Novel intranasal proteosome-based respiratory syncytial virus (RSV) vaccines elicit protection in mice without the risk of enhanced pathology or eosinophila by triggering innate immune pathways.

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For Nicholas,

Nathan and Samuel

Abstract

No safe and effective vaccine exists against respiratory syncytial virus (RSV), the main viral cause of lower respiratory tract infections in young children. Proteosome-based adjuvants, derived from the outer membrane proteins (OMP) of Neisseria species are potent inducers of mucosal and systemic immunity in humans and animals. RSV subunit vaccines based on enriched RSV proteins (eRSV) were formulated with proteosomes (Pro) or its *S. flexneri* LPS-supplemented derivative, Protollin (Prl). Administered intranasally (IN) in BALB/c mice, the vaccines elicited systemic and mucosal RSV-specific antibodies and fully protected against RSV challenge without enhanced pulmonary pathology or evidence of a Th2-biased response (eg: eosinophil infiltation or antigen-specific IL-5 production by restimulated splenocytes or lung cells). Restimulation of cells from Prl-eRSV immunized mice elicited F peptide-specific CD8+ T cells producing IFNγ and supernatant IFNγ, TNFα, IL-2 and IL-10. The Prl-eRSV vaccine was also studied in C57Bl/6 mice, to exploit the TLR2, TLR4 and MyD88-deficient (-/-) animals available on this background. Protection was significantly impaired in both TLR4-/- and MyD88 -/- mice, but not in TLR2 -/- mice following Prl-eRSV immunization and challenge. These studies revealed a role for the LPS component of Protollin in both initial (innate) cytokine release as well as dendritic cell maturation and Th1 polarization. Although antibody levels were sustained in MyD88-/- mice, the IgG1/IgG2a ratio was markedly higher in the absence of this pathway. The MyD88-/- mice also displayed elevated levels of pulmonary eosinophils following challenge, with concomitant reduction of neutrophils compared to wt mice. Using CD1d-iNKT cell-deficient mice (CD1-/-) in our BALB/c model, we also identified a significant role for the lipid component of both the Pro- and Prl-based vaccines. Responses to both vaccines in CD1-/- animals elicited lower antibody titers and reduced restimulated splenocyte supernatant cytokines (IFNγ, IL-17 and IL-10), with concomitant augmentation of neutrophil recruitment (Prl only). Pro- and Prl-eRSV vaccines may therefore exert their powerful adjuvant effects by exploiting both CD1d-iNKT and, in the case of the Prl-based formulations the TLR4-MyD88-dependant signalling pathway. These pathways not only promote stronger Th1 immune responses but also act to control pulmonary eosinophil (MyD88-dependent) and neutrophil (MyD88 and CD1d-NKT-dependent) recruitment in a murine RSV challenge model.

Résumé

Aucun vaccin n'est disponible contre le virus respiratoire syncytial (RSV), la principale cause virale d'infection des voies respiratoires inférieures chez les jeunes enfants. Les adjuvants protéosomes, dérivés des protéines de la membrane externe des espèces *Neisseria*, induisent une augmentation importante des réponses immunes mucosales et systémiques chez les hommes et les souris. Deux vaccins candidats comprenant des protéines RSV enrichies (eRSV) furent formulés avec des Protéosomes (Pro) ou son dérivé contenant en plus des LPS de *S. flexneri*, nommé Protollin (Prl). Ces vaccins administrés par voie intranasale (IN) chez les souris BALB/c induirent des anticorps systémiques et mucosaux spécifiques au RSV et une protection complète contre l'infection expérimentale au RSV, et cela sans causer une augmentation de la pathologie pulmonaire ou de la réponse Th2 (recrutement eosinophilique pulmonaire ou production de l'IL-5 suivant la restimulation de splénocytes ou cellules pulmonaires). La restimulation des splenocytes de souris immunisées avec Prl-eRSV élicita de l'IFNγ, du TNF-α, de l'IL-2 et de l'IL-10 dans le surnageant et des cellules CD8+ spécifiques au peptide F capable de produire de l'IFNγ. L'efficacité du vaccin Prl-eRSV fut ensuite analysé chez les souris C57Bl/6, ainsi que dans les souris TLR2, TLR4 et MyD88-déficientes (-/-). La protection fut réduite de façon significative chez les souris TLR4 et MyD88-/-, mais non pas chez les souris TLR2-/- après immunisation avec Prl-eRSV. Ces études démontrèrent le rôle crucial joué par les LPS de Protollin dans la production de cytokines pro-inflammatoires, l'augmentation de molécules co-stimulatoires ainsi que la polarisation des cellules Th1. Même si le niveau d'anticorps spécifiques au RSV fut maintenu chez les souris MyD88-/-, le ratio IgG1/IgG2a fut dramatiquement plus élevé que celui des autres groupes. De plus, ces souris MyD88-/- développèrent un niveau beaucoup plus élevé d'éosinophilie pulmonaire suivant l'infection expérimentale, parallèlement à une réduction des neutrophiles recrutés en ce site. En utilisant des souris déficientes en CD1d-iNKT (-/-) dans notre modèle BALB/c, nous avons aussi identifié un rôle important pour les composantes lipidiques des vaccins Pro- et Prl-eRSV. Les réponses immunitaires élicitées à la suite de ces deux vaccins démontrèrent des niveaux réduits d'anticorps et de cytokines (IFNγ, IL-17 et IL-10) spécifiques au RSV en plus d'un recrutement élevé de neutrophiles pulmonaires (Prl-eRSV seulement). Pro- et Prl-eRSV peuvent donc éliciter une immunogénicité optimale en utilisant l'axe CD1d-NKT,

et dans le cas des vaccins Prl, la signalisation dépendante à TLR4-MyD88. Ces voies de signalisations contribuent non-seulement à promouvoir des réponses Th1 plus puissantes, mais aussi contribuent à la régulation du recrutement des éosinophiles (dépendant de MyD88) et neutrophiles (dépendant de MyD88 et CD1-NKT) pulmonaires chez unmodèle murin d'infection expérimentale au RSV.

