide of 16.74 MW and Pluronic L121 at 6.5:3.5 weight ratio were spray dried under standard conditions. The mean diameter of the microparticles is 1.21  $\mu\text{m}$ , and the shape and surface are irregular.

As shown in Fig. 3.8, the 2% feed concentration yielded spherical microparticles with smooth surfaces for all polymers (Fig. 3.8 A-L). Particles size analysis revealed that the microparticle diameter was within the optimal range (1.5 to 2.6) for all polymers. The results also clearly demonstrated that the molecular weight of the polymers is significantly positively correlated to the diameter of the particles (Fig. 3.9; Table 3.2). Importantly, only molecular weight, but not composition of the polymers (PL versus PLG), influenced the microparticle diameter (Table 3.2).

In sum, this particle size optimization experiment clearly demonstrates that the concentration of total solids in feed solution and the molecular weight of the polymers have a significant impact on the size of 6.5:3.5 polymer:Pluronic L121 microparticles, but not the type of the polymer. However, a feed solution with 2% total solids containing any DL-PL or DL-PLG at different co-polymer ratios with a molecular weight range of 10 to 100 kDa will produce microparticles within the optimal size range (1 to 3  $\mu\text{m}$ ) and shape (spherical and smooth) for phagocytosis, if the microparticles are synthesized in a Büchi B-190 spray-dryer with standard setting for the remaining parameters.



**Figure 3.8. Scanning electron micrographs of spray-dried microparticles synthesized from 2% (w/v) feed solutions using different polymers.** The microparticles were composed of either one of six different polymers and Pluronic L121. Polymer and Pluronic L121 (6.5:3.5 ratio) were dissolved in DCM at 2% final solid concentration. Each produced particle is shown at low (upper row) and high (lower row) magnification.



**Figure 3.9. Mean diameter of spray-dried microparticles synthesized from 2% (w/v) feed solutions.** The diameter of random particles ( $n = 100$ ) of each type as shown in Fig. 3.8 was determined by ImageJ analysis. Error bars indicate 95% CI.

**Table. 3.2. Differences in mean diameters of the spray-dried microparticles synthesized from 2% feed solutions analyzed by one-way ANOVA.**

Polymer	Mw (kDa)	Mean Diameter (μm)	<i>P</i> value (Tukey HSD test)					
50:50 DL-PLG, IV 0.39	16.05	1.45		0.368	<0.001	<0.001	<0.001	<0.001
100:0 DL-PL (R202S), IV 0.20	16.74	1.69	0.368		0.159	0.002	<0.001	<0.001
100:0 DL-PL (R203S), IV 0.32	33.47	1.98	<0.001	0.159		0.692	<0.001	<0.001
50:50 DL-PLG, IV 0.59	36.33	2.16	<0.001	0.002	0.692		0.001	0.0229
50:50 DL-PLG-PEG, IV 0.79	80.20	2.66	<0.001	<0.001	<0.001	0.001		0.960
75:25 DL-PLG, IV 0.69	105.80	2.55	<0.001	<0.001	<0.001	0.023	0.960	

**Degradation Kinetics of Microparticles Composed of Different Polymer Carriers.** As the next step, we sought to evaluate adjuvant/antigen release potential of microparticles produced with different PLG polymer. To this end, we studied the degradation kinetics of polymer:Pluronic L121 (6.5:3.5) microparticles of the six polymers as aforementioned (Fig 3.8, Table 3.2). Repeated measurement of the pH of suspensions of the microparticles served as an indicator of polymer degradation and reduction in molecular weight by determining the release of lactic and glycolic acid during breakdown of the polymers.

The erosion of PLG polymers starts with degradation, i.e., the process of polymer chain scission. For biodegradable polymers, chain scission occurs by hydrolysis of the functional groups in an aqueous environment. Then, degradation products such as oligomers and monomers are released from the polymer matrix into the surrounding medium leading to the characteristic mass loss during the erosion process. Hydrolysis starts with the penetration of water into the polymer matrix. As polymer, PLG and PL are neutral (i.e. have no influence on hydronium ion concentration in an aqueous solution). However, with the hydrolytic uptake of water, an acid molecule is released per broken bond. This release of acidic hydrolysis products leads to a reduction of the pH of the aqueous medium (Boimvaser *et al.*, 2016). The pH of mixtures of lactic acid/glycolic acid monomers is dependent to the concentration of these acids (Fig. 3.10A). Therefore, using the pH of aqueous suspensions of PLG or PL polymers, the concentration of free acids in aqueous suspensions of these polymers, and therefore the polymer degradation, can be determined. Here, we investigated the degradation kinetics of six different microparticles

as shown in Fig. 3.10 by determining the pH of a suspension of these microparticles in suspension buffer over a time period of 112 days.

As shown in Fig. 3.10B, suspensions of all 50:50DL-PLG polymers, but most strongly of 50:50DL-PLG-PEG, showed a sharp decline in pH within the first week by 0.5-1.4 pH units from the starting pH of 4.5-5.3, followed by a more gradual decline to below pH 3.0 by day 35-42. The intermediate 75:25DL-PLG showed a less dramatic pH decline from 4.3 to 4.0 in the first week, but then reached pH 3.0 only at 91 days in suspension. These data are in contrast to those of the polylactide polymers, which declined only slowly and continuously until day 112 from starting a pH of ~6.0 to 5.2 (DI-PL R203S) and 3.8 (DI-PL R202S).

These pH changes correlated with a breakdown of the polymers and translated in precipitous reduction in molecular weights within the first week for the 50:50DL polymers, and initial rapid decline followed by gradual reduction for the 75:25DL-PLG microparticles (Fig. 3.10C). In contrast, the polylactide polymer microparticles did not show an initial rapid decline in molecular weight, but only a gradually reduced molecular weight. The threshold for solubility of PLG/PL oligomers is at a molecular weight of approximately 1 kDa. Thus, when a polymer has degraded to a MW of 10 kDa or lower, a sizeable fraction of the normally-distributed polymer molecules will be at or below the solubility limit, and will diffuse away from the microparticle, initiating the physical breakdown of the microparticle. As a measure of the start of physical breakdown, we used the calculated MW of 10 kDa, since calculation accuracy from pH for lower molecular weight declines precipitously at low pH. The 50:50DL-PEG polymers reach the 10 kDa MW threshold within 3-21 days, in dependence of starting molecular weight. Complete



dissolution of these microparticles follows within 10-20 days, as observable by the clarification of the microparticle suspension. The intermediate the 75:25DL-PLG polymer with the very high 105.08 kDa MW reached the 10 kDa MW threshold by day 91, and by termination of the pH measurements on day 112, only little of the microparticles were left in suspension, while most of the material had become soluble.

The MW trends of the polylactide polymers differed from the mixed lactide-glycolide polymers by showing minimal, but continuous reduction. The lower MW DL-PL R202S polymer reached the 10kDa threshold, from the 16.74 kDa starting point, after 63 days in suspension. But even at that point it degraded more gradually than the mixed polymers, and residual microspheres were still present in suspension 7 weeks later on day 112. The higher MW DL-PL R203S polymer never reached the 10kDa threshold, and consequently much material was still present on day 112.

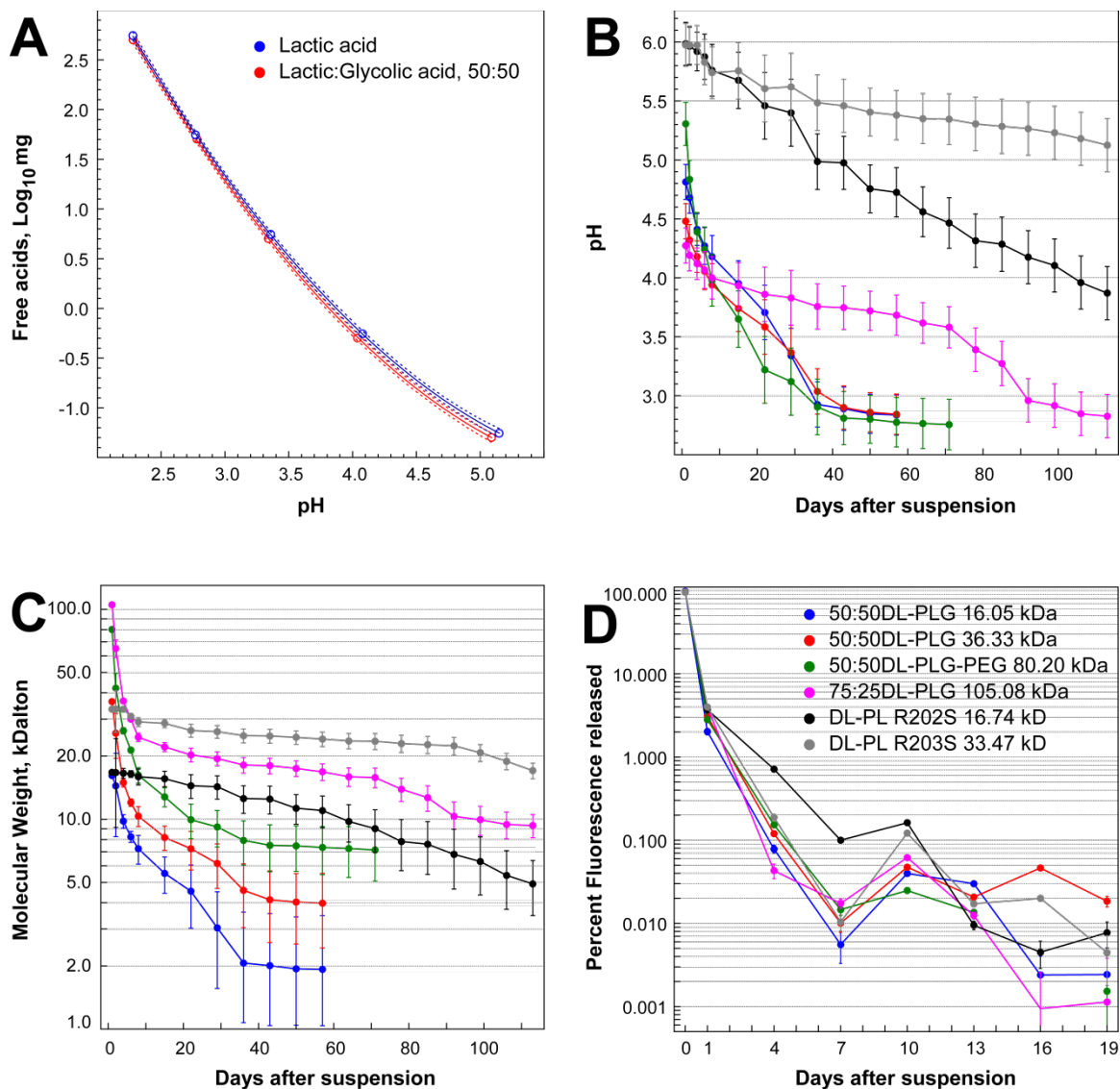
As an initial gauge of the release of embedded API molecules, we incorporated a hydrophilic Alexa Fluor 488-labeled *C. abortus* DnaX peptide in PLG-Pluronic L121 microparticles and measured fluorescence released into solution. Surprisingly, with a range of 95.27-97.80%, almost all of the fluorescence was released with the first hour after suspension of the microparticles (Table 3.3, Fig. 3.10D). Most of the remaining fluorescence, 2.02-3.99%, was released within the next 24 hours. The remaining approximately 0.3% were released from most microparticles within the next 3 days. The exception was DL-PL R202S, which released 0.71% during that time and maintained a measurable release of fluorescence up to day 10.

This surprisingly rapid release from the microparticles may be explained by the short diffusion path for water into the particles, the very high hydrophilicity of the peptide

and fluorescence label, and by partial separation of peptide molecules from hydrophobic microparticle solids during spray drying (low incorporation efficiency). Therefore, to obtain a clear picture of the release of a variety of API, more hydrophobic molecules must be tested as well. However, it is clear that most an embedded API or antigenic peptides may be released into solution even before phagocytosis of administered microparticle suspensions, and that only a fraction of them will be released intracellularly. In addition, polylactide-based microparticles may be better suited for retention and protracted released of API than mixed lactide-glycolide polymers. In these polylactide polymer microparticles, diffusion is the exclusive release mechanism because polymer degradation occurs much later. In contrast, release from lactide-glycolide co-polymers will be dictated by both diffusion and matrix degradation, since these polymers start to degrade immediately upon contact with water, losing molecular weight and cohesion precipitously.

**Table. 3.3. Percent fluorescence of total fluorescence released from microparticles early after suspension.**

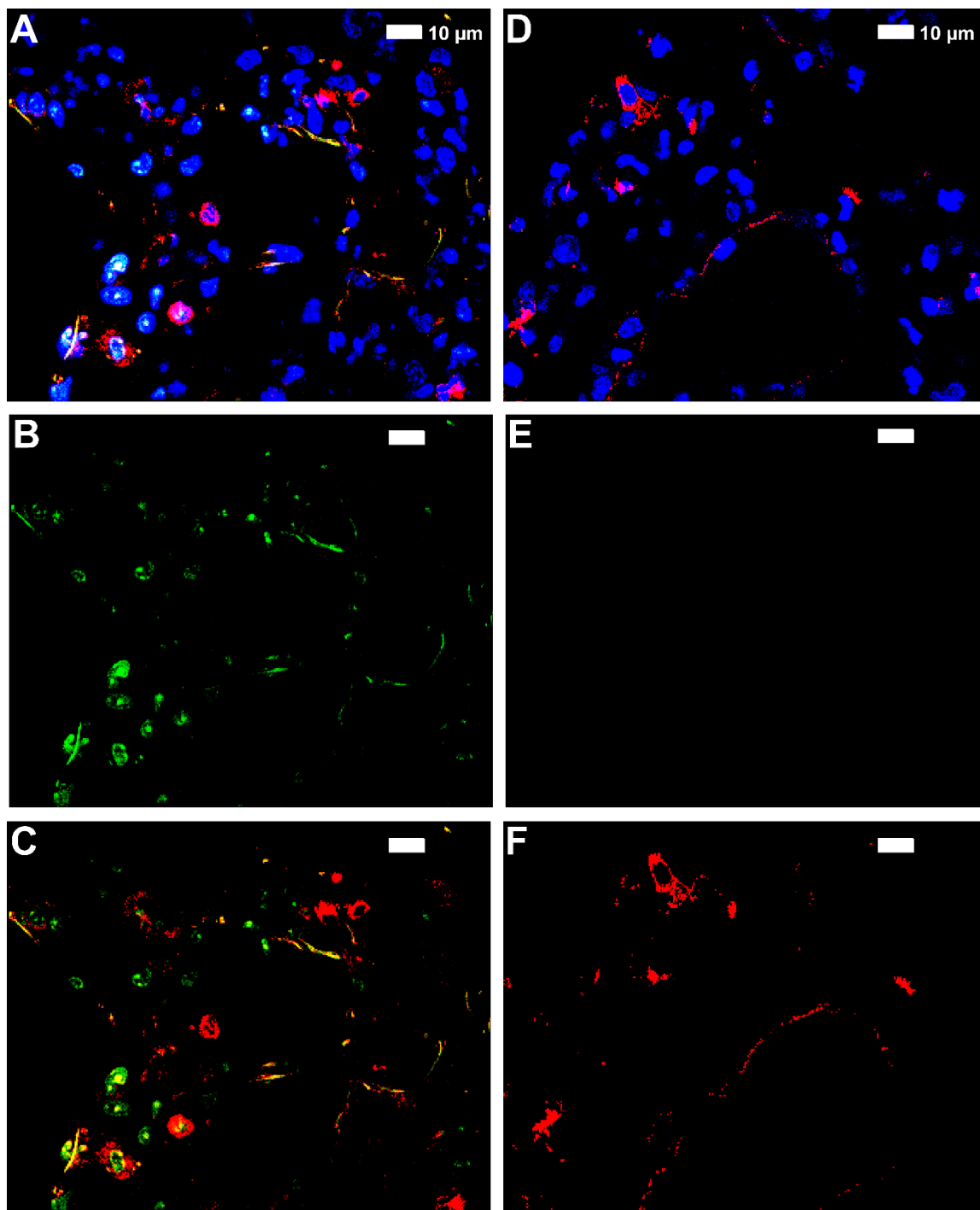
Polymer	Percent fluorescence released after suspension		
	Day 0 (0-1 hr)	Day 1 (1-24 hr)	Day 4 (25-96 hr)
50:50 DL-PLG 16.05 kDa	97.80	2.02	0.08
50:50 DL-PLG 36.33 kDa	96.57	3.08	0.12
50:50 DL-PLG-PEG 80.20 kDa	96.98	2.81	0.15
75:25 DL-PLG 105.08 kDa	95.89	3.97	0.04
DL-PL R202S 16.74 kDa	95.27	3.70	0.71
DL-PL R203S 33.47 kDa	95.62	3.99	0.19



**Figure 3.10. Kinetics of degradation and release of fluorescently-labeled peptide of different polymeric microparticles.** Microparticles were composed of polymer and Pluronic L121 adjuvant at a 6.5:3.5 ratio in 2% total solids in the DCM spray drying feed solution. For determination of peptide release an Alexa Fluor 488-labeled 20-mer *C. abortus* peptide was added (1 nM/mg solids). (A) Determination of the concentration of free lactic/glycolic acids ( $\pm 50\%$  CI) in dependence of the pH of 50 mL of the unbuffered suspension solution. (B) Microparticles were suspended in 50 mL suspension buffer under continuous agitation, and the pH was determined 1 hour after suspension (day 0), then on days 1, 3, 5, 7 and weekly thereafter for additional 15 weeks. (C) Molecular weight deduced over time from pH-dependent polymer degradation and scission of polymer molecules, based on the pH data of the suspension solution as shown in panel B. (D)

Release of fluorescently labeled peptide for each time point (1 hr = day 0, 24 hr = day 1, 3 day1-day4 = day 4). Percent release is based on total release from day 0-46.

***In vivo* Uptake of Microparticles by Macrophages.** After characterization of the DL-PL and DL-PG spray-dried microparticles, we investigated the uptake of these particles by macrophages in an *in vivo* mouse model. The spray dried DL-PLG-PEG:Pluronic L121 microparticles incorporating fluorescently labeled peptide were intranasally inoculated into mice. Twenty four hours later, the lungs were collected, zinc-formalin fixed, sucrose-dehydrated, cryosectioned, immunostained, and observed under a confocal fluorescence microscope. As shown in Figure 3.11, lung macrophages efficiently engulfed the majority microparticles as evident by the presence of green fluorescence (Alexa-Fluor 488-conjugated peptide) inside macrophages with high surface expression of the F4/80 macrophage marker (Fig. 3.11 D-F).