Introduction, Rationale and Objectives

The rationale of this thesis was based on the following general hypothesis: "*An intranasally delivered respiratory syncytial virus vaccine formulated with proteosome-based adjuvants will elicit protective immunity and prevent pulmonary eosinophilic inflammation by modulating the virus-specific immune response towards a T-helper 1 phenotype.*" To test this hypothesis, a number of Specific Aims guided my research:

1. *To produce enriched antigen RSV preparations and optimise vaccine formulation.*
2. *To test optimised vaccine for immunogenicity and characterise immune phenotype.*
3. *To establish animal models for protection and the induction of eosinophilia.*
4. *To determine protective efficacy of the optimised vaccine.*
5. *To exploit well-defined gene knock-out mice to examine the roles of specific vaccine components in the adaptive and maladaptive immune responses to RSV vaccination.*

In addition to the literature review (Chapter 1), this thesis is divided in four manuscript-style chapters, three of which have been submitted for publication (Chapter 2, published; Chapter 3, published; Chapter 4, submitted; Chapter 5, in preparation). Chapters 2 and 3 fulfill our Specific Aims #1-4 using different mouse strains. Chapter 2 describes the protective efficacy and safety of proteosome- and Protollin-eRSV vaccines in BALB/c mice. Chapter 3 establishes Protollin-eRSV vaccine efficacy in C57Bl/6 mice, paving the way for mechanistic studies in TLR knock-out animals on this genetic background (Chapter 4). The last chapter (Chapter 5) describes the possible involvement of the CD1d/Natural killer T (NKT) cell pathway in the upregulation of immunogenicity following Proteosome- or Protollin-eRSV vaccination. These last two chapters fulfill our Specific Aim #5.

Contributions of authors

Experimental conception and design were determined by the author with minimal guidance from advisors, especially in the later years of study. Throughout the work reported in this thesis, a number of reagents, processes and assays are used which were executed and established in the laboratory by the author: RSV propagation in host cells (including bioreactor growth); RSV antigen enrichment process; rapid Proteosome-eRSV vaccine formulation process; RSV challenge model; viral lung titration assay and neutralization titers, both with immunofluorescence detection of RSV antigens; RSV-specific serum and bronchoalveolar lavage (BAL) ELISAs; MHC-restricted peptide restimulation of splenocytes; restimulations of lung cell preparations; pentamer analysis by FACS; histopathology slide analysis; and BAL leukocyte differential counts. Some of these assays were performed by junior laboratory personnel once they were established.

Assays used in this thesis that were already established in the laboratories prior to this work or that were established in parallel efforts include (with their respective originator): intranasal immunization technique (Taff Jones); histopathology slide preparation (Nathalie Bellerose); BAL collection and splenocyte restimulation assay (Martin Plante); cytospin of BAL leukocyte technique (Karen Dubé).

Manuscripts and co-authors contributions

The writing of the manuscripts was performed entirely by the author with editorial contributions from senior industrial and academic advisors. Minimal contributions from advisors were required for the later manuscripts. As a Research Director at ID Biomedical and subsequently GSK Biologicals, David Burt contributed with guidance and vision to make this project a reality and a success. Similarly, as a senior scientist at ID Biomedical, GSKBio and now MedImmune/AstraZeneca, as well as an adjunct McGill professor in the Department of Microbiology and Immunology, Taff Jones acted as my thesis co-supervisor and provided daily supervision in the early days as well as mentorship. Finally, throughout my thesis, Brian Ward, Principal Investigator and thesis

supervisor, brought support, scientific direction and mentorship. Ioana Stoica-Popescu provided technical help. The contributions of authors not mentioned above is described below with the corresponding manuscript title.

Intranasal proteosome-based respiratory syncytial virus (RSV) vaccines protect BALB/c mice against challenge without eosinophilia or enhanced pathology.
Vaccine. 2007 Jul 20;25(29):5378-89

Sonya L. Cyr, Taff Jones, Ioana Stoica-Popescu, Angela Brewer, Sophie Chabot, Michèle Lussier, David Burt, Brian J. Ward.

Author contributions: Sophie Chabot participated in the early vaccine design discussions. Angela Brewer helped with the virus growth in the bioreactor and Michèle Lussier performed a restimulation assay following immunization.

C57Bl/6 mice are protected from respiratory syncytial virus (RSV) challenge and IL-5 associated pulmonary eosinophilic infiltrates following intranasal immunization with Protollin-eRSV vaccine.
Vaccine. 2007 Apr 20;25(16):3228-32.
Sonya L. Cyr, Taff Jones, Ioana Stoica-Popescu, David Burt, Brian J. Ward.