**Figure 3.11. Confocal microscopic image showing *in vivo* macrophage uptake of spray dried PLG-PEG-Pluronic L121 microparticles incorporating a peptide labeled with Alexa-Fluor 488.** (A) Merged triple-color image of mouse lung after intranasal instillation of microbeads containing a peptide labeled with Alexa Fluor 488. The blue color is derived from DAPI fluorescence from DNA staining of cellular nuclei, the green color corresponds to microparticles

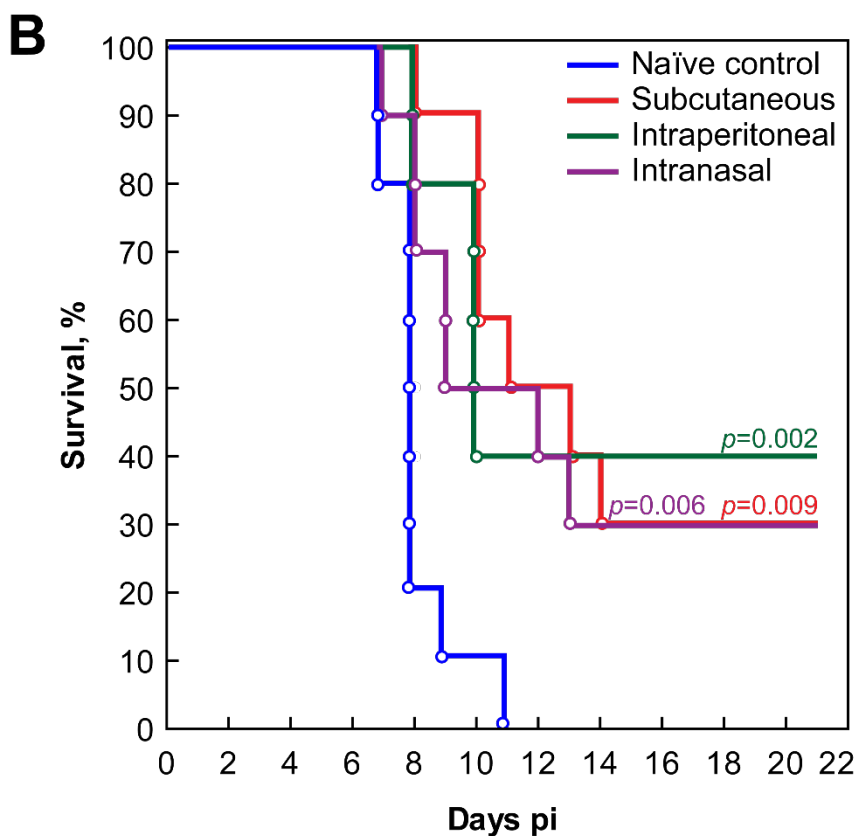
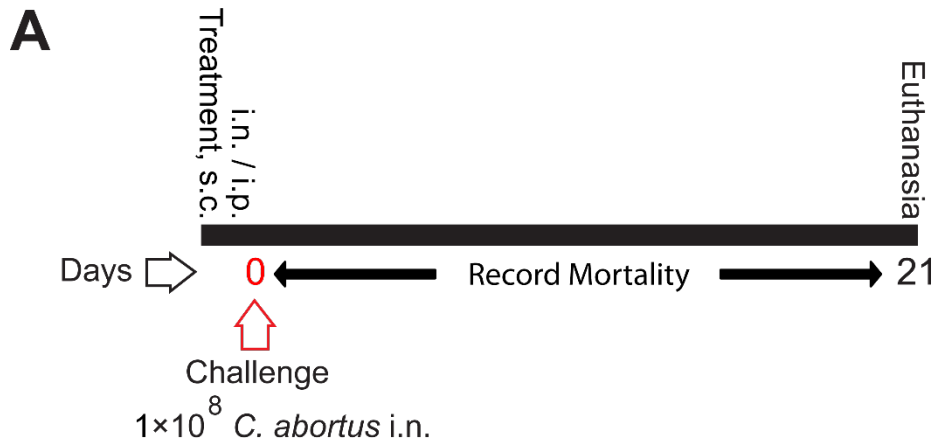
labeled with green-fluorescent peptide, and the red color indicates fluorescence associated with binding of Alexa Fluor 594-labeled antibodies against F4/80, a macrophage cell membrane marker protein. The empty spaces between aggregated cells indicate lung alveolar cavities. (B) Isolated green fluorescence indicating the location of microbeads and cytosolic peptide that diffused out of microbeads. (C) Merged green and red fluorescence indicating the co-localization of the majority of microbeads with macrophages. This demonstrates that the majority of microbeads are phagocytosed by macrophages within 24 hours. (D-F) Corresponding photomicrographs of a control specimen from a mouse that received unlabeled microbeads.

**Immunopotentiating Effect of Optimal PLG-PEG:Pluronic L121 Microparticles when Used in Different Routes of Administration.** After the extensive optimization of spray drying procedures and subsequent analyses of *in vitro* degradation kinetics and *in vivo* phagocytosis of the 1 to 3  $\mu\text{m}$  microparticles, we evaluated the immunostimulating effect of such BRM microparticles when administered by different routes. C3H/HeJ mice received 10  $\mu\text{g}$  of DL-PLG-PEG-Pluronic L121 microparticles either via subcutaneous (s.c.), intraperitoneal (i.p.), or intranasal route (i.n.). Two days (s.c.) or one day (i.p. and i.n.) after treatment, the mice were i.n. challenged with a lethal dose of  $1 \times 10^8$  *C. abortus* EBs and monitored for three weeks (Fig. 3.12A). The naïve control received i.n. PBS one day before challenge.

The mortality kinetics and survival rate were highly significantly different between naïve control mice and the mice in all three different treatment groups (Cox's F test  $p=$  0.006, 0.009, and 0.002 for the difference between naïve and mice with i.n., s.c., or i.p., BRM administration, respectively; Fig. 3.12B). The naïve control showed rapid mortality - starting from seven days after challenge inoculation. Eighty percent of the mice were dead on day 8 post challenge, and the remaining mice died by day 11 after challenge inoculation (Fig. 3.12B). In contrast, mice of all treatment groups tended to die later. In the intranasal group, mice died between days 7 and 13, and the remaining 30% mice survived (Fig. 3.12B). Similarly, 30% of the mice in the subcutaneous group survived, the dead mice, however, succumbed to the challenge infection later than in the intranasal group. The best protected group were the mice that received the BRM microparticles intraperitoneally, although not statistically significant different from the other administration routes, with mortality only between days 8-11, and 40% surviving. The



findings clearly demonstrated that the PLG-PEG-Pluronic L121 microparticles had an immunostimulating effect irrespective of the route of administration.

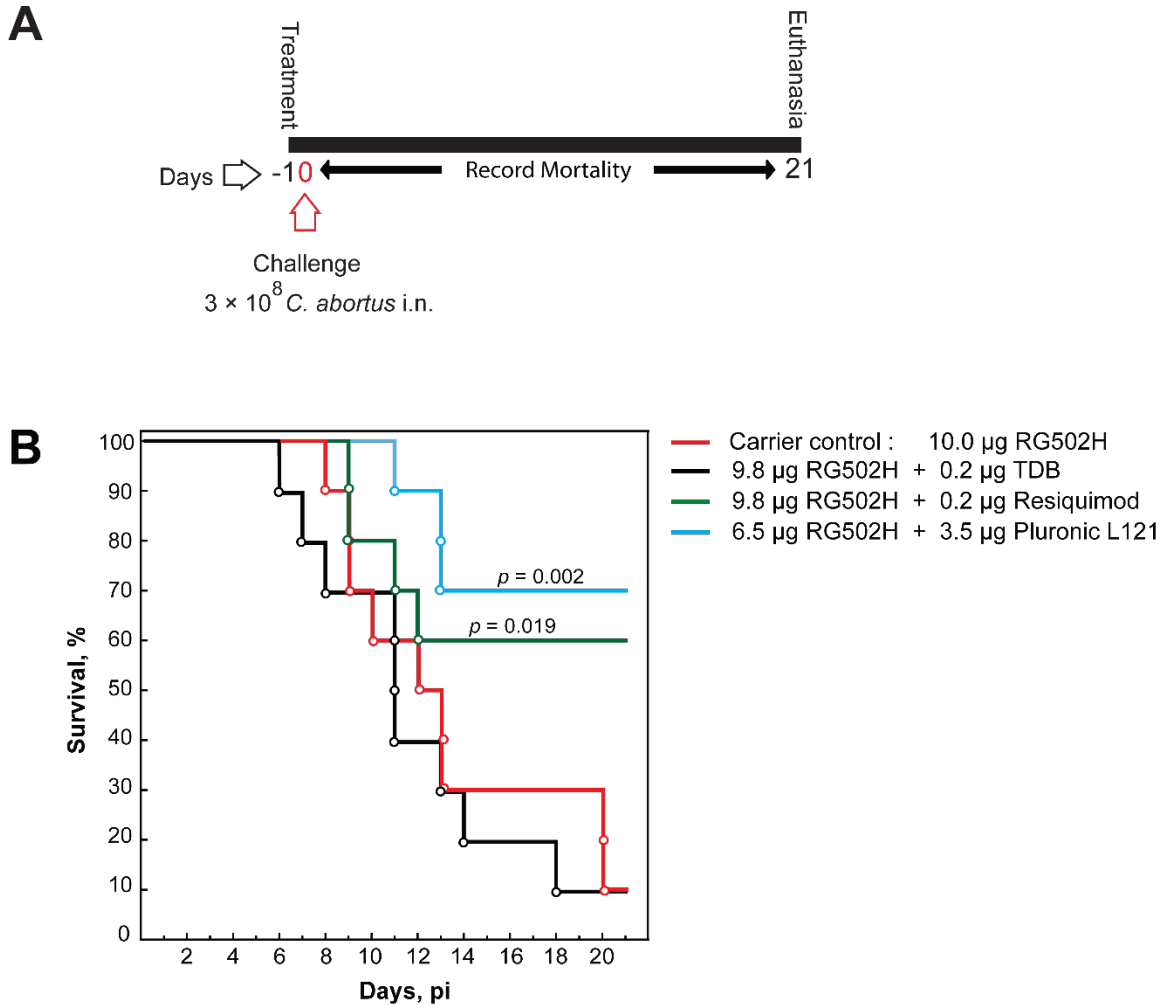


**Figure 3.12. BRM effect of DL-PLG-PEG microparticles via different routes of administration.** (A) Schematic representation of the BRM experimental protocol. C3H/HeJ mice received 10  $\mu$ g of PLG-PEG-Pluronic L121 BRM microparticles suspended in PBS/0.1% Kolliphor HS 15 via i.n., s.c., or i.p. administration. Two days (s.c.) or one day (i.n. and i.p.) after treatment all mice were i.n. challenged with  $1 \times 10^8$  *C. abortus* organisms. The naïve control group received i.n. only PBS/0.1% Kolliphor HS 15 one day before challenge. (B) Survival analysis (Kaplan-Meier survival estimate; Cox's F test; n=10 mice / group).

### **Immunopotentiating Effect of BRM Microparticles Containing Different Th1**

**Adjuvants.** AS a final step, we evaluated different Th1 adjuvants in an intranasal immunopotentiator experiment with DL-PLG BRM microparticles. C3H/HeJ mice were i.n. inoculated with 10 µg of either only RG502H microparticles (carrier control), or RG502H microparticles containing one of three Th1 adjuvants – Pluronic L121, Trehalose-di-behenate (TDB), and Resiquimod (Table 2.1). Three days after treatment, the mice were i.n. challenged with a lethal dose of  $3 \times 10^8$  *C. abortus* EBs and monitored for three weeks (Fig. 3.13A).

Starting from four days after challenge inoculation, the mice carrier control group (received only RG502H microparticles) lost body weight, became progressively sicker, lost approximately 28% of their weight, and 90% of the mice died within 21 days after challenge infection (Fig. 3.13B). The mice that received the TDB adjuvanted microparticle also followed a similar trend and lost approximately 30% body weight, and 90% of the mice died within 3 weeks after challenge (Fig. 3.13B). In contrast, mice that received either Pluronic L121- or Resiquimod adjuvanted microparticles were comparatively healthier. Starting from day 8 they experienced a minor body weight loss until day 12 of approximately 8% in Pluronic L121 group and 10% in the Resiquimod group. However, beginning day 14 after challenge inoculation, surviving mice in both groups of this group stabilized and steadily gained weight again. Importantly, mice in both groups showed significantly lower mortality by day 21 than the carrier control and TDB groups – 30% mortality for Pluronic L121 ( $p= 0.002$  vs carrier control) and 40% mortality for Resiquimod ( $p=0.019$  vs carrier control).



**Figure 3.13. Immune modulation by BRM microparticles with Th1 adjuvants Pluronic L121, TDB, and Resiquimod.** (A) Schematic presentation of BRM experimental protocol. Three days before i.n. challenge inoculation with  $3 \times 10^8$  *C. abortus*, C3H/HeJ mice were i.n. inoculated with 10  $\mu$ g spray-dried RG502H microparticles (carrier control) suspended in 20  $\mu$ l PBS/0.1% Kolliphor HS 15, or with RG502H microparticles containing either one of the three Th1 adjuvants – TDB, Resiquimod, or Pluronic L121. After challenge mice were monitored daily, and the surviving mice were euthanized on day 21. (B) Survival analysis of mice in different groups (Kaplan-Meier survival estimate; Cox’s F test (n=10 mice / group)).

## 2.4. DISCUSSION

Limitations of current vaccines, particularly against intracellular pathogens and cancer, have encouraged the application of various vaccine delivery systems in attempts to improve vaccine efficacy. Among proposed vaccine delivery systems, synthetic biodegradable polymeric microparticles have been gaining more attention, specifically regarding their advantages as an antigen/adjuvant delivery vehicle. These advantages include: their inherent features that can be tuned according to the desired antigen release profile, the ease of charge or hydrophobicity modification, and the ability to target uptake by APCs. However, the physicochemical properties of these particulates depend on a number of factors such as: preparation technique, polymer composition, hydrophobicity, molecular weight, and particle size (Allahyari and Mohit, 2015; Lima and Junior, 1999; Tracy, *et al.*, 1999). In this study, through a series of optimization approaches we developed optimal PLG microparticles, entrapped the Th1 adjuvant Pluronic L121 by spray dry technology, and investigated their immunopotential effect in an immunosuppressed C3H/HeJ mouse model. The optimization of microparticles and the *in vivo* mouse model studies were performed in simultaneous and stepwise approaches to develop a microparticulate delivery platform that can be utilized in low dose vaccine delivery against *Chlamydia abortus*.

To synthesize microparticles, we used industrially scalable spray drying technology and demonstrated that it is a perfectly suited technique to synthesize suitable microparticles for vaccine delivery. However, as previously described, microparticles characteristics are dependent on different process parameters such as inlet and outlet temperatures, spray-rate of feed, polymer concentration in the organic solvent (Conte *et*

*al.*, 1994), and the nature of the organic solvent (Gander *et al.*, 1995). Through a series of optimization approaches we standardized different spray dry parameters (feed spray rate at 3.2 mL/min, inlet temperature  $58\pm 2^{\circ}\text{C}$ ; outlet temperature  $38\pm 2^{\circ}\text{C}$ ; spray gas flow 6 bar; atomization air flow rate 500 l/h; and aspirator setting 20) to synthesize our desired microparticles with the Büchi-190 spray dryer.

We then performed an in-depth investigation on the size of the microparticles as it is a criterion of overriding importance for phagocytotic uptake of microparticles by APCs. In an early study conducted in the phagocytosis of polystyrene microspheres (0.5–4.6  $\mu\text{m}$ ) by mouse peritoneal macrophages, it was reported that maximal phagocytosis occurred for an intermediate particle size of 1.7  $\mu\text{m}$  (Tabata and Ikada, 1988). A recent study by Champion *et al.* (2008) demonstrated that 2-3  $\mu\text{m}$  polystyrene particles were optimally phagocytosed by rat peritoneal macrophages. Therefore in this study we were interested to synthesize 1 to 3  $\mu\text{m}$  PLG microparticles that would be easily phagocytosed by APCs. We found that the concentration of total solid in the feed solution as well as molecular weight of the polymer greatly influence the size of particles. We performed a concentration titration experiment of solids in feed solution and determined that a 2% concentration is ideal to synthesize optimal microparticles of 1 to 3  $\mu\text{m}$  diameter with a wide range of DL-PL or DL-PLG polymers. In a subsequent *in vivo* mouse model experiment we found that DL-PLG-PEG microparticles with 2.66  $\mu\text{m}$  diameter were efficiently engulfed by macrophages in the lung.

The ultimate goal of our approach was to utilize the microparticles as vaccine delivery vehicle in our low antigen dose vaccine platform against *Chlamydia abortus*. Therefore in this study we investigated their potential as immunostimulant while

delivering a Th1 adjuvant. To evaluate the immunopotentiating effect we used C3H/HeJ mice in the *C. abortus* respiratory disease model. As this mouse has a truncated Tlr4 protein, they fail to respond to LPS, including chlamydial LPS. This results in a lack of a protective innate immune response against chlamydial challenge inoculation, and subsequent disease with high mortality within 2 to 3 weeks. We found that administration of DL-PLG-PEG or DL-PLG (RG502H) microparticles containing the Th1 adjuvant Pluronic L121, one or three days before challenge inoculation of lethal *C. abortus*, significantly reduces the mortality of these mice. Therefore these microbeads at least partially substituted for the absent LPS response in these mice. We also demonstrated that the immunopotentiating effect can be obtained by administration either by the subcutaneous, intraperitoneal, or intranasal route. However, the effect is highly dose dependent, with an optimal dose range of 5 to 10  $\mu\text{g}$  per mouse, and lower as well as higher doses were actually counterproductive. Similarly, only one treatment, one or three days before challenge inoculation was sufficient, while multiple dosages failed to induce protection, and even exacerbated disease, most likely due to an aberrant inflammatory response derived from overstimulation. Finally, we also demonstrated that the particulate delivery is effective with resiquimod, a different Th1 adjuvant.

In conclusion, microparticles based on synthetic biodegradable polymers are an extensive area of research for effective delivery of vaccine antigens and pharmaceutical drugs in human and veterinary medicine. In the current study we focused on the synthesis of DL-PL and DL-PLG microparticles using spray drying technology. The optimization of spray drying parameters and *in vitro* and *in vivo* characterization of microparticles that

have been performed in this study will significantly benefit in immune studies of polymeric microparticles synthesized by an industrially scalable spray dry technology.



### 3.5. MATERIALS AND METHODS

#### **Preparation of Microparticles with or without an Adjuvant.**

Chemicals and reagents. The following chemicals were obtained from commercial suppliers and used as received: dichloromethane (Sigma Aldrich, St. Louis, MO, USA), Kolliphor<sup>®</sup> HS 15 [synonym: Macrogol (15)-hydroxystearate, Polyethylene glycol (15)-hydroxystearate, Polyoxyethylated 12-hydroxystearic acid, Solutol<sup>®</sup> HS 15] (BASF Corp., Germany), Lactopress<sup>®</sup> Anhydrous Microfine (DFE Pharma, Germany), Benzalkonium chloride (Sigma Aldrich, St. Louis, MO, USA).

Adjuvants. The adjuvants used in this experiment were: Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Synonym- PEG-PPG-PEG, Pluronic L121) (a product of BASF Corp., Germany, obtained from Sigma Aldrich, St. Louis, MO, USA), Trehalose Dibehenate (TDB) (Avanti Polar Lipid, Inc., Alabaster, AL 35007); and Resiquimod (R848) (Invivogen, San Diego, CA, USA).

Polymers. A list of synthetic biodegradable polymers used in this study is shown in Table 3.4, with their physiochemical properties as indicated by the manufacturers.

Spray dryer. The microencapsulating experiments were carried out by use of a bench-top Büchi mini spray dryer model B-190 (Büchi Labortechnik AG, Flawil, Switzerland). The main components of the system are the feed system of the of the microencapsulating formulation, constituted by a peristaltic pump, a two fluid atomizer (nozzle diameter of 0.7 mm) and an air compressor; the feed system of the drying gas, constituted by a blower, an air filter and a temperature control system. The dried product was collected in a Büchi high performance glass cyclone. Fig. 3.14 shows the schematic representation of process diagram of the spray dryer used.

*Microparticle production.* Stock solutions of polymers and adjuvant were prepared separately with DCM at 50 mg/ML. Feedstock solutions of adjuvants and / or polymers were prepared in DCM by adding the desired volume of the stock solution at the concentration of either 1%, 1.2%, 2.4%, or 4.8% of total solids, as required for different experiments. The ratio of the polymers and adjuvants were either 2:1 or 6.5:3.5, as needed.

Microparticles were obtained by spraying the prepared feedstock solution through the nozzle of the spray dryer. The spray rate of feed was constant at 3.2 ml/min except for one optimization experiment when 8 ml/min was used. Identical drying conditions (inlet temperature  $58\pm 2^{\circ}\text{C}$ ; outlet temperature  $38\pm 2^{\circ}\text{C}$ ; spray flow 6 bar; atomization air flow rate 500 l/h; and maximum aspirator setting 20) were used for all samples. The spray-dried microparticles obtained were collected in glass vials sealed with Parafilm and stored in a desiccator at room temperature until further characterization.

**Table 3.4. Lactic / glycolic acid polymer samples used in this study**

Chemical Name	Abbreviation	Source	Synonym	IV(dL/g)	Mw (Da) (average)
50:50 Poly(DL-lactide-co-glycolide)	50:50 DL-PLG	Durect Corp.	Lactel <sup>®</sup> B6010-1	0.39	16,050
50:50 Poly(DL-lactide-co-glycolide)	50:50 DL-PLG	Durect Corp.	Lactel <sup>®</sup> B6010-2	0.59	36,330
Methoxy poly(ethylene glycol)- 50:50 DL-poly(lactide-co-glycolide)	50:50 DL-PLG-PEG	Evonik	--	0.79	64,760
75:25 Poly(DL-lactide-co-glycolide)	75:25 DL-PLG	Durect Corp.	Lactel <sup>®</sup> B6007-1	0.69	105,080
Poly(D,Llactide)	50:50 DL-PL	Evonik	Resomer <sup>®</sup> R202S	0.20	16,740
Poly(D,Llactide)	0:50 DL-PL	Evonik	Resomer <sup>®</sup> R203S	0.32	33,470
50:50 Poly(D,Llactideco-glycolide)	50:50 DL-PLG	Evonik	Resomer <sup>®</sup> RG502H	0.20	12,000

IV-Intrinsic viscosity; Mw- Molecular weight; -- not found

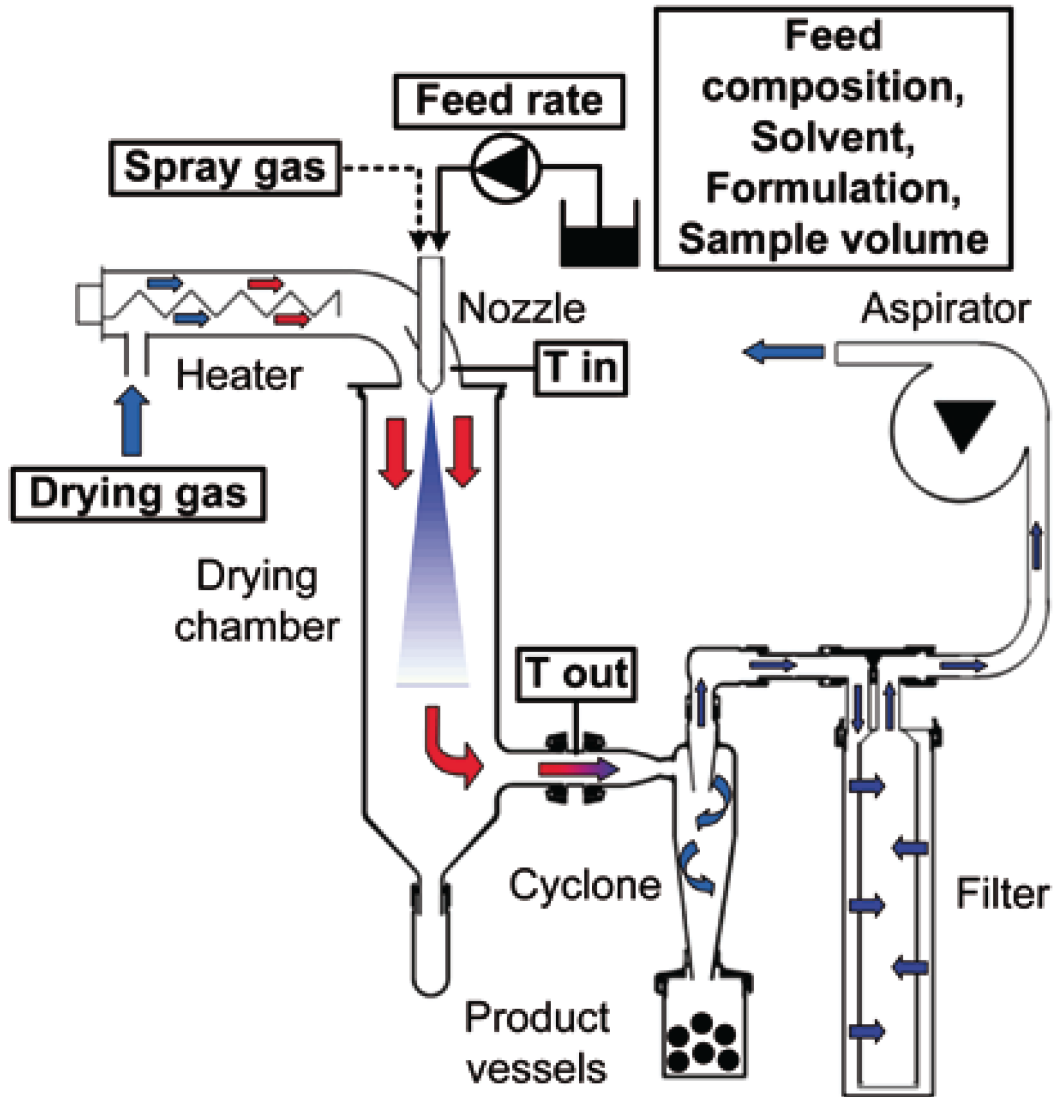


Figure 3.14. Process diagram of the Mini Spray Dryer B-190 model with process parameters. (Reproduced from Arpagaus *et al.*, 2010)

## **Microparticle Characterization.**

*Morphology.* The surface morphology and shape of the spray-dried microparticles was visually assessed by the Zeiss EVO 50 scanning electron microscope (Carl Zeiss, Germany). Briefly, spray-dried microparticles were placed on adhesive carbon tapes mounted on aluminum stubs followed by sputter coating with a thin layer (5 nm) of gold particles for 2 minutes with an EMS 550x auto sputter coater (Electron Microscopy Sciences, Hatfield, PA) under an Argon gas purge. The specimens were then imaged at an accelerating voltage of 20 kV energy with magnification of 3000, 5000 and 10000 times.

*Particulate size analysis.* To quantify the size of the microparticles, the image analysis software, ImageJ, was used to measure the Feret's diameter (area-based diameter of non-spherical particles) of at least 100 randomly distributed microparticles per sample. Due to high agglomeration of the particles, as observed in scanning electron microscope (SEM) image, the Feret's diameter of each particle was manually measured in the ImageJ software instead of using automatic measurement procedures.

*In vitro degradation kinetics.* Microparticles were synthesized from the feed solution containing 2% of total solid comprised of either 50:50 DL-PLG or 50:50 DL-PLG or 50:50 DL-PLG-PEG or 75:25 DL-PLG or 50:50 DL-PL (R202S) or 50:50 DL-PL (R203S) and Pluronic L121 at 6.5:35, respectively, in DCM. Fifty to 100 mg of each of six different polymeric microparticles were suspended in unbuffered suspension solution (0.001% benzalkonium chloride and 0.5% Kolliphor HS-15 in dH<sub>2</sub>O) in a 50 mL glass vial, sonicated for 10 minutes, and incubated at 37°C on the shaking incubator at 50 rpm. The pH of the medium of each preparation was measured after 1 hour incubation (day 0), then 24 later, then on days 3, 5, and 7, and after this first week once every week by a digital pH

meter. The pH of lactic acid/glycolic acid is correlated to the concentration of these acids (Fig 3.10A). Therefore, using the pH of poly-lactic-co-glycolic acid polymers, the concentration of free acids in aqueous suspensions of these polymers can be determined. Using the total amount of polymer added to 50 mL suspension buffer, the pH measurement over time, after correction for residual free acid in the polymer, can be used to determine the fraction of degraded polymer (free acid) in total polymer (bound acid). Further correlation to the initial molecular weight of the polymer using the formulas below allows determination of the reduction in molecular weight of the polymer over time by degradation.

$$\text{End MW}_{\text{polymer}} = \frac{\text{starting MW}_{\text{polymer}}}{1 + \log_2\left(\frac{\text{starting molecules} + \text{broken bonds}}{\text{starting molecules}}\right)}$$

$$\text{starting molecules} = \frac{\text{starting weight polymer (g)} \times \text{Avogadro's number}}{\text{starting MW}_{\text{polymer}}}$$

$$\text{fraction degraded} = \frac{10^{\text{Log\_mg\_monomer\_ratio\_formula}}}{\text{starting weight polymer (mg)}}$$

$$\text{broken bonds} = \frac{\text{fraction degraded} \times \text{starting weight polymer (g)} \times \text{Avogadro's number}}{\text{average MW}_{\text{monomer}}}$$

*In vitro API release kinetics.* Microparticles were synthesized from the DCM feed solution containing 2% of total solid comprised of either 50:50 DL-PLG or 50:50 DL-PLG or 50:50 DL-PLG-PEG or 75:25 DL-PLG or 50:50 DL-PL (R202S) or 50:50 DL-PL (R203S) and Pluronic L121 at 6.5:35 ratio, respectively, along with Alexa Fluor 488 conjugated to a 20-mer *C. abortus* peptide to (50 nM of peptide QQQEPSKPSIQPEK<sup>AF488</sup>HYQDQS added to 50 mg microparticle solids). Twenty mg of each of the six spray-dried microparticles were suspended in 5 mL suspension buffer (0.001% benzalkonium chloride and 0.5% Kolliphor HS-15 in PBS) and incubated at 37°C on a rotating mixer. After 1 hour (day 0), 24 hours (day 1), and then every 3 days for a

total of 46 days, the beads were sedimented at  $2,600\times g$  for 15 min, and the complete supernatant was removed, a 1.5 mL aliquot was stored at  $-80^{\circ}\text{C}$ , and the beads were re-suspended in new suspension buffer. The 521 nm fluorescence of the supernatant was measured in a spectrophotometer. Peptide released at each sampling time point was calculated as the fraction of the total release over 46 days.

*In vivo macrophage uptake.* Mice received intranasally PLG-PEG-Pluronic L121 microparticles with entrapped *C. abortus* peptide conjugated with Alexa Fluor 488. One day later, mice was anesthetized by intraperitoneal injection of ketamine and xylazine, and the lung was perfused with PBS and fixed with Z-Fix. The fixed lung was treated under vacuum with successive sucrose gradient solutions (10%, 20%, and 30%) for dehydration and cryoprotection. The sucrose infiltrated lung was embedded with 2 ml Neg-50 (Richard-Allan Scientific) in a cryomold boat and the prepared lung cryomold was stored at  $-80^{\circ}\text{C}$ . Using a cryostat (HM 550 Series, Richard-Allan Scientific), lung sections were cut to 8  $\mu\text{m}$ , mounted onto Superfrost/Plus microscope slides (Fisher Scientific), air dried in the dark (to avoid photobleaching of fluorescent beads), and blocked with antibody dilution buffer (5% BSA and 10 % donkey serum in PBS). Sections were stained with F4/80 rat IgG2b monoclonal antibody (1:500 overnight; Fisher), followed by donkey anti rat IgG Alexa Fluor 594 (1:500 for 1 h; Fisher). Nuclei were counterstained with DAPI (4, 6-diamidino-2-phenylindole) in mounting medium (Invitrogen). Immunofluorescence was examined and digital micrographs were taken using a Nikon Eclipse TE2000-E confocal microscope and analyzed using NIS-Elements software (Nikon).

### **Immunopotentiating Effect of BRM Microparticles.**

Preparation of BRM microparticles suspension. One mg of the BRM microparticles were mixed with 3 mg of lactose anhydrous microfine ((DFE Pharma, Germany) in a glass vial, then dissolved in 1 mL of suspension buffer (0.001% benzalkonium chloride and 0.5% Kolliphor HS-15 in PBS), and sonicated for 15 minutes in cold water. From this initial suspension, the final BRM suspension was prepared at appropriate dilutions in suspension buffer, as required for different dosage for different experiments.

Chlamydia abortus. *C. abortus* strain B577 (ATCC V-656) was grown in Buffalo Green Monkey Kidney monolayer cell cultures, purified by differential centrifugation, and quantified as previously published (Li *et al.*, 2005). Purified infectious EBs were suspended in sucrose-phosphate-glutamate (SPG) buffer, stored in aliquots at  $-80^{\circ}\text{C}$ , and their infectivity was confirmed in female A/J mice.

Animal and BRM microparticles administration. Inbred female C3H/HeJ mice were sourced from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age. Udel “shoebox” type cages with spun fiber filter tops were maintained in static air or ventilated cage racks. Five to ten animals were housed per cage in a temperature-controlled room on a 12-hour light/dark cycle, with ad libitum access to water and standard rodent chow. All animal experiments were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Each group was consisted of 10 mice. Mice received the BRM preparation under light isoflurane inhalation anesthesia by intranasal administration of 20  $\mu\text{l}$  or subcutaneous (between the shoulder blades) or intraperitoneal injection of 200



µl of BRM microparticles dissolved in suspension buffer at 6 weeks of age at different time and dosage as required for different experiment set up.

Control animals. Mice that received either only PBS (naïve) or microparticles without adjuvant (carrier control) served as controls.

Intranasal *C. abortus* Challenge and Monitoring. All mice were challenged with  $1 \times 10^8$  or  $3 \times 10^8$  *C. abortus* elementary bodies suspended in 20 µl sucrose-phosphate-glutamate buffer. In the first experiment for model analysis, all animals were weighed during challenge infection and every second subsequent day until euthanasia on day 10 post challenge. Mice were monitored every day and death, if any, was recorded. Ten days after challenge, mice were sacrificed by CO<sub>2</sub> inhalation and weighed. Lungs were collected, weighed, snap frozen in liquid nitrogen, and stored at -80°C until further processing. Percent lung weight increase was based on naïve lung weights of 134 mg for adult female C3H/HeJ mice. Subsequently, in all experiments mice were monitored daily after challenge, mortality was recorded, and surviving mice were euthanized 21 days after challenge inoculation.

**Data Analysis.** All analyses were performed with the Statistica 7.1 software package (StatSoft, Tulsa, OK). Results were analyzed by survival analysis and Cox F test, Student's t-test, linear regression, and one-way and repeated measures ANOVA with Tukey's honest significant differences test for correction of the p value in multiple comparisons. P values  $\leq 0.05$  were considered significant.

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## CHAPTER 4

### BIODEGRADABLE MICROPARTICLE LOW-DOSE PEPTIDE VACCINE AGAINST *C. abortus*

#### 4.1 INTRODUCTION

***Chlamydia abortus* and Infection.** *Chlamydia abortus*, formerly known as *C. psittaci* serotype I, is a non-motile, coccoid, obligate intracellular, Gram-negative bacterium that belongs to the family *Chlamydiaceae* (Everett *et al.*, 1999; Sachse *et al.*, 2015). It is considered a very homogeneous species with low genetic heterogeneity (Sait *et al.*, 2011; Laroucau *et al.*, 2009) with a genome size of 1,144,377-base pairs that contains 961 predicted coding sequences (Thomson *et al.*, 2005).

The organism can efficiently colonize the placental trophoblasts and is one of the major causative agents of abortion and fetal loss in sheep, goats, and cattle in many countries (Borel *et al.*, 2004; Campos-Hernández *et al.*, 2014; Kalender *et al.*, 2013). In 1936, Greig first described chlamydial abortion in sheep in Scotland and named it enzootic abortion in ewes (EAE) (Greig, 1936). The infection of susceptible pregnant ewes may cause abortion, still births, or weak lambs (Aitken, 1990). Usually, immunologically naïve flocks receive the infection from a latently infected animal with the agent being subsequently transmitted from aborting ewes via shedding of large amounts of infectious *Chlamydia* in the foetal membranes and in vaginal discharges (Aitken, 1993). In newly infected flocks, up to 30% of ewes may abort in the last trimester of gestation or give birth

to weak or dead lambs. After abortion, ewes in these flocks may develop a protective immunity, with the incidence remaining at 5-10% in further lambing seasons, with essentially all aborting ewes being primiparous animals (Rodolakis *et al.*, 1998; Aitken, 2007). *C. abortus* also been associated with cases of abortion in pigs, horses, rabbits, guinea pigs, and mice (Everett *et al.*, 1999). Its association with bovine infertility (De Graves *et al.*, 2004) and mastitis (Corner *et al.*, 1968; Rønsholt and Basse, 1981) has also been reported. Finally, the pathogen represents a threat to human health because it can cause zoonotic infections; pregnant women who are exposed to infected animals are also at risk of abortion and life-threatening illness (Longbottom and Coulter 2003; Pospischil *et al.*, 2002; Walder *et al.*, 2003 and 2005).

*C. abortus* in the United States. In the United States chlamydial abortion was first reported in 1958 in Montana sheep and the organism was first isolated from the outbreak in 1960 (Young *et al.*, 1958; Parker *et al.*, 1960). Subsequently, the prevalence and distribution of the disease was reported in sheep flocks in Idaho (Frank *et al.*, 1962), California, and Oregon (McKercher *et al.*, 1964). During that time, a similar chlamydial agent was also isolated from abortion in cattle (Storz *et al.*, 1960) and in goats (McCauley *et al.*, 1968) in California. Currently, it is the most common cause of abortion in goats in the USA (Merck Veterinary Manual).

**Economic Significance.** *C. abortus* is endemic in ruminants throughout the world and is considered as one of the economically most important animal pathogens of domesticated animals (Gokce *et al.*, 2007). It is recognized as a major cause of abortion and lamb loss throughout the world, except for in Australia and New Zealand (Kerr *et al.*,

2005; Longbottom *et al.*, 2013; Nietfeld, 2001). In the United Kingdom, it is estimated to cost the sheep industry more than £20 million a year (Aitken *et al.*, 1990).