Intranasal, Protollin-based RSV vaccination in mice: TLR4/MyD88 signaling is required both for protection and the control of pulmonary granulocyte recruitment following challenge.
Submitted for publication.
Sonya L. Cyr, Isabelle Angers, Loic Guillot, Ioana Stoica-Popescu, Michèle Lussier, Salman Qureshi, David S. Burt, Brian J. Ward.

Author contributions: Salman Qureshi laboratory; Isabelle Angers performed the mouse breeding and helped with the genotyping. Loic Guillot was instrumental in getting the mice in the laboratory and performed genotyping. Salman Qureshi provided scientific leadership and support. Michèle Lussier helped with sample analysis.

CD1d signaling and natural killer T (NKT) cell activation may contribute to the immunogenicity of proteosome-based Respiratory Syncytial Virus (RSV) vaccines in mice.

Sonya L. Cyr, Ioana Stoica-Popescu, Brian J. Ward.

Contributions to original knowledge: Demonstration that adjuvants which contain lipids may utilize this pathway to enhance immune responses. Hypothesis conception by the author.

List of Abbreviations

BAL, bronchoalveolar lavage

CMI, cell-mediated immunity

DC, dendritic cells

eRSV, enriched respiratory syncytial virus antigen

FI-RSV, formalin-inactivated RSV vaccine

HA, hemagglutinin

IFN, interferon

IL-, interleukin-

IN, intranasal

IM, intramuscular

IRAK, interleukin-1 receptor-associated kinase

IRF-3, IFN regulatory factor 3

LPS, lipopolysaccharide

LS, lung cell supernatants

M (cells), microfold

MHC, major histocompatibility complex

mt, mutant

MyD88, myeloid differentiation protein 88

NALT, nasopharynx-associated lymphoid tissue

NF-κB, nuclear factor kappa B

NKT, natural killer T cells

OMP, outer membrane protein

PAMP, pathogen-associated molecular pattern

PE, phosphatidylethanolamine

PG, phosphatidylglycerol

PIP2, phosphatidylinositol 4,5-biphosphate

PorA, Porin A

PorB, Porin B

Prl, Protollin

Pro, proteosome

RSV, respiratory syncytial virus

SS, splenocyte supernatants

TBK1, TNFR associated NF-κB kinase (TANK) κB binding kinase-1

TCID, tissue culture infective dose

Th1, T-helper 1 cells

Th2, T-helper 2 cells

TIRAP, Toll/IL-1 receptor (TIR) domain-containing adaptor protein, also known as MAL

TLR, Toll-like receptor

TNF, tumor necrosis factor

TNFR, tumor necrosis factor receptor

TRAF-6, tumor necrosis factor receptor (TNFR) associated factor

TRAM, Toll/IL-1 receptor (TIR) related adaptor molecule

TRIF, Toll/IL-1 receptor (TIR) domain-containing adaptor-inducing IFN-β

wt, wild type

Table of contents

Chapter 1. Literature Review

1.1. Respiratory Syncytial Virus.

1.1.1. History

First isolated in a laboratory chimpanzee during an epidemic of respiratory illness in 1956, respiratory syncytial virus (RSV) was originally coined chimpanzee coriza agent (CCA) [1]. Within a year, it had been recovered from two young patients suffering from either pneumonia or croup, at which time the characteristic syncytial cytopathic effect was first observed [2]. The virus was rapidly identified as the most important cause of viral lower respiratory tract illnesses in young children [3].

1.1.2. Epidemiology

To this day, RSV remains a major cause of infant and childhood morbidity and mortality worldwide [4,5]. Sizable RSV outbreaks occur every year with a biannual rhythm [6]. Although there are two major circulating strains of RSV (A and B), no particular clinical or epidemiologic features distinguish between the two strains [7]. In a recent epidemiology study performed in Valladolid, Spain, it was determined that even though epidemic onset and conclusion can vary, the peak prevalence typically occurs in January and the length (8 months) remains constant [8]. Similarly, in Fortaleza, Brazil, the length of recent epidemics was observed to be relatively constant (6-8 months) with onset (January or February) corresponding to the beginning of the rainy season [9]. Risk factors associated with severe RSV morbidity and mortality include premature birth and pre-existing respiratory or cardiac deficiencies [10]. For example, in a recent outbreak in a neonatal intensive care unit in Nashville TN, infants requiring intubation had

significantly lower birth weights and gestational ages (eg: 28 versus 35 weeks) than non-intubated infants [11]. Although severe RSV disease resulting in hospitalization usually occurs in neonates and infants, infections continue to occur throughout life, with the severity and frequency decreasing with age until the onset of senescence. The risk of severe RSV diseases in the elderly has only recently been recognized [12,13]. Immunocompromised patients are also at particularly high risk for severe RSV disease [14-16].

In all of the groups at particular risk for severe RSV disease, several associations have been observed with other pathogens such as Pneumococci [17] and Parainfluenza virus [18]. Prior RSV is the most common virus infection associated with acute otitis media (AOM) caused by bacterial pathogens such as *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis.* AOM is a disease of low severity but very high prevalence in the paediatric age group [19]. Although the potenial for epidemiological interference between influenza viruses and RSV viruses exists, no such interference has been observed. In fact, both viruses can infect simultaneously, with influenza virus and RSV typically causing upper respiratory tract symptoms (URTI) and lower respiratory tract symptoms (LRTI), respectively [20].

1.1.3. Virology

1.1.3.1. Classification

RSV is classified in the order of the *mononegavirales*, possessing a non-segmented, negative-sense, single-strand RNA genome and is a member of the pneumovirus genus of the *paramyxoviridae* family [21,22]. Particular features of *mononegavirales* virions include the tight association of the entire viral genome with nucleocapsid-associated proteins and polymerase. Genomic RNA is transcribed into capped, polyadenylated positive strand mRNA by the virus-encoded RNA-dependent RNA polymerase for translation into viral proteins. For genomic RNA replication, a positive-sense copy is synthesized and encapsidated and then serves as the template for the synthesis of full-length progeny [23]. The replicative cycle occurs entirely in the cytoplasm. This process is completed with the budding of the nucleocapsid through the host-cell plasma membrane embedded with viral proteins, thus generating the viral envelope [24,25].

1.1.3.2. Genetic organization

The RSV genome encodes 10 major sub-genomic mRNA that give rise to 11 viral proteins, two of which (M2-1/M2-2) are encoded by overlapping open-reading frames in the same M2 mRNA [26] (Figure 1). The remaining encoded proteins are the non-structural proteins 1 and 2 (NS1 and NS2); nucleocapsid protein (N); phosphoprotein (P); matrix protein (M); small hydrophobic protein (SH); attachment protein (G); fusion protein (F); transcription antitermination factor (M2-1), RNA synthesis factor (M2-2) and the large polymerase protein (L). The paramyxovirus family includes three other genera, containing well known viruses such as Sendai, human parainfluenza and measles viruses

which all have significant general and regional amino acid and nucleotide sequence similarities [26]. Compared to these other viruses, RSV, the only known human virus of the pneumovirinae, encodes for the unique non-structural proteins NS1 and NS2, the small hydrophobic surface protein (SH) and a second matrix protein M2-2 [26].

1.1.3.3. Protein functions

The NS proteins of some *mononegavirales* have been demonstrated to interfere with the anti-viral immune response. This effect was first demonstrated for RSV in a Bovine model in which mutant viruses lacking the NS2 protein were unusually strong inducers of IFN-α/β [27]. Recently, several studies have implicated the RSV NS proteins with inhibition of type-1 interferon signal transduction and cytotoxic T cell responses [28,29], with suppression of premature apoptosis by an NF-kappaB-dependent, interferon-independent mechanism [30] and with the degradation of STAT2 [31]. In some respects, these actions of the NS proteins in RSV infection parallel those of the C and V genes of other Paramyxoviridae [32,33]. The N protein contributes to the formation of an Rnase resistant nucleocapsid of helical structure. This RNA-N complex serves as a template for the polymerase complex formed by the phosphoprotein (P), major polymerase subunit (L), and the M2-1 and M2-2 cofactors [34]. During genome transcription and replication, P is believed to position L on the RNA–N template and to help translocation of the polymerase complex along the helical nucleocapsid [35]. It has recently been demonstrated that the nine C-terminal amino acids of protein P are both necessary and sufficient for binding to RNA-N ribonucleoprotein complexes [36].

F, G and SH are all transmembrane surface glycoproteins. Similar to the surface proteins of many other viruses, all have been shown to co-localize at lipid rafts to facilitate viral budding and production of infectious virions [37,38]. However, it has recently been demonstrated that the RSV F protein targets lipid rafts without a requirement for the other surface proteins [39]. In addition, although the RSV F protein is related both structurally and functionally to the F proteins of other paramyxoviruses [26,40], it can be self-sufficient for cell-to-cell transmission [41-43]. Synthesized as a precursor type I glycoprotein, F_0 is cleaved by a cellular protease of trypsin-like specificity at its N-terminus, leading to the formation of disulfide-linked F_2 and F_1 domains, anchored via the C-terminus of the F_1 domain [44]. Due to its relatively high level of conservation within the circulating strains and its requirement for syncytia formation [45], the F protein is thought to be an extremely important antigen for vaccine development.

The role of the G protein as an RSV attachment protein is well-characterized, although no sequence relatedness or structural similarities are found with the HN or H attachment proteins of other paramyxoviruses [46]. In fact, the only similarity between RSV G and the attachment proteins of other paramyxovirus members is their glycoprotein type. All are type II glycoproteins, with uncleaved N-terminal anchor sequences and extracellular C-termini [46]. Heavily glycosylated (both *N*- and *O*- linked sugars constitute about 60% of its total weight) [47], the RSV G protein has been demonstrated to interact with glycosaminoglycan (GAG) on host cells via its heparin binding domains [48]. The G protein also possesses a CX3C chemokine motif within its non-glycosylated conserved core region that can bind CX3CR1 and modify CXCL1-mediated responses [49].

Moreover, recent studies have shown that RSV G may also interact with L-selectin on lymphocytes (CD62L) and annexin II on epithelial cells [50]. Like the F protein, antibodies directed against the RSV G protein can neutralize this virus, making it an attractive antigen for vaccine development [51]. The function of the remaining surface glycoprotein, SH, remains unclear although it is known to accumulate in the Golgi complex in some in vitro systems [52].