In the United States, there are 5.32 million head of sheep (USDA, NASS, Sheep and Goats, Jan. 29, 2016). According to the American Sheep Industry Association, in 2010 the United States produced approximately 163 million pounds of lamb and mutton, and 30.6 million pounds of greasy wool are produced. There were 2,621,514 goats in the United States as of 2012 (USDA, NASS, Sheep and Goats, 2012). Thus, considering the severity of economic losses by EAE in the sheep industry, its importance in the USA cannot be underestimated. Furthermore, its zoonotic importance also demands strict measures to control and prevention of this disease.

**Treatment and Control of *C. abortus* Infection.** When signs of EAE are first observed or if there is perceived threat of EAE, tetracycline is usually used in pregnant sheep flocks to reduce the incidence of abortion and lamb losses (Aitken *et al.*, 2007). However, this combined therapeutic/prophylactic approach does not guarantee to prevent abortion or to prevent the shedding of *Chlamydiae* from infected ewes at parturition (Longbottom and Coulter, 2003). Moreover, employing a prophylactic strategy always risks the development of antibiotic resistance. While there is no report of tetracycline resistance in *C. abortus*, it has been found in *Chlamydia suis* strains isolated from pigs (Lenart *et al.*, 2001; Dugan *et al.*, 2004). However, although antibiotic resistance in *Chlamydiae* remains a relatively rare clinical occurrence *in vivo*, the potential to evolve resistance through the accumulation of point mutations under selective antibiotic pressure has been shown to occur *in vitro* (Sandoz and Rockey, 2010). Furthermore, tetracycline

resistance can be transferred horizontally, suggesting that maintained antibiotic pressure could drive the spread of resistance (Suchland *et al.*, 2009). In addition, the prolonged use of antibiotics in livestock is also a consumer issue due to public concerns over residues in the food chain. In general the use of antibiotic is not an effective way to manage EAE, it being more desirable to control infection by management and vaccination.

**Vaccination against *C. abortus*.** It has been mentioned earlier (Chapter I) that live-attenuated vaccines are commercially available against *C. abortus* and extensively used in the sheep industry. However, failure of this vaccine has been reported in two recent studies and DNA sequencing linked the live-attenuated vaccine strain of *C. abortus* with cases of EAE (Wheelhouse *et al.*, 2010; Laroucau *et al.*, 2009). In their studies, both groups used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to identify single nucleotide polymorphisms (SNPs) associated with the 1B vaccine strain of *C. abortus*. These SNPs were found to be unique to the vaccine strain when compared to its parent strain AB7 and thus used to develop PCR-RFLP markers for discriminating the vaccine strain from wild-type *C. abortus* (Burall *et al.*, 2009; Wheelhouse *et al.*, 2010). Subsequent analyses of placental clinical samples derived from sheep that had experienced OEA revealed the presence of sequences that matched the vaccine strain.

Moreover, since it is a live attenuated vaccine, there are a number of recommendations and restrictions on its use. Neither its administration in pregnant ewes is allowed, in case it reaches the placenta, nor should pregnant women handle the vaccine or handle sheep that have been recently vaccinated to avoid possible zoonotic transmission. In addition, with the current serodiagnostic tests it is not possible to discriminate infected



from vaccinated animals (DIVA), which could be achieved only with a sub-unit vaccine as opposed to the live-attenuated strain. Therefore, development of a safe and efficacious subunit vaccine against *C. abortus* is urgently needed.

In our previous study we found that, using femtoMoles doses of synthetic overlapping 20-mer peptides from three vaccine candidate proteins of *C. abortus* provides significant protection against *C. abortus* challenge. In a subsequent study we also developed a biodegradable polymer based microparticle delivery platform which showed significant immunopotentiating effect when tested against *C. abortus* with the Th1 adjuvant, Pluronic L121. In this study we examined whether a delivery of femtoMole-dosed of peptide of vaccine candidate antigens via this microparticulate delivery platform would induce complete and consistent protection against *C. abortus*.

## 4.2. HYPOTHESIS

We hypothesize that low dosage of protective peptide antigens of *Chlamydia abortus* will induce a protective Th1 response against *C. abortus* challenge, if delivered with a safe and efficient adjuvant in a biodegradable synthetic polymeric particulate delivery vehicle.

## 4.3. OBJECTIVES

The aim of this investigation was to examine in a murine model of *C. abortus* respiratory disease the effect of

- 1) varying the dosage of vaccine antigen by subcutaneous and intranasal administration via the spray-dried biodegradable microparticulate delivery vehicle on the protection efficacy against *Chlamydia abortus* challenge, and
- 2) to develop a biodegradable microparticulate vaccine that induces protective Th1 immune response against *C. abortus*.

#### 4.4. RESULTS

**129S6 mouse model of chlamydial respiratory disease.** In the initial low-antigen-dose vaccine platform experiments (Chapter 2) we used A/J mice as a model for chlamydial respiratory disease. However, in later experiments the phenotype of these A/J mice had changed, and naïve mice showed strong resistance to a lethal dose of *C. abortus* inoculation, essentially equal to *C. abortus* live-vaccine immunized mice. We attributed this consistent change to genetic drift due to random point mutations in the genome of founder mice, a phenomenon that is frequently observed in inbred mouse strains. This genetic drift can eventually affect the whole mouse colony and result in a shift or even complete reversal of the phenotype of this inbred mouse strain for specific disease models (Casellas, 2011). Due to the lack of a differential *C. abortus* disease response of naïve versus immunized A/J mice we sought to identify another suitable inbred mouse strain. We attempted to find a strain with a deficiency in the innate immune response that is essential for a disease phenotype of naïve mice, and therefore would demarcate unprotected naïve mice from mice with vaccine-induced protection.

In the BRM experiments (chapter 3) we used C3H/HeJ mice which were highly susceptible and frequently succumbed to *C. abortus* challenge due to their deficiency in Tlr4 (LPS ligand)-mediated innate immune recognition. However, the complete lack of an early innate response also compromises any adaptive response during challenge infection. Therefore minimal or no protection of these mice by adaptive immunity is achievable, even after low-dose *C. abortus* infection (live-vaccination), followed by sustained antibiotic suppression of chlamydial replication after one week that prevents a lethal outcome of the low-dose infection. Therefore, these mice are not a suitable model for a vaccine study.

Another inbred mouse strain with a known functional deficiency of the innate immune response is the 129 mouse strain. These mice have a defect in the effector arm of the innate immune system with aberrant DAP12 signaling in NK cells that prevents interferon- $\gamma$  secretion by these cells (McVicar *et al.*, 2002). We anticipated that due to the ineffective NK cell functionality the naïve mice would not be able to effectively control chlamydial multiplication with the innate immune response. A corollary of high chlamydial loads would be that these mice may be prone to develop non protective Th2 immunity and would therefore may not be able to successfully clear the bacteria after high-dose challenge inoculation.

Because of ready availability, we decided to examine the 129S6 mouse strain provided by Taconic Labs as potential model for chlamydial respiratory disease. We performed an experiment similar to that of the chlamydial respiratory disease in the A/J mouse model (Chapter 2). We first examined the 129/S6 mouse strain as chlamydial lung challenge model by contrasting the disease outcome 10 days after challenge inoculation between the negative controls – naïve mice which received only PBS and carrier control mice that received microparticles containing adjuvant but no vaccine antigens. As a potential positive control we also examined mice that had been previously infected (live-vaccinated) with a low-dose of the *C. abortus* challenge strain. All mice were euthanized after 10 days of challenge inoculation. As readouts of disease outcome, changes in the post-challenge body and lung weights, and the lung burden of *C. abortus* as determined by qPCR, were evaluated.

As shown in Fig. 4.1A, following *C. abortus* challenge inoculation, mice in the naïve and carrier control groups progressively lost body weight which was in average 5%

and 9%, respectively, after 10 days of challenge inoculation. In contrast live-vaccine mice showed an initial body weight loss of 2% on day 2 post challenge, however, subsequently they gained weight, up to 3% on day 10 post challenge. Analysis of individual mouse body weight loss data revealed a strong bimodal response in both naïve and carrier control mice. In the naïve group, two mice became highly diseased, sharply lost body weight following challenge inoculation and lost >30% of weight and finally died on day 10 days after challenge. Among the remaining 10 mice, five mice had an average 5% body weight loss whereas the others maintained body weight with an overall median body weight loss of 2.02 % (Fig. 4.1B). Although no mice died in carrier control group, 60% of the mice lost 5 to 10% body weight, with a median body weight loss of 9.05% after 10 days of challenge (Fig. 4.1B). In contrast, none of the live vaccine mice showed any sign of disease and steadily gained body weight after challenge inoculation as indicated by negative median value of 1.81% for the body weight loss after 10 days of challenge (Fig. 4.1B). Due to such bimodal responses, the data did not follow a normal distribution and required non-parametric statistical analyses. Therefore, we analyzed the data with the non-parametric Kruskal-Wallis (K-W) test which is based on rank-transformed data and is analogous to one-way ANOVA. The initial data are transformed to their normally-distributed ranks and then analyzed by ANOVA. Although, K-W analysis showed no significant differences ( $p=0.441$ ; Fig 4,1B) among the groups, the mean rank of 17.5, 18.4, and 13.4 for the naïve, carrier control, and live vaccine group, respectively (Fig. 4.1E), indicated that the naïve and carrier control groups were relatively more sick than the live-vaccine control.

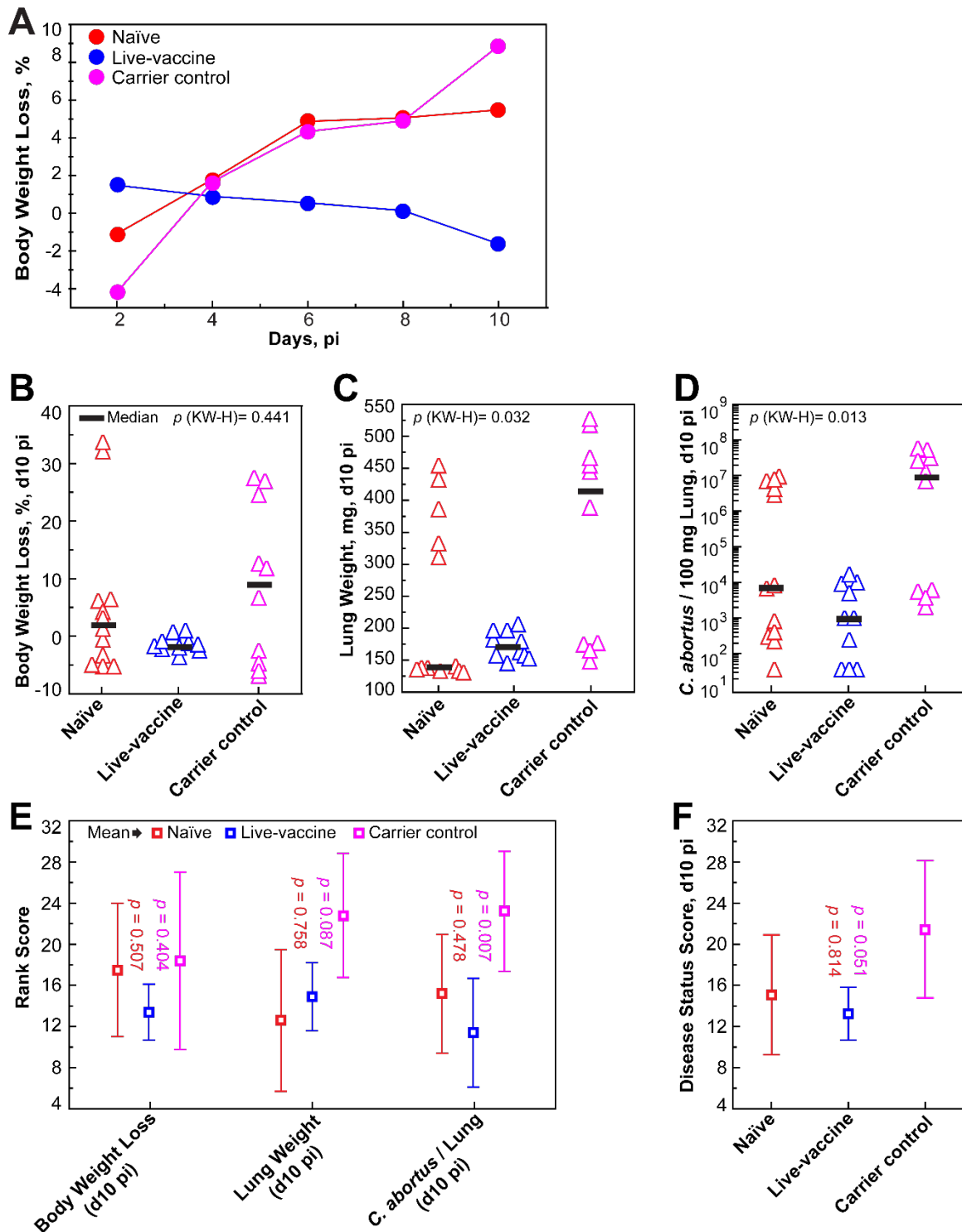
Analysis of 10 days post challenge lung weight data also revealed a strong bimodal response for naïve and carrier controls (Fig. 4.1C). Ten days after challenge inoculation,

40% of the naïve mice had lung weights of more than 300 mg. However, the remaining 60% mice had lung weights similar to unchallenged control mice (~140 mg) that resulted in the overall median value of 138 mg (Fig. 4.1 C). In the carrier control group, 60% of the mice had lung weights of more than 350 mg with a median of 415 mg after 10 days of challenge (Fig. 4.1C). None of the live vaccine mice showed any lung inflammation and all had consistently similar and slightly elevated lung weights with a median of 169.5 mg (Fig. 4.1C). The K-W analysis showed significant differences among the groups ( $p=0.032$ ; Fig. 4.1C) with mean rank of 12.58, 22.28, and 14.9, respectively, (Fig. 4.1E).

All groups showed bimodal chlamydial lung burdens (Fig. 4.1D). The naïve mice had median chlamydial loads of 7,943 genomes/100mg lung, whereas the live vaccine control mice had a median chlamydial load of 1,047 genomes/100mg lung (Fig. 4.1D). Although, the live-vaccine mice cleared the bacteria relatively better than the naïve mice, they did not differ significantly ( $p^{Dunnnett}=0.478$ ), as indicated by the K-W mean rank score of 15.16 and 11.4, respectively (Fig. 4.1E). However, the carrier control group mice had a very high chlamydial burden with a median of 9,332,543 genomes/100mg lung and the mean K-W rank score of 23.20 which was highly significantly higher than the live-vaccine control ( $p^{Dunnnett}= 0.007$ ; Fig. 4.1E).

To assess the overall disease status, we combined the individual rank score of body weight loss, lung weight increase, and chlamydial loads and defined it as disease status score. As shown in Fig. 4.1F, the carrier control group mice were significantly more sick than the live-vaccine control with the mean rank score of 21.47 and 13.23, respectively, ( $p^{Dunnnett}= 0.051$ ; Fig. 4.1F). However, the naïve mice showed a rank score of 15.08 which was similar to that of live-vaccine control.

In sum, the naïve 129S6 mice show bi-modal susceptibility against *C. abortus*, and therefore do not represent a sharp contrast to the live-vaccine protective control. However, administration of adjuvanted microparticles without vaccine antigen aggravated the disease outcome after challenge inoculation, due to innate immune stimulation by the carrier control microparticles. Since the innate immune system of the 129S6 mice has the NK cell defect in its effector function, the resultant inflammation is ineffective at clearing the chlamydial infection, but exacerbates disease. Due to this pronounced contrast of the carrier control mice to vaccine-protected mice, the carrier controls allow maximum readout amplitude between presence and absence of protection in the chlamydial lung disease model.



**Figure 4.1.** *C. abortus* respiratory disease in 129S6 mice. (A) Percentage of daily body weight loss from day 2-10 after intranasal challenge with  $3 \times 10^8$  *C. abortus* elementary bodies. (B-D) Distribution of individual mouse data for body weight loss, lung weight, and *C. abortus*, respectively, 10 days after challenge. (E) Rank scores (mean  $\pm$  95% CI) as obtained following rank



transformation of the data, starting at 1 for the lowest rank, as shown in B-D. (F) Mean combined scores for body weight loss, lung weight, and chlamydial lung burden as shown in E. The differences of the live-vaccine control to naïve and carrier controls was determined by post-hoc Dunnett test. n = 12 (naïve) or 10 (live-vaccine and carrier control).

**Rationale Dosage of Peptides in Vaccine Microparticles.** As demonstrated in Chapter 3, that polymer and the Pluronic L121 adjuvant as a ratio of 6.5:3.5 produced optimal microparticles, and 10  $\mu\text{g}$  of total spray dried microparticles per mouse dose elicited a substantial immunopotentiating response. A mixture of 6.5 parts any PLG polymer and 3.5 parts Pluronic L121 has a specific weight of 1.22  $\text{g}/\text{cm}^3$  (equals 1.22  $\mu\text{g}/10^6 \mu\text{m}^3$ ). Therefore, a mouse dose of 10  $\mu\text{g}$  of PLG:Pluronic L121 (6.5:3.5) has a volume of  $8.2 \times 10^6 \mu\text{m}^3$ . As shown in Chapter 3, optimal spray dry parameters resulted in 1.5 to 2.6  $\mu\text{m}$  spherical DL-PL and DL-PLG particles, normally distributed within the range from 1 to 4  $\mu\text{m}$  (Fig. 3.3B). The volume of a bead with diameter of 2  $\mu\text{m}$  is 4.19  $\mu\text{m}^3$  (volume of sphere =  $4 \pi r^3/3$ ). Thus, a mouse dose of 10  $\mu\text{g}$  of PLG:Pluronic L121 (6.5:3.5) beads of 2  $\mu\text{m}$  diameter contains  $(8.2/4.19) \times 10^6 \mu\text{m}^3 = 1.96 \times 10^6$  microparticles.

As vaccine antigens, we used 20-mer overlapping peptide antigens derived from five vaccine candidate proteins of *C. abortus*. One femtoMol ( $10^{-15}$  Mol) of an average 20-mer peptide has a molecular weight of  $\sim 2,200$  Dalton, and hence contains  $\sim 6 \times 10^8$  molecules. For assured antigenic stimulation, each microsphere should contain a minimum number of peptide molecules. Given a Poisson distribution of the peptides in microparticles and the normal distribution of microparticle diameter around 2  $\mu\text{m}$ , at least 6 peptide molecules per microparticle should be present to assure with more than 95% probability the presence of a peptide in a microparticle. An amount of 0.02 femtoMoles (fM) of a 20-mer peptide contains  $12 \times 10^6$  peptide molecules. Thus, when 0.02 fM of each peptide is added to a 10  $\mu\text{g}$  mouse dose of a microparticle vaccine containing  $1.96 \times 10^6$  of 2  $\mu\text{m}$  diameter microparticles, each microparticle will contain on average 6.122 molecules of this peptide. Therefore, the 0.02 fM 20-mer peptide antigen dose is the minimum

required for consistent peptide antigen delivery in a 10 µg mouse dose of a low antigen-dose vaccine with microparticles of an average 2 µm diameter.

**Inclusion of Peptides in Microparticles Reduces Disease.** As an initial approach for microparticle peptide vaccine development, we examined whether inclusion of femtoMoles of *C. abortus* vaccine candidate 20-mer overlapping peptide antigens influenced the disease outcome in the 129S6 mouse strain. The mice received subcutaneously three different dosages of peptide antigens in 10-fold increasing dilution - starting from the possible lowest dose of 0.02fM to 2.00 fM in 10 µg PLG-PEG:Pluronic L121 (6.5:3.5) microparticles, or microparticles without peptide antigens as carrier controls. Six weeks after a single vaccination, the mice were intranasally challenged with  $3 \times 10^8$  *C. abortus* organisms, and euthanized 10 days later.

As shown in Fig. 4.2, microparticles containing peptide antigens reduced the disease as indicated by lowered body weight loss, lowered lung weight, and lowered chlamydial lung loads in all three peptide vaccine mice. Ten days after *C. abortus* challenge, the carrier control mice had a median body weight loss of 9.05%, whereas the mice which received microparticles with 0.02 fM and 2.00 peptides gained body weight with the negative median of 3.87% and 2.34%, respectively (Fig. 4.2A). The 0.2 fM peptide dose mice had a marginal body weight loss of 0.20% (Fig. 4.2A). Linear regression analysis showed a trend towards negative correlation of body weight loss with peptide dose, although the correlation failed to reach significance ( $r=0.226$  and  $p=0.160$ ; Fig. 4.2A). The K-W analysis of the data revealed no significant difference among the groups, due to the strong bimodal disease outcome.

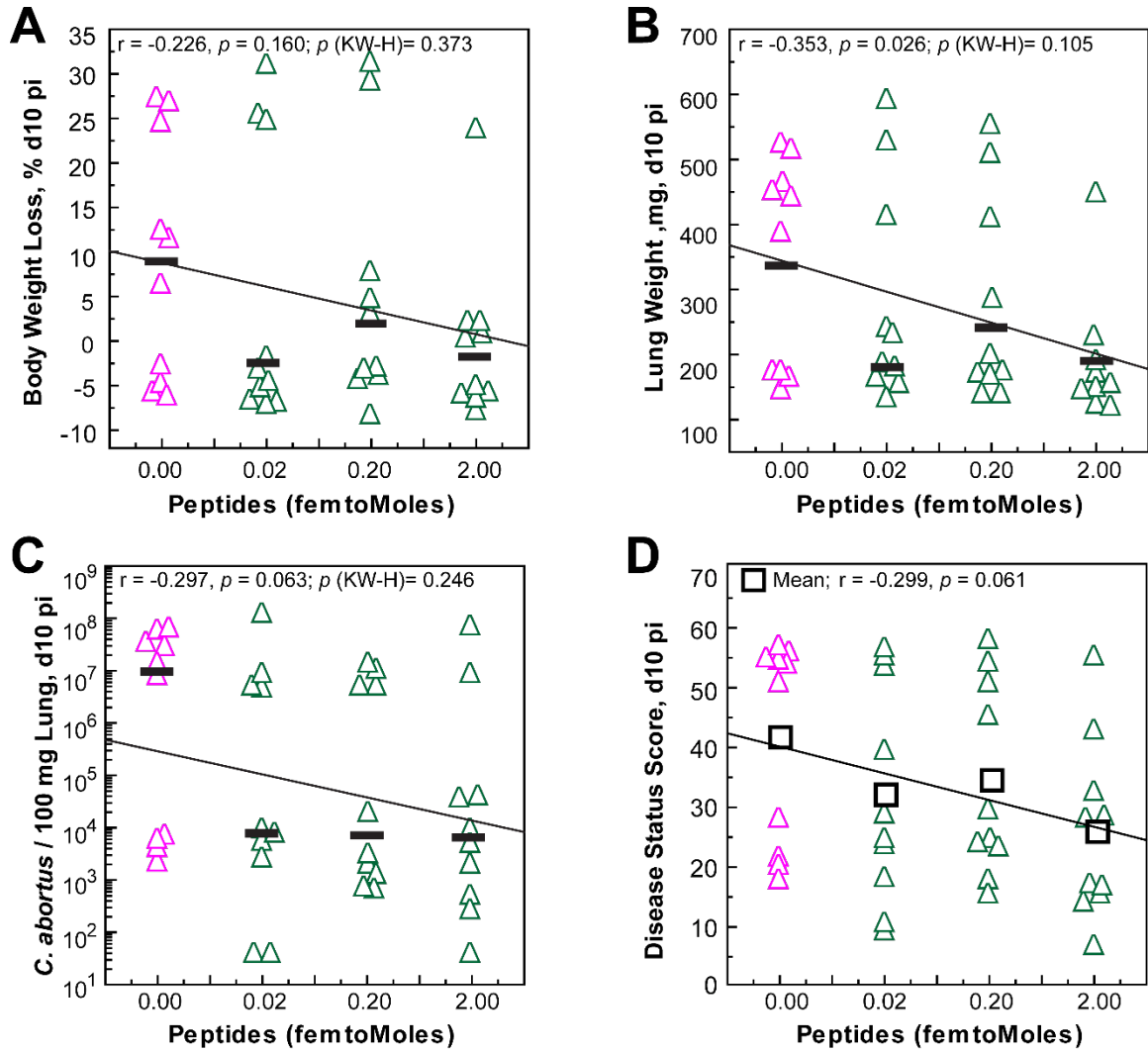
Similarly, carrier control mice showed highly inflamed lungs as indicated by high lung weight after 10 days of challenge with a median of 415 mg. Conversely, the median lung weights of the 0.02, 0.20, and 2.0 fM peptide mice were 208, 188, and 152 mg, respectively. Importantly, linear regression revealed a significant negative correlation for the lung weight with peptide dose ( $r=0.353$  and  $p=0.026$ ; Fig. 4.2B).

The three peptide groups also had substantially lowered levels of *C. abortus* in the lungs as compared to the carrier control. The lungs of the carrier control mice were heavily loaded with *C. abortus* with a median of 9,332,543 genomes/100mg lung (Fig. 4.2C). In contrast, the median lung chlamydial burdens in the 0.02, 0.20, and 2.0 fM peptide mice were 8,128 genomes, 7,413 genomes, and 6,606 genomes per 100 mg of lung, respectively. However, due to the strong bimodal response, the lung chlamydial burden did not differ significantly among the groups ( $p^{KW-H} = 0.246$ ). The linear regression model analysis demonstrated a trend towards negative correlation for the lung chlamydial burden with peptide dose that marginally failed to reach significance ( $r=0.297$  and  $p=0.063$ ; Fig. 4.2C). The linear regression analysis of overall disease status scores – the composite rank scores of body weight loss, lung weight, and chlamydial burden in lung – clearly demonstrated a negative correlation for the disease outcome with the peptide dose which marginally failed to reach at significance ( $r=0.299$  and  $p=0.061$ ; Fig. 4.2D).

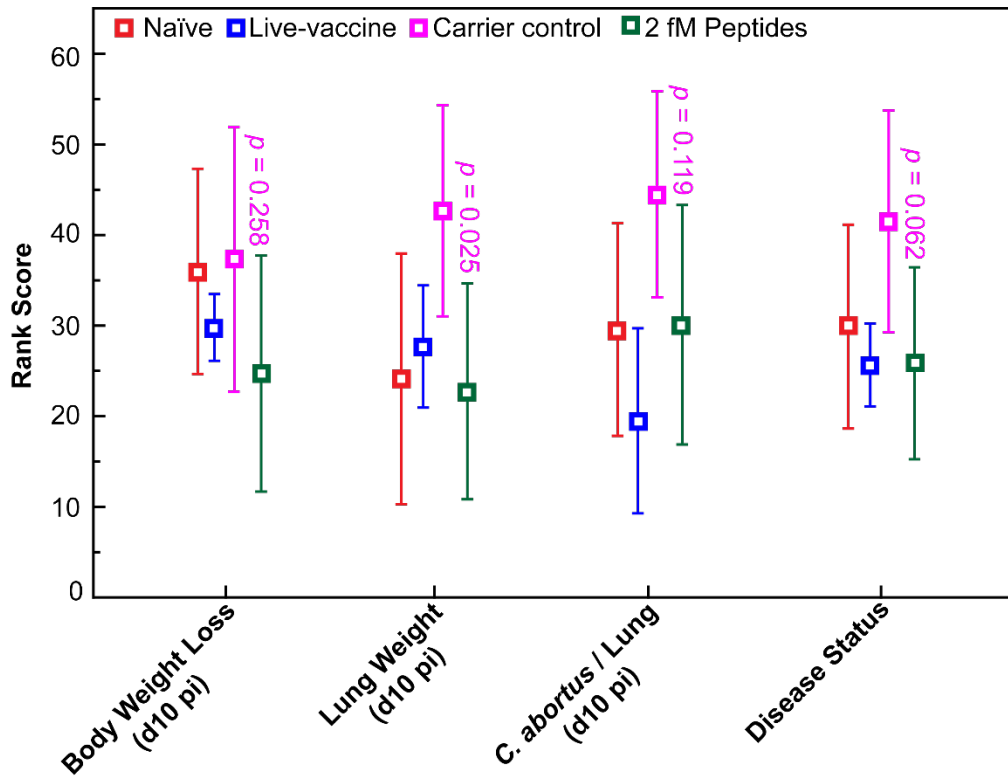
Overall, this experiment clearly demonstrated that vaccination with PLG-PEG microparticles containing femtoMoles of peptide antigen reduced *C. abortus* disease as compared to the PLG-PEG-PluronicL121 carrier without peptide antigens. All three peptide dosages - 0.02, 0.2, and 2.0 fM each peptide per vaccine, resulted similar responses,

however, the 2.0 fM peptides yielded the best response as indicated by lowest disease score (Fig. 4.2D).

Analysis of the rank scores for body weight loss, lung weight, and chlamydial burden in the lung revealed that the best protected 2fM peptide group was even more protective than the live-vaccine control in terms of body weight loss and post challenge lung weight, with the mean rank scores of 24.7 and 22.75, respectively (Fig. 4.3). In contrast, the mean rank scores for the live-vaccine control were 29.8 and 27.7, respectively (Fig. 4.3). However, the mean rank score for chlamydial burden was substantially higher in the 2.0 fM peptide group (mean= 30.1) than the live vaccine control (mean=25.6). The disease status scores of 25.85 and 25.66 were similar for both 2 fM vaccine and live-vaccine, respectively. Thus, vaccination with microparticles containing low peptide antigen doses effectively reduced disease by reducing inflammation, but did not effectively eliminate the *C. abortus* bacteria.



**Figure 4.2. Inclusion of peptides in microparticles correlates negatively with the disease outcomes.** Data are presented as scatter plots with least-square distance distribution fitting (n=10 mice). Slope of linear regression fit, and significance of correlation and Kruskal-Wallis-H statistics for the complete dataset are indicated.



**Figure 4.3. Disease outcome comparisons between peptide microparticle vaccines with the carrier controls.** Rank scores were obtained after rank transformation of the 10 days post challenge data from body weight losses, lung weights, and *C. abortus* burdens in lung. The disease status score is the mean of these three rank scores. Naïve and live-vaccine mouse data serve as reference points, but statistical comparisons by post-hoc Dunnett test was performed only between carrier controls and the 2 fM peptide vaccine group.

**Inclusion of an Inhibitor of Apoptosis Enhances Chlamydial Elimination and Induces Full Protection Against *C. abortus*.** It was evident from the previous experiment that the inclusion of femtoMoles of vaccine candidate peptides in the PLG-PEG-Pluronic L121 microparticulate delivery vehicle substantially reduced *Chlamydia*-induced inflammation, but did not effectively eliminate the chlamydial burden. We assume that the vaccine microparticles induced a protective Th1 response against chlamydial challenge that was not strong enough to fully eliminate the bacteria.

Based on the release data of fluorescently labeled peptide from PLG-PEG-Pluronic L121 microparticles, it is clear that more than 99.5% of the peptide is released from these microparticles within 24 hours after administration, with the remaining 0.5% released over the subsequent 10 days. Peptide molecules that bind to the MHC-II complex of APC with ingested microparticles will remain bound and able to stimulate cognate T-cells for approximately one week. The PLG-PEG microparticles also degrade rapidly and release substantial amounts of lactic and glycolic acid within the APC during the first few days after ingestion (Chapter 3, Fig. 3.10). This acid stress may induce apoptotic death of such APC, thus reducing the time of antigen presentation and potentially resulting in under-stimulation of the T-cell response.

In addition, it has recently been reported that antigen presentation in the context of infected apoptotic cells leads to the synthesis of TGF- $\beta$  and IL-6 and favors Th17 cell differentiation (Torchinsky *et al.*, 2009). Although it is not microbe induced apoptosis, the vaccine microparticles have been designed to release intracellular PLG-PEG oligomers and Pluronic L121 adjuvant that act as danger signals to mimic chlamydial PAMPs. Thus, PLG-PEG microparticle-induced apoptosis of APC may also promote Th17 cell



development, thus induce an inflammatory T-cell immune response that is inefficient at eliminating chlamydiae. Yet another possibility is the induction of immunosuppressive T regulatory cells ( $T_{reg}$ ) in the presence of apoptotic cells, but an incomplete PAMP response to the vaccine microparticles (Wan and Flavell, 2007). A third possibility would be a combined induction of ineffective Th17 and  $T_{reg}$  cells by these mechanisms. Therefore, PLG-PEG microparticles-induced APC apoptosis may partially suppress the Th1 cell activation and trigger ineffective and/or inflammatory T-cell responses. We hypothesized that delaying the acid induced apoptotic death of APCs by an apoptotic inhibitor would suppress Th17 induction and would trigger strong Th1 response that would effectively eliminate *C. abortus*.

To prevent apoptosis of antigen presenting cells, we incorporated an apoptosis inhibitor -Q-VD-OPh, the amino acids valine and aspartate with N-terminal quinolyl and a C-terminal di-fluorophenoxy methylketone group- in the PLG-PEG-PluronicL121-peptide microparticles. Q-VD-OPh is a broad spectrum pan-caspase inhibitor that irreversibly binds to activated caspases to block apoptosis. It is highly permeable and non-toxic *in vitro* and *in vivo*, even at extremely high concentrations (Caserta *et al.*, 2003).

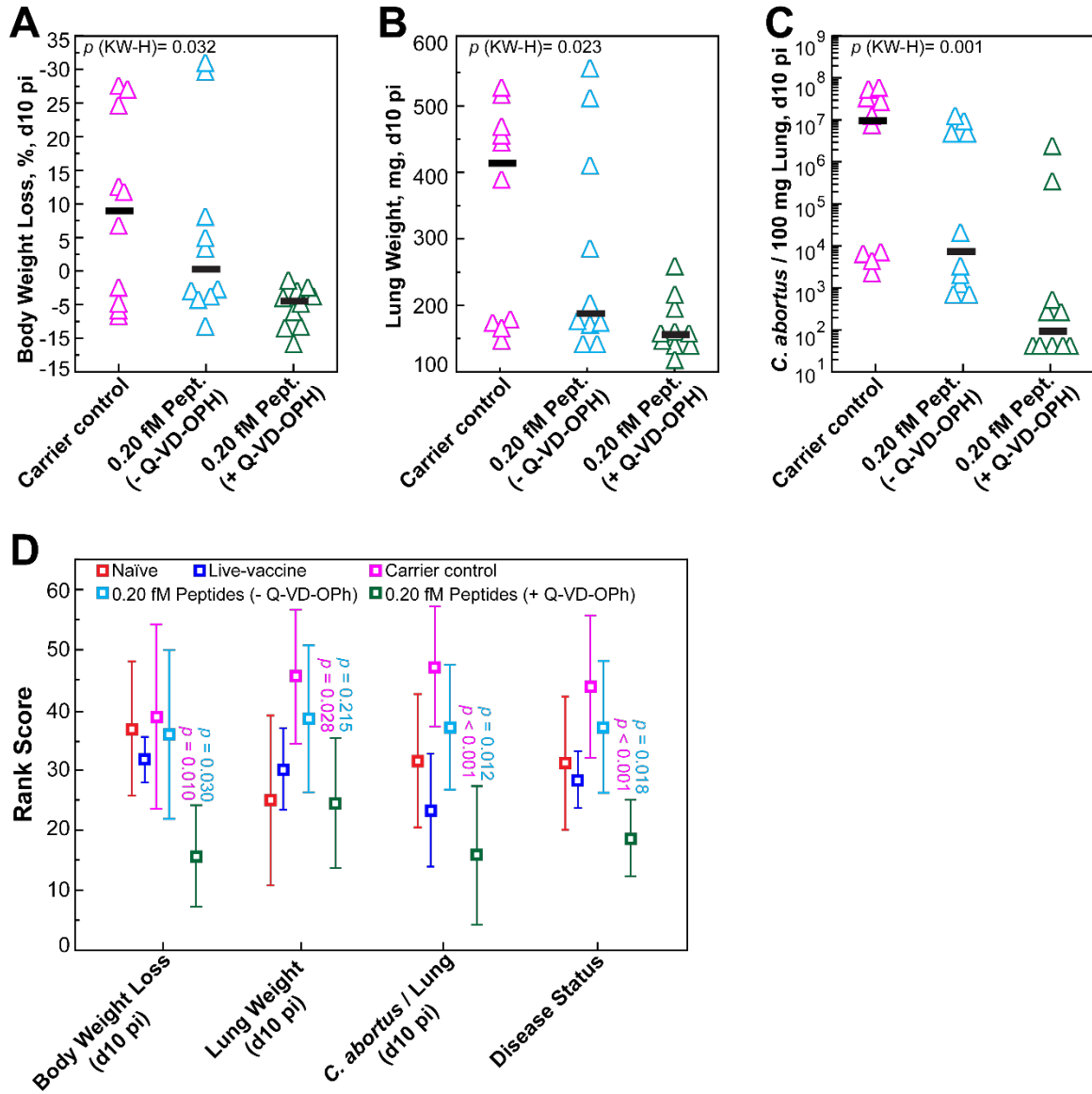
To evaluate our hypothesis, we designed an experiment where we used only the 0.2 fM peptide loading dose at which the microsphere vaccine induced an immune response that did not effectively eliminate *C. abortus*. As shown in Fig. 4.3, inclusion of 0.2  $\mu$ g Q-VD-OPh per mouse dose in the peptide vaccine microparticles completely reversed the disease outcome into a fully protective phenotype against chlamydial challenge in the 129S6 mouse strain.

Analysis of the body weight data on day 10 after chlamydial challenge demonstrated that mice that received 10  $\mu$ g vaccine microparticles containing 0.20 fM peptides and 0.2  $\mu$ g Q-VD-Oph consistently gained body weight, with a negative median 4.58% body weight loss (Fig. 4.3A). In contrast, mice that received the carrier control or 0.20 fM peptide-loaded microparticles without Q-VD-Oph had a median body weight loss of 9.05% and 0.580%, respectively. K-W statistics revealed significant differences among the three groups ( $p=0.032$ ). Most importantly, the body weight gain in the Q-VD-OPH-treated group was highly uniform, and none of the mice showed any sign of disease. In contrast, the carrier control as well as Q-VD-Oph-untreated vaccine mice showed a strong bimodal response with healthy as well as severely diseased mice. The Q-VD-Oph vaccine mice also showed healthy normal lungs with a 156 mg median lung weight that differed significantly from the carrier control group ( $p^{KW-H} = 0.023$ ; Fig. 4.4B).