1.1.4. Clinical disease

RSV infections in otherwise healthy infants normally cause respiratory symptoms limited to the upper respiratory tract (eg: rhinorrhea). However, in 25-40% of cases, infections can progress to below the larynx in conjunction with decreased appetite [53]. When this occurs, cough usually follows 1 to 3 days later, with sneezing and low-grade fever. In mild cases, the disease may not progress further and the chest X-ray remains normal. Clinically, there can be moderate tachypnea, diffuse rhonchi, fine rales and wheezes [54,55]. Importantly rhinorrhea, intermittent fever and otitis media are also common. Recovery generally occurs within 7 to 12 days [56]. However, in severe cases, progression of coughing and wheezing can cause dyspnea, chest hyperexpansion, and both intercostal and subcostal retraction may be evident. Severe tachypnea can supervene, leading to hypoxia and respiratory failure in advanced disease [57,58]. In immunocompromised patients, RSV infection may cause rapid respiratory failure leading to mortality rates exceeding 70% [59].

Although the typical manifestations of RSV occur in the respiratory tract, extrapulmonary manifestations are common in children with severe RSV infection [60]. Life-threatening

extrapulmonary manifestations of RSV infection include central apnoea [61], febrile convulsions [62], ventricular tachycardias, fibrillation and pericardial tamponade [63-65]. Hyponatraemia is common among children suffering from severe RSV infection and can lead to seizures [62]. RSV RNA is detectable by RT-PCR in the peripheral blood of a significant proportion of RSV infected neonates and infants (63 and 20%, respectively) [66]. Based on the low percentage of bacterial infections identified in admitted infants (0.6-1.2%), it is unlikely that these manifestations are due to systemic co-infections [67].

1.1.5. Host response to RSV

1.1.5.1. Innate immunity

Host-defense against RSV depends on the interaction of the innate and adaptive immune responses, which include many overlapping systems over the course of the infection. The primary contact of RSV with pattern recognition receptors (PRR) located in the airway epithelium causes the release of a number of factors that induce the innate anti-viral response including soluble PRRs include surfactant proteins [68] and CD14 [69]. Surfactant protein A (SP-A) can specifically bind to the highly glycosylated F2 subunit of the F glycoprotein [70], and neutralize RSV infection by inducing macrophage-mediated opsonization of viral particles [71]. Toll-like receptors (TLRs) act as cell-associated PRRs to signal the presence of a foreign invader by activating downstream signalling cascades resulting in cytokine expression. RSV was one of the first viruses to be identified as a TLR4 ligand, following the demonstration that TLR4-deficient mice had impaired capacities to clear the virus (reduced IL-12 expression, NK and CD14+ cell pulmonary trafficking activity) [72]. Although TLR4 polymorphisms have been identified that confer decreased responsivity to LPS, a recent study comparing the genotypes of children with severe vs mild RSV disease found no association between the Asp299Gly polymorphism and disease severity [73]. Further studies are required to decipher the molecular basis for the implication of TLR4 in RSV disease [74,75].

The chemokine and cytokine expression patterns that follow RSV infection of epithelial cells have powerful effects on the balance between virus elimination and disease pathogenesis. For example, infants hospitalized with RSV infection exhibit RSV-specific

increases in both Th1 (IL-2 and IFNγ) and Th2 (IL-4 and IL-6) cytokines, as well as CC chemokine (MIP-1α, RANTES) mRNA expression [76]. The innate immune cells that respond to chemokine and cytokine signals, which include macrophages, eosinophils, neutrophils, and NK cells, all have important roles in controlling RSV infection, but may exacerbate inflammation and contribute to disease pathogenesis during an imbalanced response [77]. For instance, anti-viral mediators released by eosinophils (eg. eosinophil cationic protein) and neutrophils (eg. defensins) are associated with bronchial hyperresponsiveness [78] and damage to respiratory epithelial cells [79], respectively, following severe RSV infection.

1.1.5.2. Adaptive Immunity

The activation of innate immune cells such as NK cells contributes to the recruitment and maturation of DCs to regulate the downstream development of antigen-specific T and B lymphocytes via major histocompatibility complex (MHC) antigen presentation. Cell-mediated immunity plays an important role in the clearance of RSV as suggested by markedly increased RSV disease severity in immunosuppressed patients [80,81]. In addition to antigen-specificity, T and B lymphocytes help to protect against RSV through their ability to recognize a variety of antigens and mount memory responses [82]. Relative resistance to RSV infection in the upper respiratory mucosa is thought to be mediated, in part, by transient local expression of secretory IgA [83]. More durable resistance however, is associated with neutralizing IgG antibodies [84]. Interestingly, although serum neutralizing antibodies correlate well with protection of the lower respiratory tract, high titres of serum neutralizing antibodies do not alter virus replication

in the nasopharynx [85,86]. Moreover, even though RSV reinfection of the upper respiratory tract is common, reinfection of the lower airways is rare, suggesting that the mediators of immunity may differ between the upper and lower airways, with more transient local immunity and memory-efficient humoral immunity dominating at these two sites, respectively [87].