The most striking finding of this experiment is the effective elimination of *C. abortus* by the Q-VD-Ph vaccine. As shown in Fig. 4.4C, the median *C. abortus* lung load per 100 mg was only 97.7 genomes in the Q-VD-Oph-positive mice, whereas it was 9,332,543 and 7,413 in the carrier control and Q-VD-Oph-negative vaccine mice, respectively. Chlamydial elimination was significantly lower in the Q-VD-Oph group as indicated by  $p^{KW-H} = 0.023$  (Fig. 4.4C). It should be noted again that this chlamydial elimination was highly uniform, and in fact five out of 10 mice were completely negative and three had minimal *C. abortus* numbers (<2 copies per 100 mg lung) as determined by qPCR.

The mean rank score for body weight loss, lung weight, and *C. abortus* lung burden in the Q-VD-Oph-positive vaccine group was 9.6, 17.36, and 9.28, respectively (Fig.

4.4D). These scores were even lower than those of the live vaccine group, and significantly differed from the carrier control ( $p^{Dunnnett}$  = 0.010, 0.028, and <0.001, respectively, Fig. 4.4D). It should be noted that the chlamydial load rank score of the Q-VD-Oph vaccine group was also significantly lower than that of the naïve controls ( $p$  = 0.053). The overall disease status demonstrated a highly significantly lowered disease in Q-VD-Oph vaccine group (mean = 18.67) as compared to the carrier control (mean = 43.86,  $p$  < 0.001) as well as the Q-VD-Oph-negative vaccine group (mean = 36.78,  $p$  = 0.018; Fig 4.5). In sum, the inclusion of Q-VD-Oph in the 0.20 fM peptide vaccine group completely reversed the disease outcome into a fully protective healthy phenotype against *C. abortus* challenge inoculation in 129S6 mice.



**Figure 4.4. Reversal of disease outcome by inhibition of apoptosis.** 129S6 mice received vaccines without peptides (carrier control), with 0.2 fM *C. abortus* vaccine candidate peptides, or with 0.2 fM *C. abortus* peptides plus 0.2  $\mu$ g of the apoptosis inhibitor Q-VD-Oph. Day10 post challenge data from body weight losses (A), lung weights (B), and *C. abortus* burdens in lungs (C), as well as ranks scores for these parameters and the combined disease status score (D) are shown. Naïve and live-vaccine mouse data serve as reference points, but statistical comparisons by post-hoc Dunnett test were performed only between carrier controls and the 0.2 fM peptide vaccine groups.

### **Intranasal Administration Q-VD-OPh-0.2fM Peptide Microparticle Vaccine**

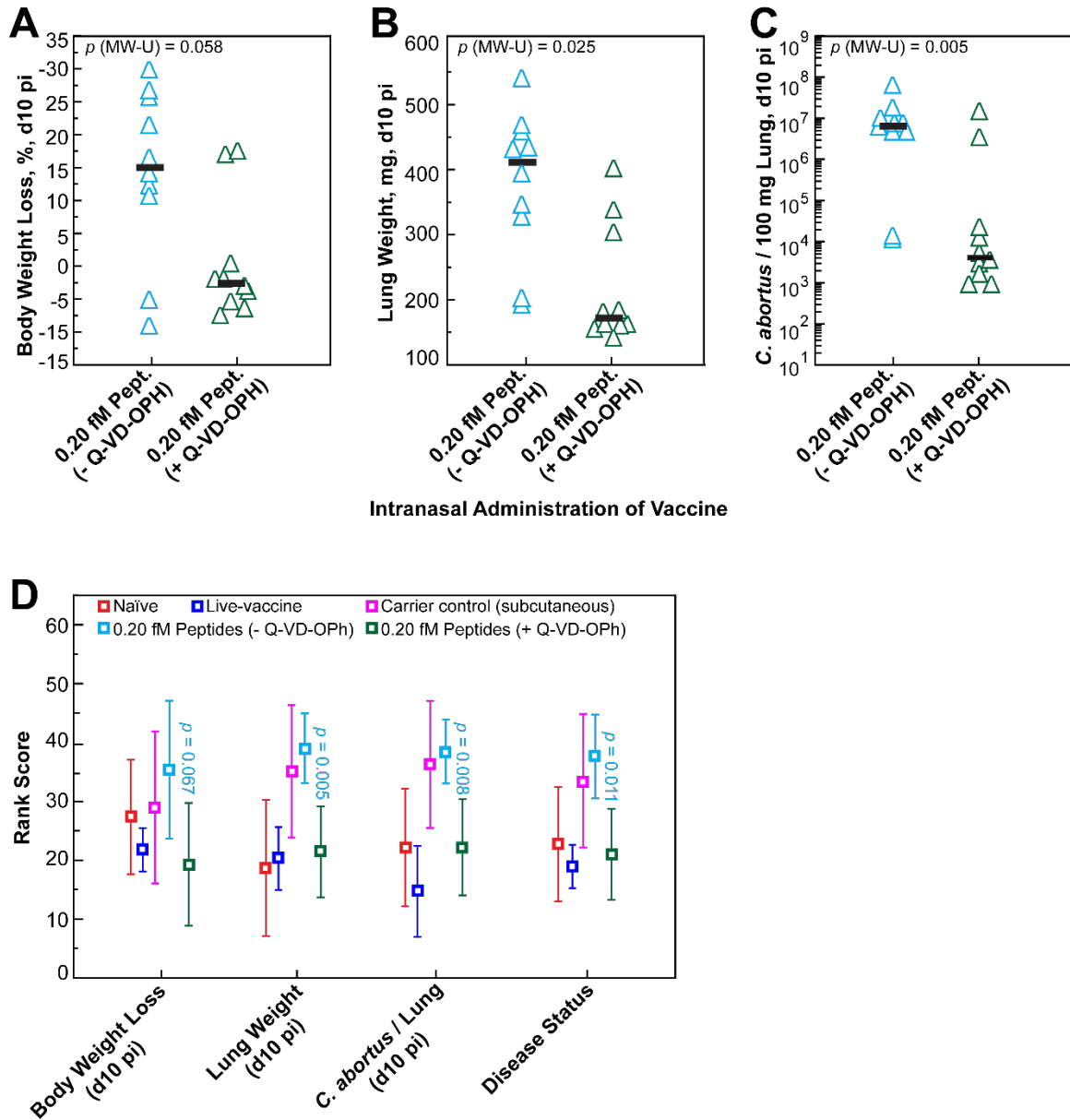
**Provided Protection.** In the previous experiments, mice were vaccinated via the subcutaneous route. In this experiment, we examined whether mucosal administration of the microparticle vaccine also provided protection, and compared the effect of vaccine microparticles directly as contrast experiment.

As shown in Fig. 4.6, intranasal administration of 0.20 fM peptide loaded PLG-PEG-PluronicL121 microparticles without Q-VD-OPh failed to induce protection as evident by high body weight loss with a median of 15.10% 10 days after *C. abortus* challenge (Fig. 4.6A). Similarly, these mice had 400.5 mg median lung weights (Fig. 4.6B) and a median 6,025,595 *C. abortus* burden per 100 mg lung (Fig. 4.6C) 10 days after challenge. In contrast, inclusion of Q-VD-OPh in this vaccine highly significantly reduces the disease and effectively eliminated the bacteria. The Q-VD-OPh vaccine group had an overall body weight gain as indicated by a 4.58% negative median (Fig. 4.6A) which barely failed to reach significance in the Mann-Whitney U test ( $p^{MW-U} = 0.058$ ; Fig. 4.6C). These mice had also significantly lower lung weight (median=167.5 mg) than the Q-VD-OPh negative vaccine mice ( $p^{MW-U}=0.025$ ), and a highly significantly lower bacterial load (median = 3,630 *C. abortus* genomes/100 mg lung;  $p^{MW-U}=0.005$ ; Fig. 4.6C).

The disease rank scores analysis demonstrated, particularly in reference to carrier control, naive, and live-vaccine mice, that the Q-VD-OPh-negative vaccine group actually showed exacerbated disease, as indicated by high rank scores with the means of 35.30, 38.95, and 38.40, for body weight loss, post challenge lung weight, and chlamydial lung burden, respectively. In contrast, the Q-VD-OPh vaccine group had the mean scores of 19.20, 21.35, and 22.10, respectively, that were significantly lower than those of the Q-

VD-Oph negative group ( $p^{\text{Dunnett}}=0.067$ , 0.005, and 0.009, respectively; Fig. 4.7). The overall disease status scores were also significantly reduced in Q-VD-Oph-positive vaccine group (mean=20.88) as compared to the Q-VD-Oph-negative group (mean=37.55,  $p^{\text{Dunnett}}=0.011$ ; Fig. 4.7).

Thus, intranasal administration of the 0.2fM peptide loaded PLG-PEG-PluronicL121 microparticle vaccine with 0.2  $\mu\text{g}$  Q-VD-Oph also significantly reduced disease. In sum, the 0.20 fM *C. abortus* peptide vaccine with the apoptotic inhibitor Q-VD-Oph incorporated into PLG-PEG-PluronicL121 microparticles has a great potential to be used as vaccine against *C. abortus* infection.



**Figure 4.5. Protection by intranasal administration of a Q-VD-OPh-0.2fM peptide microparticle vaccine.** Microparticle vaccines with 0.2 fM *C. abortus* vaccine candidate peptides and with 0.2 fM *C. abortus* peptides plus 0.2  $\mu$ g of the apoptosis inhibitor Q-VD-OPh were directly compared. Naïve, live-vaccine, and carrier control mouse data serve as reference points, but statistical comparisons by post-hoc Dunnett test were performed only between the 0.2 fM peptide vaccine groups.

**Microparticulate Vaccines with the R202S (DL-PL) Carrier Protect against *C. abortus* in Jax 129X1/SvJ mice.** In the previous experiments, we used 50:50DL-PLG-PEG, one of the most rapidly degrading polymers (degradation timeframe ~4 weeks), as carrier for the vaccine microparticles. Upon encountering lack of protection or even disease exacerbation in certain groups, we hypothesized that such rapid degradation and intracellular release of lactic/glycolic acids may induce apoptotic death of APCs. This in turn may drive a Th17 and/or T<sub>Reg</sub> response, and suppress the vaccine-induced Th1 response. In fact, subsequent experiments clearly demonstrated that the inclusion of an inhibitor of apoptosis (Q-VD-OPh) in the 50:50DL-PLG-PEG-PluronicL121-0.2fM microparticles resulted in full protection against *C. abortus* challenge. Therefore, in the next experiment we investigated the use of an alternative polymer carrier, the low-molecular weight, but slowly degrading polylactide polymer DL-PL R202S with a degradation timeframe of ~80 days. This carrier would release essentially no acid for the first 3 weeks after phagocytosis, thus lie inert in APC, and releases incorporated peptides within 10 days after uptake exclusively by diffusion (Fig. 3.10). Therefore, we anticipated minimal or an absent apoptotic stimulus from these vaccine microparticles after uptake by APCs.

Due to ready availability, we performed this experiment with the 129 mouse strain from the Jackson Laboratories – 129X1/SvJ. As an initial approach to develop a live-vaccine control group, we inoculated naïve 129X1/SvJ with low dose of  $3 \times 10^7$  *C. abortus* elementary bodies. Interestingly, this Jackson 129 mouse strain showed a much higher susceptibility to *C. abortus* than the Taconic 129S6 mouse. After inoculation with the low dose *C. abortus* inoculum, the mice did not exhibit any disease symptoms for the first week,



then became progressively sick, and started to die from 12 days of post inoculation. As an attempt to stop the death, we introduced the antibiotic enrofloxacin in water on day 13 after *C. abortus* inoculation. However, even the antibiotic treatment could not prevent the mortality, and 80% of the mice died within 4 weeks after the low dose *C. abortus* inoculation (Fig. 4.6). Due to this extreme susceptibility of 129X1/SvJ mice to *C. abortus*, protection was not possible by inoculation of live bacteria, and we could not develop a live-vaccine control. Furthermore, there was no need to include a carrier control in the vaccine experiment, since the naïve mice developed severe disease, and therefore any protection would be a contrast to the severe disease of naïve mice.

In the subsequent vaccine experiment, the 129X1/SvJ mice received subcutaneously three different dosages of peptide antigens in 2.5-fold increasing concentration - starting from 0.2 fM to 1.25 fM in 10 µg DL-PL R202S:Pluronic L121 (6.5:3.5) microparticles. Six weeks after a single vaccination, the mice were intranasally challenged with  $3 \times 10^8$  *C. abortus* organisms, and euthanized 10 days later.

As shown in Fig. 4.7, the R202S based vaccine microparticles at the dosage of 0.5 and 1.25 fM peptide antigens significantly reduced the disease, but not with the 0.20 fM peptides. In fact, in the 0.2 fM peptides followed the same trend as naïve mice, whereas the 0.5 and 1.26 fM peptide vaccine mice differed significantly from the naïve mice.

After *C. abortus* challenge, the naïve control mice became very sick and showed a median body weight loss of 31.21% after 10 days of challenge. The 0.20 fM peptide mice were also very sick with a median body weight loss of 24.95%. In contrast, the mice that received microparticles with 0.05 fM and 1.25 fM peptides showed no visible sign of sickness and had marginal median body weight loss of 3.59 and 3.78%, respectively, after

10 days of challenge, a difference that barely failed to reach significance in comparison with naïve and 0.20 fM peptide groups ( $p^{KW-H}=0.069$ ; Fig. 4.7A).

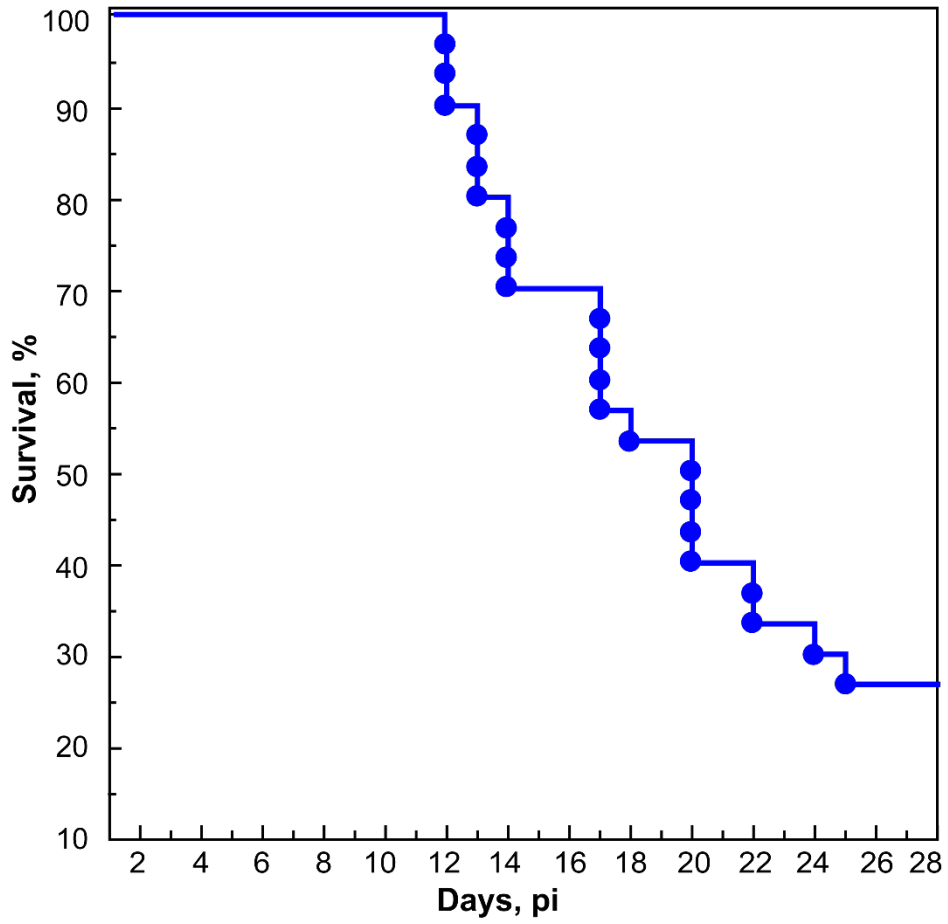
Similar to the profound body weight loss, the naïve control mice had very high lung weight with the median of 535.5 mg after 10 days of challenge (Fig. 4.7B). The 0.2 fM peptide dose mice also had a similar lung weight with a median of 456 mg. Conversely, the 0.50 and 1.26 fM peptide mice had median lung weights of 232 and 190 mg, which were significantly lower than the naïve control ( $p^{KW-H}=0.035$ ; Fig. 4.7B).

Analysis of the *C. abortus* loads in the lungs demonstrated that the naïve and 0.20 fM peptide group had uniformly higher chlamydial loads with medians of 11,906,935 and 23,307,731 genomes/100 mg lung, respectively. In contrast, although both the 0.50 and 1.26 fM peptide mice showed a pronounced bimodal response, they had significantly lower chlamydial loads (median = 56,053 and 86,556, respectively) than the naïve and 0.20 fM peptide mice ( $p^{KW-H}<0.001$ ). It is important to note that one mouse in the 0.50 fM peptide group and 2 mice in the 1.25 fM peptide group were negative for lung chlamydiae as determined by qPCR, but had very high lung weights (490, 633 and 325 mg) and substantial body weight losses (23.37%, 36.63 and 30.13 %). This indicates that the vaccine induced Th1 response fully eliminated *C. abortus*. However, due to extreme susceptibility of this mouse strain to *C. abortus* challenge, they had strong inflammatory response, similar to antibiotic-treated mice that continued to die even 10 days after initiation of antibiotic administration.

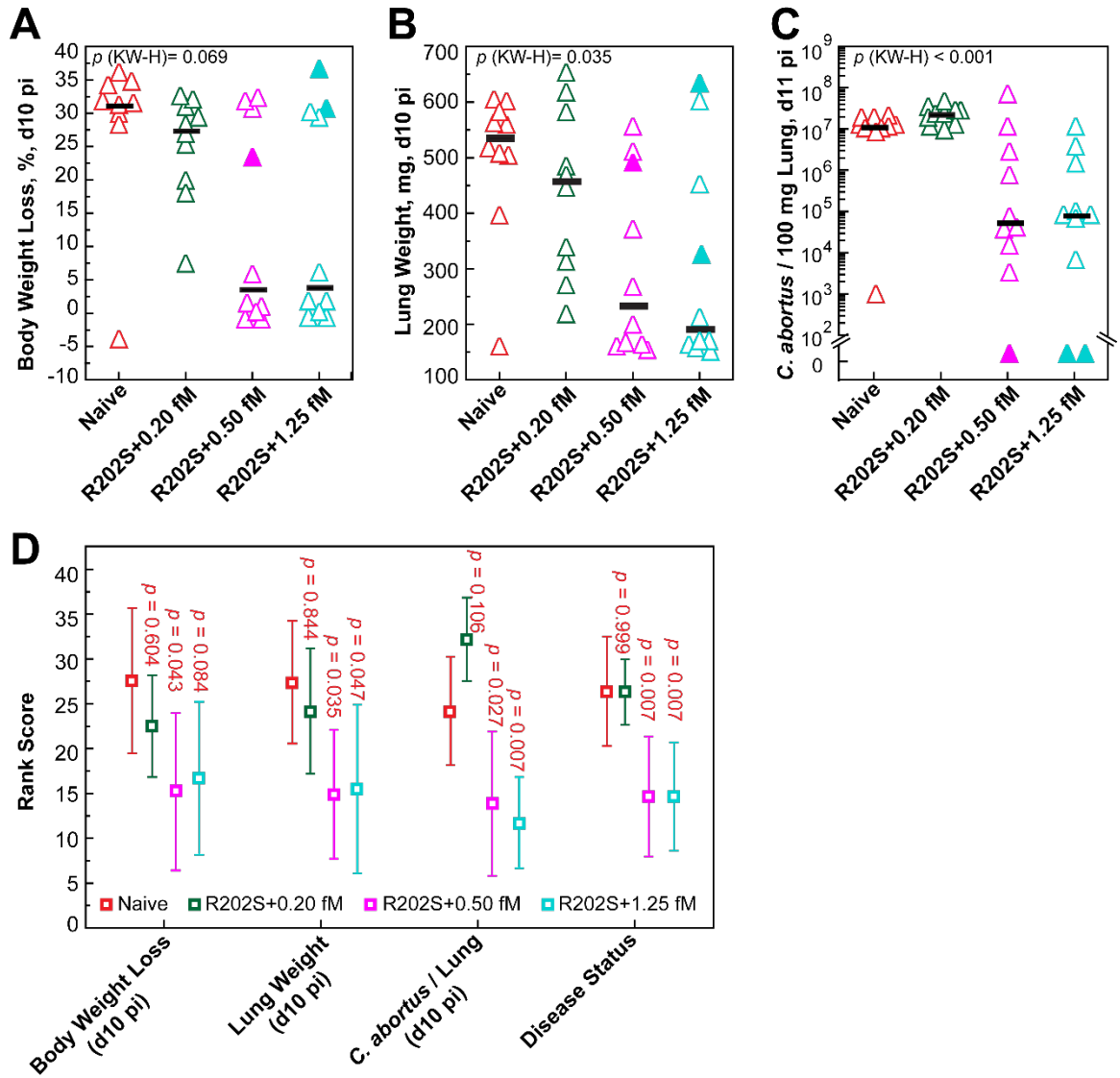
Analysis of the rank scores for body weight loss, lung weight, and chlamydial burden in the lung revealed significantly lower scores for the 0.50 and 1.25 fM peptide groups than for the naïve controls (Fig. 4.7). The overall disease status scores demonstrated

that the 0.50 fM and 1.26 fM peptide groups were highly significantly healthier than the mortally diseased naïve controls (mean = 13.85, 14.65, and 26.50, respectively;  $p^{\text{Dunnett}}=0.007$  for both; Fig. 4.7). Interestingly, the 0.2 fM peptide vaccine co-segregated in all parameters with the naïve mice. This indicates a failure of the 0.2 fM peptide dosage to induce protective immunity. A likely explanation for this lack of protection may be the delayed antigen release from the DL-PL R202S carrier as compared to the 505:50DL-PLG-PEG carrier.

In sum, a microparticle vaccine comprising slow degrading DL-PL R202S as carrier, Pluronic L121 as adjuvant, and 0.5-1.25 femtoMole dosage of *C. abortus* peptide antigens, induces highly significant protection against chlamydial challenge without triggering an unwanted Th1 suppressive or inflammatory immune response in the *C. abortus* respiratory mouse disease model.



**Figure 4.6. Jax 129X1/SvJ mice show strong susceptibility to the low dose inoculum of  $3 \times 10^7$  *C. abortus* organisms.** Survival analysis (Kaplan-Meier survival estimate; n=30 mice).



**Figure 4.7. Microparticle vaccines with the R202S DL-PL carrier mediate protection against lethal *C. abortus* challenge.** Microparticle vaccines composed of the DL-PL R202S polylactide polymer and Pluronic L121 were loaded with 0.2, 0.5, or 1.25 fM *C. abortus* vaccine candidate peptides and compared to naïve mice. Statistical comparisons of the vaccine groups with naïve mice were performed by post-hoc Dunnett test.

#### 4.5. DISCUSSION

Currently, one of the main problems associated with chlamydial vaccine development is the necessity for the induction of strong Th1 immune responses, as well as delivery of the vaccine candidate. In our previous study, we demonstrated that femtoMole dosage of 20-mer synthetic peptides derived from three vaccine candidates of *C. abortus* induced significant protection in chlamydial respiratory disease model. In this present study, we developed vaccine microparticles comprising a biodegradable polymer as carrier, Pluronic L121 as Th1 adjuvant, and femtoMole dosage of *C. abortus* peptides as antigen, and tested its efficacy in a murine *C. abortus* respiratory disease model. Our results demonstrate that the protection is achieved by microparticulate delivery of femtoMole peptide per vaccine dose, however complete protection is greatly influenced by type of polymeric carrier used.

We performed our studied in the 129 mouse strain which has a defect in its NK-cell effector arm and hence is incompetent to generate a successful innate immune response. In our initial studies we used 129S6 mice from Taconic. Although, high dose of chlamydial challenge could not induce severe disease in these mice, inoculation of PLG-PEG microparticles with Pluronic L121, but no antigen, resulted in severe disease. Thus, we compared the vaccine induced protection with the carrier control.

In an initial approach we have shown that inclusion of femtoMoles of peptides in PLG-PEG-Pluronic L121 microparticles provided substantial protection as compared to carrier control without peptides. Peptide dosage in 10-fold increments from the possible lowest dose of 0.02 fM to 2.0 fM showed a trend of better protection as the dosage of peptides increased. However, there was no significant differences among the three peptide

groups and none of them could significantly reduce the chlamydial loads as compared to the carrier control. We anticipated that such ineffective chlamydial elimination might be due to an insufficient Th1 immune response. We thought that acids released by the rapidly degrading PLG-PEG might induce apoptosis of the APCs and therefore favor a Treg and or Th17 environment, hence the vaccine suppressed a strong Th1 response required for protection against *C. abortus*.

To inhibit potential PLG-PEG-induced apoptosis of APCs, we incorporated per 10  $\mu\text{g}$  vaccine dose 0.2  $\mu\text{g}$  of the pan-caspase inhibitor, Q-VD-OP-h, in the microparticles along with Pluronic L121 and 0.2 fM peptides. We used 0.20 fM peptides in this experiment, because this dose resulted in relatively more disease than the 0.02 and 2 fM peptide in the previous experiment. Interestingly, inclusion of Q-VD-Oph not only significantly reduced disease in after chlamydial challenge, but also highly significantly enhanced clearance of the bacteria as compared to the carrier control. While this protection was received by subcutaneous immunization of vaccine microparticles, strong protection was also observed when same vaccine microparticles were mucosally administered by intranasal instillation into the nostrils.

Next, we hypothesized that the apoptotic death of APCs could be avoided by using a slowly degrading polymer instead of the rapidly degrading PLG-PEG polymer. We examined whether the slow degrading polylactide polymer DL-PL R202S can induce significant protection, but without inclusion of Q-VD-Oph. We performed this experiment with 12X1/SvJ mice from The Jackson Laboratory which were highly susceptible to *C. abortus*. With the anticipation that a 0.20 fM peptide dose may be too low to induce substantial stimulation with this low degrading polymer, we tested three different dosages

of peptides in 2.5-fold increments from 0.20 to 1.25 fM per vaccine dose. Our findings, clearly demonstrated that subcutaneous vaccination of 129X1/SvJ mice with microparticle vaccine comprising R202S-Pluronic L121, with either the 0.50 or 1.25 fM peptides highly significantly reduced disease as compared to highly diseased naïve controls. We also observed that the 0.20 fM peptide dose did not provide protection and showed similar disease as the naïve control. Thus, degradation kinetics of the vaccine carrier polymer greatly influence the protective response. While vaccine microparticles composed of the rapidly degrading PLG-PEG polymer require the addition of the apoptotic inhibitor Q-VD-Oph to induce complete protection, the slowly degrading polylactide R202S polymer induces protection without the inhibitor of apoptosis.

In sum, in the present study we developed a fully synthetic biodegradable polymer-based, femtoMoles-dosed peptide microparticle vaccine that induced protection in a single vaccination against *C. abortus*. This vaccine can be used by both subcutaneous and mucosal routes, and its formulation can be optimized based on the polymeric particles used as carrier.



#### 4.6. MATERIALS AND METHODS

**Peptides.** Twenty amino acid long peptide antigens of *C. abortus* vaccine candidate proteins were commercially synthesized by Thinkpeptides®, Inc., Bradenton, FL. *C. abortus* DnaX2 comprised 44 peptides, *C. abortus* GatA 49 peptides, *C. abortus* GatC 9 peptides, *C. abortus* OmpA (PompB) 83 peptides, and *C. abortus* Pbp3 64 peptides. Each of the peptides were 20 amino acids (aa) in length, with 10 aa overlaps between sequential peptides and spanning the entire consensus sequences of the 3 *C. abortus* proteins (Fig. 4.5). To prevent potential *in vitro* polymerization, all peptides were synthesized with N-terminal and C-terminal amide and used as crude preparation with >70% purity.

The peptides were collected in a deep-well 96-well polypropylene plate, and each peptide was dissolved in ~400 µl of dimethyl-sulfoxide (DMSO; Amresco, OH, USA) to create a  $10^{-6}$  M solution of each peptide, calculated from MW and mg yield of each peptide. For preparation of the  $10^{-8}$  M vaccine stock of all peptides of each protein, 5 µl of each peptide of a protein were pooled and the solution filled up to 500 µl with DMSO. The plates were stored at -80°C. Required volumes of combined peptides for each protein were further diluted in DCM for spray drying to obtain 0.02 fM, 0.20 fM, 0.50 fM, 1.25 fM, or 2.0 fM of each of combined peptide of each protein in 200 µl of per mouse vaccine dose. Since the average molecular weight of a 20-mer peptide is 2,200 Daltons, the average amount of the 0.02 femtoMoles of each peptide corresponds to 0.044 picograms (pg).

**Adjuvants.** The adjuvant used in this experiment was Pluronic L121 (Sigma Chem. Co., St. Louis, MO, USA).

**Q-VD-OPh.** The caspase inhibitor Q-VD-OPh (Quinolyl-valyl-O-methylaspartyl-[2,6-difluoro-phenoxy]-methyl ketone) was obtained from SMB Biochemical, Santa Ana, CA, and a stock solution was made by dissolving 10 mg of Q-VD-OPh in 1 mL of DCM and stored at -80°C. Appropriate volumes of this stock solution were used in the feed stock solution for spray drying so that a 10 µg final vaccine dose contains 0.2 µg of Q-VD-OPh.

***Chlamydia abortus.*** *C. abortus* strain B577 (ATCC V-656) was grown in Buffalo Green Monkey Kidney monolayer cell cultures, purified by differential centrifugation, and quantified as previously published (Li *et al.*, 2005). Purified infectious EBs were suspended in sucrose-phosphate-glutamate (SPG) buffer, stored in aliquots at -80°C.

**Vaccine Microparticle Synthesis.** Microparticles were synthesized with the optimized spray dry procedure as described in Chapter 3. In brief, 2% feedstock solutions composed of polymer (50:50 PLG-PEG or DL-PL R202S), Pluronic L121, peptides, with or without Q-VD-OpH were prepared in DCM by adding the desired volume of the previously prepared stock solution, as required for different experiments. The ratio of the polymers and adjuvants was 6.5:3.5. Microparticles were obtained by spraying the prepared feedstock solution in Büchi mini spray dryer model B-190 (Büchi Labortechnik AG, Flawil, Switzerland).

**Preparation of Microparticles Vaccine Suspension.** One mg of the vaccine microparticles were mixed with 3 mg of lactose anhydrous microfine (DFE Pharma, Germany) in a glass vial, then dissolved in 1 mL of suspension buffer (0.001%

benzalkonium chloride and 0.5% Kolliphor HS-15 in PBS), and sonicated for 15 minutes in cold water. Finally, 1:20 fold (subcutaneous vaccine) or 1:1 fold (intranasal vaccine) of this initial suspension was prepared in suspension buffer solution to obtain a 10 µg of vaccine microparticle per mouse dose of 200 µl (S.C.) or 20 µl (i.n.) suspension.

**Animal and Immunization.** Inbred female 129S6 mice were sourced from the Taconic Bioscience (One Hudson City Centre, Hudson, NY) and 129X1/SvJ mice from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age. Udel “shoebox” type cages with spun fiber filter tops were maintained in static air or ventilated cage racks. Five to ten animals were housed per cage in a temperature-controlled room on a 12-hour light/dark cycle, with ad libitum access to water and standard rodent chow. All animal experiments were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Each group was consisted of 10 mice. Mice received the single vaccine under light isoflurane inhalation anesthesia by subcutaneous injection of 200 µl vaccine microparticles between the shoulder blades or intranasal inoculation of 20 µl vaccine microparticles at 6 weeks of age.

**Positive and Negative Controls.** Naïve, mock-vaccinated mice served as controls for a complete lack of protective immunity against *C. abortus*. During vaccination, the mice that received the microparticles without peptide antigens also served as negative control and termed as carrier control. Mice that received a low intranasal dose of  $3 \times 10^7$  *C. abortus* bacteria (genomes) 4 weeks before the high-dose challenge infection served a controls for protective immunity (live-vaccine controls).

**Intranasal *C. abortus* Challenge and Monitoring.** Mouse intranasal inoculation was performed as previously described (Huang *et al.*, 1999), and optimal doses for live immunization and challenge inocula were determined in preliminary experiments. All mice were challenged 4 weeks after the second vaccination under light isoflurane anesthesia intranasally with  $3 \times 10^8$  *C. abortus* elementary bodies suspended in 20  $\mu$ l sucrose-phosphate-glutamate buffer. All animals were weighed during challenge infection and every second subsequent day until euthanasia on day 10 post challenge. Mice were monitored every day and death, if any, was recorded. Ten days after challenge, mice were sacrificed by CO<sub>2</sub> inhalation and weighed. Lungs were collected, weighed, snap frozen in liquid nitrogen, and stored at -80°C until further processing. For mice that died before sacrificing on day 10, body weight losses, lung weight increases and chlamydial lung loads of the mouse in any group were taken as the highest of each of these parameters prior to death for the day 10 values.

**Mouse Lung Nucleic Acid Extraction.** Mouse lungs were homogenized in guanidinium isothiocyanate Triton X-100-based RNA/DNA stabilization reagent by shaking with a BeadRaptor device to create a 10% (wt/vol) tissue suspension. This suspension was used for total nucleic acid extraction by the High Pure® PCR template preparation kit (Roche Applied Science, Indianapolis, IN) (DeGraves *et al.*, 2004; Wang *et al.*, 2004).

**Analysis of *C. abortus* Lung Loads by Quantitative PCR (qPCR).** The PCR primers and probes were custom synthesized by Operon, Alameda, CA. The *C. abortus*

genomes copy number per lung was determined by *Chlamydia* genus-specific 23S rRNA FRET (fluorescence resonance energy transfer) qPCR.

**Data Analysis.** All analyses were performed with the Statistica 7.1 software package (StatSoft, Tulsa, OK). Data of *C. abortus* genome copies were logarithmically transformed. Results were analyzed by non-parametric Mann-Whitney U and Kruskal-Wallis H tests, linear regression, Kaplan-Meier survival curve, and one-way ANOVA with Dunnett's test for correction of the  $p$  value in multiple comparisons.  $P$  values  $\leq 0.05$  were considered significant.

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## CHAPTER 5

### OUTLOOK

#### 5.1. INTRODUCTION

**What we have achieved so far.** The experiments described in Chapters 2-4 have been explicitly designed to create a Th1 vaccine platform that can be directly moved from the mouse model to production animals of agricultural importance, i.e. chickens, swine, and ruminants (cattle, sheep, goats). It is also for this reason that completely synthetic components for the vaccine were selected, the cost-effective synthesis of which can be tightly controlled. Furthermore, carrier and adjuvant are composed exclusively of carbon, oxygen, and hydrogen, thus have virtually no potential for creation of toxic residues. They are also completely, but non-enzymatically, hydrolytically degradable, and thus have leave minimal, if any, degradation products in animal tissue. And they have standing approval by the Food and Drug Administration (FDA) for medical use. Spray drying was chosen as production method because of its continuous rather than batch manufacturing process, and the easy control of production parameters and scalability to industrial quantities, resulting in highly cost-efficient production.

We can also formulate this vaccine to modulate the immune response to achieve complete protection by adjusting the vaccine carrier polymer, antigen loading, and addition of an inhibitor of apoptosis. The achievable protection is better than previous vaccination with live *C. abortus*, but the vaccine can also be formulated to cause severe, near lethal

disease. So we can use this platform not only to develop real-life vaccines, but also as a tool to model and understand chlamydial immuno-pathogenesis by manipulating the vaccine immune response.

**What next?** The next logical step is the experimental use of this vaccine platform against an appropriate disease in a production animal species. The obvious choice would be immunization against *C. abortus*-induced abortion in ruminants. However, the production animal models for this disease are cumbersome, time-consuming, and unreliable. Therefore, testing in an epidemiological study of prevention of naturally occurring *C. abortus* abortion is preferable, once the scaling of the vaccine platform has been demonstrated.

An approach more amenable to scaling for production animals is the use in pigs against porcine reproductive and respiratory syndrome, a debilitating herd disease. It is caused by PRRS virus (PRRSV), a 15 kb single-stranded enveloped RNA *Arterivirus*, and is the most prevalent disease of swine in the world, resulting in US annual losses to the swine industry of \$600 million, more than any other disease in swine (Dokland, 2010). The advantage of testing our vaccine platform against a disease caused by a virus with a small genome is that discovery of vaccine candidate proteins is not required since the proteome is so small that all peptides comprising the proteome can be economically incorporated into the vaccine. The PRRSV proteome consists of 6 structural and 8 non-structural proteins that comprise a total of 5,151 amino acids. Therefore, complete antigenic coverage of the PRRSV proteome with 20-mer peptides with 10 amino acid overlap requires 511 such peptides.

**How to scale-up the vaccine dose for production animals.** An essential question is the amount of vaccine required for animals with larger body weights than laboratory mice. Given that the “vaccine quantum unit” of a single microparticle presumably will act similarly or identically on the cellular level, irrespective of the vaccinee species, only the amount of vaccine administered, but not its composition, must be adjusted for different species, and age groups among them. A good rule of thumb for relating different body weights of animals is the “power of three quarters” rule of allometric scaling, found first by Kleiber for metabolic rates (West et al., 2002). For instance, the amount of vaccine for a calf with 50 kg body weight would have to be scaled up by the ratio of calf/mouse body weight to the power of  $\frac{3}{4}$ . Since laboratory mice weigh on average 20 g at pathogen challenge at 10 weeks of age, this factor would be  $(50,000/20)^{0.75} = 354$ . Thus, instead of a linearly 2,500× scaled-up dose from 10 µg to 25 mg, only 3.5 mg of the microsphere vaccine would be required for a calf with 50 kg body weight, and an approximately 27 mg vaccine dose for a cow with 750 kg body weight. These vaccine dosages are consistent with the dose ranges of currently used vaccines, and can easily be suspended in customary volumes of 0.5-2 ml vaccine per vaccinee.