The phenotype of helper-T cell responses elicited following RSV infection is not entirely clear. A number of studies have linked Th2-biased responses to RSV disease severity, characterized by elevated antigen-specific IgE and lower IFNγ/IL-4 ratios in restimulated PBMCs of infected individuals [88,89]. Moreover, the development of Th2 responses during RSV infection has been associated with the subsequent development of wheezing and asthma [90]. However, patients who develop severe hopoxic bronchiolitis appear to have a more balanced Th1/Th2 response [90]. The latter observation is consistent with a study suggesting that macrophage MIP-1α release, not the Th2 response is the principal mediator of severe forms of RSV diseases [91]. Overall, these reports suggest that differences in T-helper cell responses to RSV infection, which may either be of Th1, Th2 or balanced Th1/Th2 phenotype, can have important consequences for RSV disease manifestations. The final pattern of response can be influenced at the onset of infection by cytokine and chemokine effectors released by epithelial cells and innate leukocytes [92]. While RSV-specific cytotoxic T cells (CTL) can contribute to protection in mice [93,94], it has been difficult to assess the efficacy of RSV-specific CTL in humans. However, the peripheral blood mononuclear cells (PBMCs) of infants with RSV infection

contain RSV-specific CTL [95,96], and CTL responses against N, M, M2, F and SH proteins are elicited in adults [97].

1.2. Vaccine History

1.2.1. Formalin-inactivated RSV vaccine

A vaccine against RSV has been sought from the moment of this virus' discovery. In the 1960s, formalin-inactivated split-virion RSV antigens were combined with an aluminum salt adjuvant and given intramuscularly to children from 2 months to nine years of age (known as formalin-inactivated RSV vaccine or FI-RSV) [98,99]. Contrary to expectations, immunization with the FI-RSV vaccine resulted in sharply increased rates of hospitalization following natural infection (85%) compared the placebo group (5%) [100]. Sadly, two children died in this pivotal study, and RSV vaccine research has progressed very slowly since that time [98]. The testing of subunit RSV vaccines in naïve infants is of great concern to this day. The unusual presence of eosinophils in the lungs of children who died was emphasized in the publications describing the incident [98], sparking great interest in this peculiar manifestation. This unusual pathology was reproduced in mice, which exhibited an altered pattern of CD4+ T lymphocyte activation characterized by eosinophil recruitment and type 2 cytokine production [101]. Exacerbated Th2 responses similar to those observed following FI-RSV immunization and infection were replicated in mice exposed to a vaccinia virus expressing the G protein (vv-G) [102]. These aberrant responses were also shown to be abrogated in the absence of the G protein [103]. Although vv-G and FI-RSV immunization both lead to similar exacerbated Th2 immune responses, these parallel manifestations are actually regulated by distinct pathways [104]. Whereby FI-RSV enhanced-disease can be improved by the

presence of Th1 immune effectors such as IL-12 [105], the response to vv-G immunization is unaffected by IL-12 [106]. This last observation suggests that the propensity of RSV antigens to elicit aberrent responses may be modulated if the correct cytokine milieu is generated during immunization.

Another factor that may have contributed to enhanced disease followng FI-RSV vaccination is the formalin inactivation process itself [107]. In a recent study, the presence of carbonyl groups on formaldehyde-treated antigens was revealed to contribute to the augmentation of Th2 responses. This finding is consistent with reports that even uninfected formalin-inactivated host-cell extracts formulated as a mock vaccine can elicit exacerbated disease similar to that seen with FI-RSV immunization after RSV challenge [108,109]. These observations also suggest that subunit vaccine antigens which are not subjected to a formalin-inactivation process might not be expected to elicit enhanced disease after infection.

Although the human and murine data are quite consistent, data from the cotton rat model [110] and a recent review of the autopsy material from the two patients who died as a result of FI-RSV vaccine immunization and subsequent infection [111] suggest that neutrophils and monocytes rather than eosinophils, are the most prominent cell types to infiltrate the lungs in this vaccine-enhanced disease. Although neutrophils may contribute to FI-RSV enhanced disease, the neutrophil infiltrates in the lungs of these children may have resulted from bacterial superinfections (*K. pneumonia* and *E.coli* were documented) or may have been a reflection of the natural response to RSV pneumonia

itself [111]. Recent cotton rat data is consistent with the mouse work, however, in that FI-RSV vaccine supplemented with the Th1-inducing adjuvant monophosphoryl lipid A (MPLA) can reduce the cytokine storm associated with FI-RSV enhanced disease [112]. Together, these observations suggest that the Th2/FI-RSV enhanced disease paradigm appears to be consistent in cotton rats and mice but further investigation may be required to reconcile all of the findings in humans and rodent models.

1.2.2. Other subunit vaccines

Considerable effort has been invested in the development of subunit vaccines containing isolated viral proteins and given with traditional adjuvants. The purified F protein (PFP) vaccine family (PFP-1, PFP-2 and PFP-3) has been shown to be safe and immunogenic in different clinical trials in children [113]. However, PFP-3 failed to protect against RSV infection in a pivotal Phase III clinical trial, conducted in children suffering from cystic fibrosis [114]. Similarly, a subunit vaccine made of co-purified F, G and M proteins elicited promising immunogenicity in adults, but the responses were short-lived [113]. Finally, recombinant G protein expressed in E. coli, created as a C-terminal binding fusion partner to the albumin binding segment (BB) of streptococci, BBG2Na, was found to be immunogenic and protective in cotton rats [115]. However, human clinical trials were halted prematurely due to the unexpected development of purpura in some adult volunteers [114].