**PPRSV Vaccine Trial.** Using the rationale and approach laid out above, we therefore initiated a trial of experimental PPRSV microparticle vaccine preparations in pigs with collaborators Drs. Fernando Osorio and Hiep Vu at the University of Nebraska-Lincoln. Freshly weaned female pigs of 3-4 weeks of age received a single subcutaneous vaccine dose, were intranasally challenged with the virulent PPRSV strain FL12 56 later, and euthanized on day 70. Scaling up the vaccine dose for an assumed weight of 40 kg at

the time of challenge, the pigs received a 3 mg vaccine microparticle dose by subcutaneous injection in 1 ml volume. Vaccine preparations used were on the basis of the rapidly degrading 50:50DL-PLG-PEG poly-lactide-co-glycolide and slowly degrading polylactide DL-PL R202S polymers and used 511 overlapping 20-mer peptides covering the complete proteome of the FL12 challenge virus.

The rationale of this trial was to obtain a gauge on vaccine dosage, its non-specific immunostimulatory effect (BRM), and its antigen-specific T cell protective immune response against PRRSV. We considered achieving full sterilizing protection against the rapidly multiplying PRRSV difficult without neutralizing antibodies. Nevertheless, we intended to observe in this trial the sum total of all vaccine effects with particular consideration of overall growth and feed conversion of the experimental pigs, pre- and post PRRSV challenge. In real life use of a vaccine in production animals, the most important effect is not maximum protection by strictly immunological parameters, but most effective achievement of the economical production goal, in essence maximum growth with minimum use of feed (maximum feed conversion). Intriguing initial results of these ongoing experiments are presented below.



## 5.2. RESULTS

**The Microparticle Carrier and PRRSV Vaccine Protect Against PRRSV Infection and Disease.** In the initial experiment we used as carrier control the slowly degrading polylactide carrier DL-PL R202S, but as vaccine the rapidly degrading 50:50DL-PLG-PEG carrier for combination with the PRRSV peptide antigens. The rationale was that by challenge on day 56 after vaccination, microparticles of the carrier control would still be present in pigs and thus exert any non-specific BRM effect for which the microparticles had been designed. In contrast, the vaccine carrier would be degraded by day 56, and therefore observed effects would be due to vaccine-evoked immunity, but not due to direct BRM effects of microparticles. Observations during vaccination (data not shown) did not reveal any detrimental side effects of the vaccine such as inappetence of vaccinees, or local inflammation and swelling at the injection site.

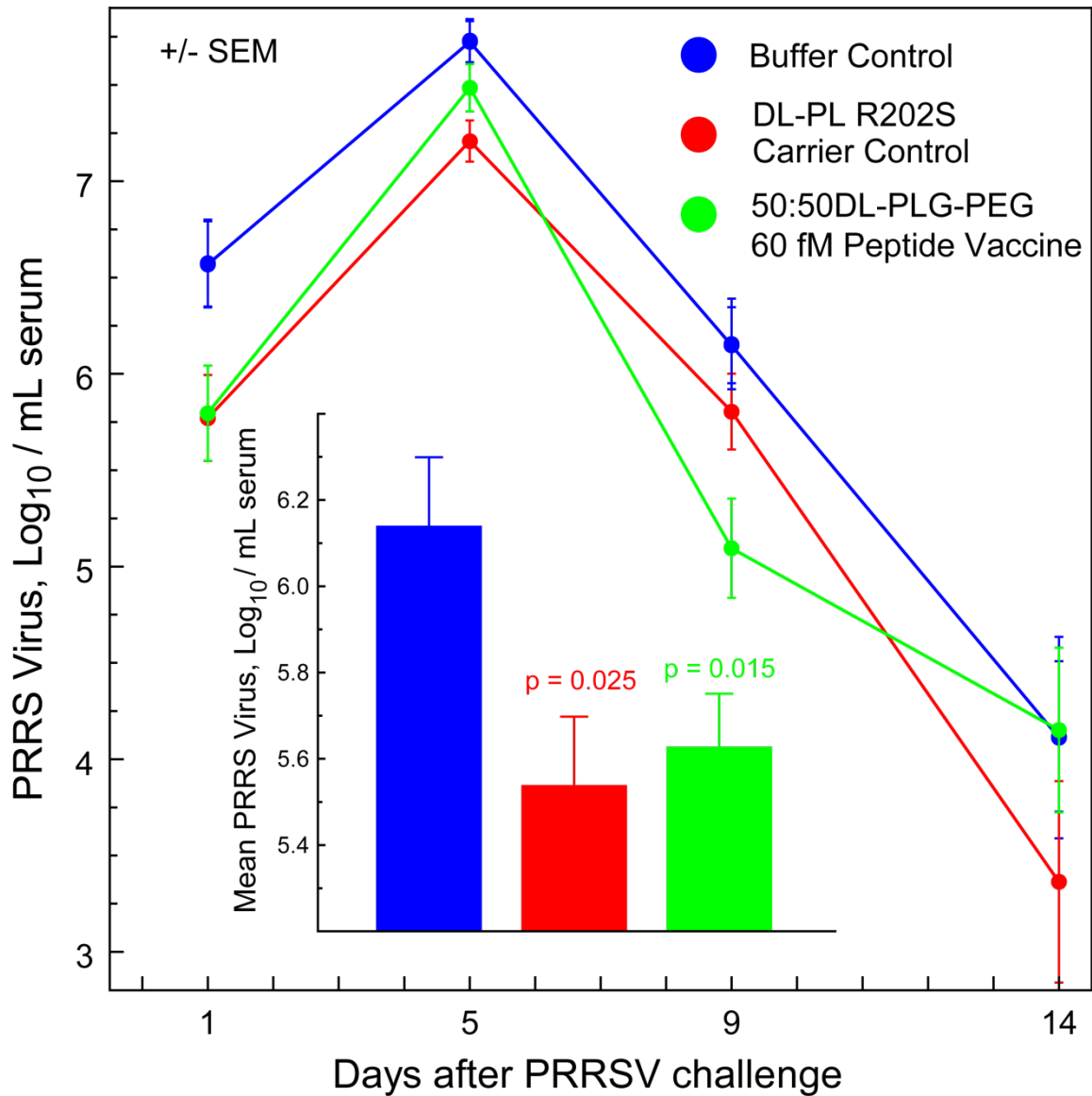
Data in Figure 5.1 and Table 5.1 show a significant reduction of PRRSV loads throughout the course of the challenge infection in both carrier control and vaccine pigs as compared to the suspension buffer controls. While the 75% and 69.3% virus suppression mediated by carrier control and vaccine, respectively, would not be considered sufficient in strict immunological terms, it is nevertheless sufficiently potent to eliminate disease, as evident by the marginal body temperature increases during challenge as compared to the suspension buffer controls (Table 5.1). The negative PRRSV pre-challenge antibody levels confirm that any vaccine response against PRRSV challenge was mediated cellular, but not by antibody immunity. Consistent with lower virus exposure due to virus suppression by cellular immunity is also the significantly reduced PRRSV antibody responses evoked by

the challenge infection in both carrier control and vaccine pigs as compared to the suspension buffer controls (Table 5.1).

**Carrier and Vaccine Microparticles Exert Strong Immunostimulatory and Growth-Promoting Effects.** The most important finding with respect to real-life beneficial use of our synthetic vaccine platform is, however, the fact that it highly significantly enhanced the growth of the vaccinated pigs (Table 5.1, Fig. 5.2). This growth enhancing effect is most significantly evident in the first two weeks after vaccination, when the pigs are under considerable stress due to transport, re-grouping with socially unknown cohorts, and the exchange of commensal and pathogenic infectious agents between these cohorts. Both carrier control and vaccine pigs show highly significantly higher body weight gains during this critical initial 2-week period than the suspension buffer controls. While the profoundly enhanced growth is not sustainable at this level once the experimental cohorts have reached social and infection equilibrium, the growth promoting effect nevertheless persists until challenge inoculation on day 56 (Table 5.1). Then again during PRRSV challenge, both carrier and vaccine groups show enhanced growth due to disease protection by BRM effect of carrier and cellular immunity of vaccine pigs. In total from day 0 through day 70, weight gains are significantly higher by 14.6% in PRRSV vaccine pigs as compared to suspension buffer controls, and marginally fail to reach significance at 10.4% in carrier control pigs (Table 5.1).

In a follow-up experiment, we further tested the growth promoting effect in more depth by doubling the groups size to 12 pigs and by also measuring feed consumption. This allowed us to determine feed conversion rates, aside from animal loss rates the

ultimate measure of economic benefit of any management procedure in production animal agriculture. We used in this experiment a single vaccine based on the slowly degrading polylactide DL-PL R202S carrier, and loaded with a 5-fold higher antigen load of 300 fM PRRSV peptides per 3 mg pig vaccine dose. This vaccine did not mediate protection against PRRSV and elicited higher body temperature during challenge than the suspension buffer control vaccine (data not shown). This was most likely due to an inflammatory immune response because of antigen overdosing, similar to observations in the mouse *C. abortus* vaccine experiments. Nevertheless, this vaccine also elicited a profound, even stronger, growth promoting effect in the first two weeks after vaccination (Fig. 5.2A), and enhanced growth by 47.7% over the suspension buffer control pigs (90.4% vs. 61.2% weight gains). Most importantly, this enhanced growth was achieved by even lower feed consumption than that of suspension buffer controls (Fig. 5.2), as indicated by 50.6% increased feed conversion (1.16 vs. 0.77 g weight gain/g feed). Again, this profoundly increased growth did not persist once the groups had equilibrated, but the trends for weight gains remained similar to the first vaccine experiment.



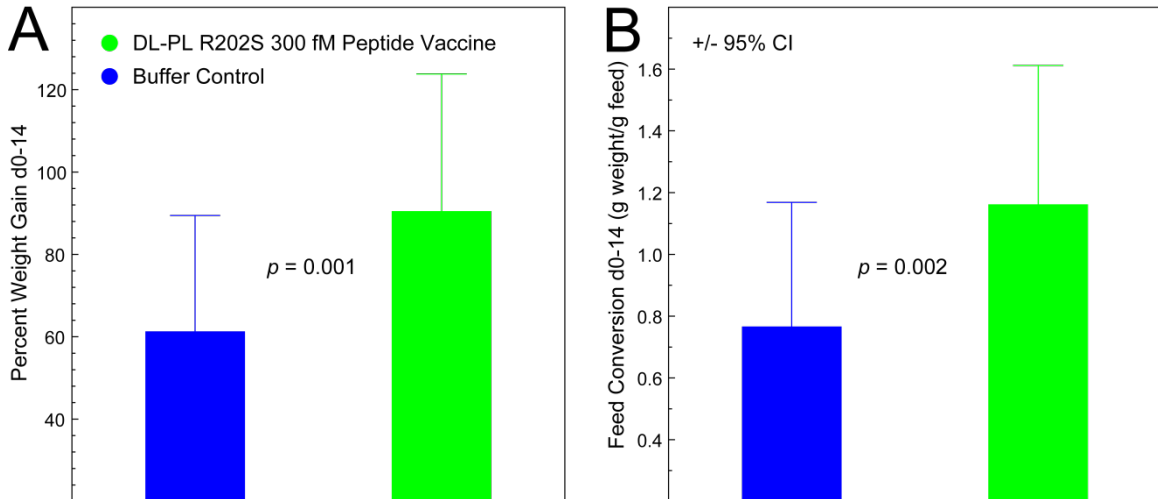
**Figure 5.1. Serum loads of PRRSV after PRRSV challenge.** Data of are shown controls that received on day 0 suspension buffer only (buffer control), or 3 mg microparticles composed of slowly degrading polylactide DL-PL R202S and Pluronic L121 adjuvant (DL-PL R202S carrier control), or 3 mg microparticles composed of rapidly degrading 50:50DL-PLG-PEG carrier, Pluronic L121, and 60 fM of PRRSV peptides (50:50DL-PLG-PEG 60 fM peptide vaccine). Pigs were intranasally challenged on day 56 with at a dose of  $10^{5.0}$  TCID<sub>50</sub> of highly virulent PRRSV strain FL12, and euthanized on day 70. Comparisons of trends and means of log-transformed PRRSV serum loads were performed by repeated measures ANOVA and Tukey's HSD post-hoc test (n = 6).

**Table 5.1. Results of PRRSV microparticle vaccine challenge experiment.**

	Suspension Buffer Control <sup>a</sup>	DL-PL R202S Carrier Control <sup>a</sup>	50:50DL-PLG-PEG 60 fM PRRSV Peptide Vaccine <sup>a</sup>
% weight gain day 0-14	41.76	<b>56.63</b>	<b>61.58</b>
day 0-56	314.48	323.92	351.39
day 57-70	17.74	25.13	20.64
day 0-70	387.71	428.15	<b>444.16</b>
Anti-PRRSV antibodies day 70 <sup>b</sup>	2.07	<b>1.66</b>	<b>1.81</b>
°F body temperature increase d57-70	1.16	<b>0.56</b>	<b>0.32</b>
Area under Log PRRSV RNA curve	85.26	<b>77.79</b>	<b>77.65</b>

<sup>a</sup> n = 6; <sup>b</sup> pre-challenge antibody levels were 0 arbitrary units

<sup>c</sup> comparison to suspension buffer control: **red**  $p \leq 0.05$ ; **red**  $p \leq 0.001$ .



**Figure 5.2. Growth and feed conversion in the first two weeks after PRRSV microparticle vaccination.** In a repeat of the preceding trial with increased group size of 12 pigs, buffer control pigs and vaccine pigs are shown that received 3 mg microparticles composed of slowly degrading polylactide DL-PL R202S, Pluronic L121 adjuvant, and 300 fM of PRRSV peptides (DL-PL R202S 300 fM peptide vaccine). Group comparisons were performed by Student's t-test (n = 12).

## 5.4. DISCUSSION

The PRRSV vaccine challenge trials have confirmed that our microparticle vaccines elicit without negative side effects cellular, but no antibody, immunity in the agriculturally relevant porcine production animal species. Implicit in this finding is also that the dose and mode of vaccine administration have been successfully scaled up from the mouse model to the pig. While the use of the long-acting and stable polylactide DL-PL R202S vaccine carrier is highly preferable, reduced antigen dosing will need to be optimized for maximal achievable protection and absence of an inflammatory response.

Economically most important is the profound growth promoting effect of the microparticle immune stimulator and vaccine platform, enhancing both growth and feed conversion during periods of stress. In contrast to other vaccines, in particular to attenuated live vaccines, the microparticle vaccines enhance, rather than suppress, growth. In experiments, the growth-promoting effect manifests itself mainly when the treatment cohorts are initially cohabitated, but recedes after the cohorts have reached social and infection equilibrium. Under industrial animal agriculture conditions with large cohorts, however, a social and, in particular, an infection equilibrium typically is never reached, because of the increasing frequency of “supershedder” animals in such large cohorts. Therefore, a long-term sustained growth promoting effect of the microparticle immune stimulator and vaccine platform can be anticipated.

In summary, we have developed a fully synthetic, biodegradable T cell vaccine platform technology that also strongly enhances growth and feed conversion in agricultural production animals. Industrial spray drying on a mid-size production facility can produce  $\geq 4$  million chicken or  $\geq 400,000$  pig or  $\geq 40,000$  cow doses per hour. This technology also

enables the rapid discovery, development, and production of new vaccines. Moreover, polylactide carrier vaccines produced by this method are substantially cheaper than current vaccines, and do not require a cold storage chain. We anticipate commercialization of this vaccine platform technology in the near future.

#### 5.4. MATERIALS AND METHODS

**Animal Experiments.** The study was conducted by collaborators Drs. Fernando Osorio and Hiep Vu at the University of Nebraska-Lincoln. Groups of 6-12 female weaned PRRSV-free pigs (3 weeks of age) were purchased from the UNL research farm. The pigs were accommodated in the BL-2 facility at UNL for one week and a serum sample collected to assure lack of viremia and sero-negativity for PRRSV. Subsequently, pigs were subcutaneously vaccinated with 3 mg of the microparticle vaccine suspended in 1 ml of suspension buffer (Chapter 3).

**Vaccine Microparticle Synthesis and Composition.** Microparticles were synthesized with the optimized spray dry procedure as described in Chapter 3. In brief, 2% feedstock solutions composed of vaccine were prepared in DCM by adding the desired volume of previously prepared stock solutions. Microparticles were obtained by spraying the prepared feedstock solution in the Büchi mini spray dryer model B-190 (Büchi Labortechnik AG, Flawil, Switzerland).

The following compositions of microsphere vaccines were used:

- 1) DL-PL R202S and Pluronic L121 (6.5:3.5) as vaccine carrier control;
- 2) 50:50DL-PLG-PEG and Pluronic L121 (6.5:3.5), and 60 fM pooled 511 FL12 challenge strain PRRSV peptides per 3 mg vaccine dose as experimental vaccine #1 (equivalent to 0.2 fM peptides per 10 µg mouse vaccine dose);
- 3) DL-PL R202S and Pluronic L121 (6.5:3.5), and 300 fM pooled 511 FL12 challenge strain PRRSV peptides per 3 mg vaccine dose as experimental vaccine #2 (equivalent to 1.0 fM peptides per 10 µg mouse vaccine dose);



Per 20 pig vaccine doses, 60 mg of the vaccine microparticles were mixed with 300 mg lactose anhydrous microfine (DFE Pharma, Germany) in a glass vial. Prior to administration, they were homogeneously suspended in 20 mL of suspension buffer (0.001% benzalkonium chloride and 0.5% Kolliphor HS-15 in PBS) by push/pulling 5× through a 20-gauge needle.

**PRRSV Challenge Infection.** At 56 day post-vaccination, pigs were challenged by intranasal spray-infection with wild-type, highly virulent PRRSV strain FL12 at a dose of  $10^{5.0}$  TCID<sub>50</sub> per pig. At 70 day post-vaccination (14 days after challenge infection, dpc), the pigs were euthanized and necropsied. Body temperature was measured daily from -3 to 14 dpc by using micro-transponders that were implanted to pigs at the beginning of the experiment. Body weight was determined in bi-weekly intervals from the day of vaccination (day 0) through the termination of the experiment at day 70. Feed consumption per group was determined by weighing of feed before adding to the feeder. Every two weeks, feed remaining in the feeder was weighed and subtracted from the starting feed weight. Serum samples were collected at 0, 56, 57, 59, 61, 65, 70 days (-56, 0, 1, 3, 5, 9, 14 dpc) and stored at -80°C. At 14 dpc, pigs were be euthanized and necropsied, and samples of tonsil and Inguinal lymph node were collected and stored at -80°C.

**PRRSV Sample Analyses.** PRRSV specific antibodies were determined in serum collected at day 0, and on days 56 and 70 by the commercial PRRSV IDEXX ELISA. PRRSV viral loads were determined in total nucleic acids extracted from 100 µl serum by a PRRSV 3'UTR single-step, quantitative, reverse-transcriptase real-time fluorescence

resonance energy transfer PCR following the design and protocol of *C. abortus* PCR methodology developed in the Kaltenboeck laboratory.

**Data Analysis.** All analyses were performed with the Statistica 7.1 software package (StatSoft, Tulsa, OK). Results were analyzed by Student's t-test and one-way and repeated measures ANOVA with Tukey's honest significant differences test for correction of the p value in multiple comparisons. P values  $\leq 0.05$  were considered significant.

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