1.2.3. Live-attenuated RSV vaccines

Following the tragic events linked to the FI-RSV vaccine, significant efforts were also invested in the creation of a live-attenuated RSV vaccine candidate. Unfortunately, the live-attenuated intranasal strategy has proven to be exceedingly difficult to implement in practice. Striking the balance between infectivity and immunogenicity has not been achieved despite decades of effort and several clinical trials [114]. For example, several generations of temperature-sensitive, cold-adapted viruses were found to be either underattenuated, leading to unacceptable disease symptoms or overattenuated and non-immunogenic [114]. These mutants were refined with the advent of reverse genetics, enabling the design of viruses with targeted mutations [116,117]. Although promising results in rhesus monkeys were obtained [118], immunization with these attenuated viruses has not yet been demonstrated to be efficacious in humans [119]. The latest strategy in live-attenuated vaccine consists of a chimeric virus based on a bovine/human parainfluenza-3 (b/hPIV-3) viral vector expressing RSV F, which can protect African green monkeys against human RSV infection [120]. This new candidate vaccine, also known as MEDI-543, is currently being tested in a clinical trial that began in the Fall of 2006, involving children 1 to 9 years of age [113].

In summary, some progress has been made but a number of hurdles remain in the path to safe and efficacious RSV immunization. Perhaps the most significant hurdle is the age of the group most at risk. Neonates possess an immature immune system, rendering the development of efficacious immunity following infection or vaccination a significant challenge [87]. Another hurdle common to immunization of this age group is that maternal antibodies can strongly interfere with the development of immunity, particularly

with live virus vaccines [87]. In addition, the immunopathogenic host responses to RSV antigens are not yet fully understood and constitute an important risk for vaccines [121]. Any vaccine candidate therefore has the daunting tasks of eliciting better immune responses than those elicited by natural infection, despite maternally-derived antibodies and without the risk of exacerbated diseases. An ideal vaccine would therefore be required to elicit the following: a balanced immune phenotype without the risk of Th2-driven lung inflammation, RSV-specific CD8+T cells, mucosal RSV-specific antibodies in the RT, neutralizing antibodies and protection, and finally, absence of vaccine induced eosinophilia or pulmonary pathology. A proteosome-based RSV vaccine may fulfill these requirements.

1.2.4. Prophylactic treatment

The development in the last ten years of humanized monoclonal antibodies directed against the F protein (Palivizumab) has provided a much needed option for the treatment of high-risk preterm infants [122]. Although Palivizumab did not offer protection to the upper airways, it has very recently been demonstrated that a novel derivative of Palivizumab, Motavizumab, can reduce both pulmonary and upper respiratory tract viral titers up to 100 times better than its predecessor [123]. Palivizumab administration has resulted in significant reduction of RSV-infection related hospitalization in the high-risk infant group [124], and it can be expected that Motavizumab will elicit even superior results. However, the cost associated with this treatment is prohibitive and this approach does not provide the herd immunity that a vaccine would offer [125].

1.3. Novel technology

1.3.1. Proteosome technology

Proteosomes are composed primarily of chemically extracted outer membrane proteins (OMPs) from *Neisseria menigitidis* (mostly porins A and B as well as class IV OMP), maintained in solution by detergent [126]. The extraction process (proprietary information) strongly influences the size of proteosome vesicles, which can reach several hundred nanometers in diameter. Reflecting the transmembrane nature of OMPs, proteosomes hydrophobic protein-protein interactions cause their self-assembly into multimolecular, membranous nanoparticles with vesicular characteristics [126]. Using detergents of various strength and properties, proteosome particles can be easily dissociated into variable multimolecular fragments and be reconstituted following detergent removal. Proteosome-based vaccine formulations exploit this property to facilitate the interaction of antigenic protein hydrophobic moieties with proteosome vesicles during the detergent removal process leading to the formation of non-covalent complexing between antigens and proteosomes [126]. Proteosomes can be formulated with a variety of antigens such as purified or recombinant proteins derived from viral or bacterial sources [127-130], or even LPS [131] by diafiltration or traditional dialysis processes. The hydrophobic moieties of amphipathic antigens may be embedded within the core of the complex, possibly contributing to the correct presentation of the antigens and the "detoxification" of the LPS antigen [131] . Proteosome-*Shigella-flexneri* 2a LPS complexes, known as Protollin, have been administered in Phase I and II clinical trials as a vaccine against dysentery, to more than 100 volonteers and were found to be safe and non-toxic [132]. Protollin was able to deliver doses of up to 1.5 mg of LPS intranasally,

without adverse events [131]. Although a very narrow portrait of the lipid content is available for proteosomes, recent unpublished observations indicate that phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are co-purified with OMPs.

1.3.2. The Th1/Th2 paradigm and cross-presentation

The differentiation of Th0 cells to either Th1 or Th2 is critically dependent on the cytokine microenvironment in which they are exposed to antigen and activated. Th1 cells typically produce IFN-γ, IL-2 and TNF-α and mediate cellular immunity to a large number of pathogens, particularly intracellular pathogens [133]. Th2 cells produce generally IL-4, IL-5 and IL-13, favour humoral responses and are crucial to mount effective immune responses against helminth infections and many extracellular bacteria [134]. Proteosome immunization with a variety of antigens has been demonstrated to favour the development Th1-biased responses. For example, proteosome-formulated baculovirus-derived Influenza hemaglutinin (HA) given to mice intranasally elicits higher levels of HA-specific IgG2a and markedly reduced levels of IL-5 compared to mice given the antigen alone [130]. More recently, mouse immunization with Protollin combined with a recombinant plague antigen, F1-V (capsular and virulence associated proteins, respectively) enhanced the release of TNF-α, while concurrently suppressing secretion of the regulatory cytokine IL-10 compared to the F1-V alone [129]. Finally, Protollin given with a split measles virus antigen skews the IgG1/IgG2a ratio towards Th1-biased responses [128]. These results suggest that the proteosome-based adjuvants can drive T-helper cell polarization away from an intrinsic Th2-response that would be elicited by the antigen alone.

Proteosome-based adjuvants, like other adjuvants that may target TLR pathways have the potential to generate strong cellular responses against viral epitopes in addition to mucosal and systemic antibody responses. The opportunity to elicit antigen-specific CD8+ T cells and CTL activity with exogenous, non-living antigens provides a novel opportunity for vaccines [135], but also provides an explanation to how robust T cell responses are raised against pathogens that do not infect APCs [136]. Although exogenous antigens are typically presented via MHC class II molecules following endocytosis, the recent discovery of fusion between ER membrane and nascent phagosomes in the peripheral compartments of DCs and macrophages suggests the presence of an organelle specialized for MHC class I cross-presentation of exogenous antigens [136]. Even though endogenous proteins are degraded in the cytosol mainly by the proteasome and transported by the TAP protein to the lumen of the endoplasmic reticulum (ER), internalized exogenous proteins may be degraded in the endocytic pathway prior to phagosome and ER fusion [137]. Endocytic degradation, involving reduction, unfolding and lysosomal proteolysis may facilitate the exchange of previously loaded MHC class I peptides in the ER with peptides from exogenous proteins [136]. Alternatively, as phagosomes form, they may fuse with the ER and thereby acquire many of the ER resident proteins. Acquired exogenous antigens can be retro-transported to the cytosol for ubiquitination and proteasome degradation, then be transferred back into the phagosome by TAP proteins [138]. It is not entirely clear however, what constitutes a trigger for cross-presentation. Recent reports have demonstrated that CpG-DNA targeting of TLR9 may enhance cross-presentation by DCs [139]. In these studies, TLR9

was not necessary for cross-presentation itself, but was essential for cross-priming of CD8 T cells [139]. Moreover, stable virus-like particles of papaya-mosaic virus have been shown to induce MHC class I presentation of influenza and melanoma antigens as well as reactive antigen-specific CD8+ T cells via a proteasome-independent mechanism [140].

1.4. Advantages of novel technology

1.4.1. Targeting the NALT

Proteosome-based adjuvants have two major advantages for subunit RSV immunization. Binding TLR ligands and intranasal delivery. Although intranasal immunization is also the route of choice for live-attenuated vaccines [119], the balance of virulence and efficacy has been impossible to achieve to date (as outlined above). Subunit vaccines given intranasally present no risk of infection and may prevent infection of the upper respiratory tract [83].

Multiple immune effectors are active at mucosal surfaces to prevent infections. Respiratory tract (RT) epithelial cells initiate host protective responses by secreting surfactant proteins, providing mucocilliary clearance and through the expression of innate immune pathogen associated molecular pattern (PAMPs) receptors such as TLRs [141]. Epithelial cells also promote the production of a variety of effectors such as cytokines and chemokines [142,143]. Among other innate immune receptors, TLR2 and TLR4 are expressed in human epithelial cells of the nasal mucosa [144]. The effectors elicited following TLR ligation may promote the migration of DCs to the narrow spaces between nasal and airway epithelial cells, where DCs can extend their dendrites directly into the

outer limit of the epithelium and sample foreign material from luminal compartments [141,145] (Figure 2). In addition to the active role played by epithelial cells, the induction of adaptive immune responses to pathogens or vaccine antigens also requires the presence of organized lymphoid tissue or follicle either within the mucosa or in draining lymph nodes [141]. The presence of mucosal lymphoid follicles, also known as nasopharynx associated lymphoid tissue (NALT) in the respiratory tract [146,147], influences the overlying epithelium by inducing the differentiation of specialized follicle-associated epithelium (FAE)[148]. Antigen sampling by DCs or microfold (M) cells in the FAE deliver antigen by transepithelial vesicular transport directly to the NALT, which contains antigen-presenting cells (APCs), B and T cells [148]. CD4+ T cells isolated from the NALT of naïve mice have a dominant Th0 cytokine profile that can differentiate into either Th1 or Th2 effectors in response to stimulation by MHC class II presentation [149,150]. Moreover, the capacity of a subunit nasal vaccine to elicit CTL responses in different mucosa and systemic immune compartment has recently been observed for HIV gp 160 [151], demonstrating the potential of the NALT to elicit functional T cell immunity. B cells that are pre-conditioned to undergo IgA class switching are also located in the organized NALT [152] and the generation of memory B cells that produce high affinity IgA has been shown to occur at the NALT [153].

Proteosome-based vaccines offer three further theoretical advantages to successfully deliver antigens within the airway luminal environment [141]. The first two advantages relate to their particulate nature: Particulate vaccines may 1) protect associated antigens from mucosal secretions and proteases, and 2) enhance M cell uptake [141]. M cells are

particularly accessible to microparticles (<1 μm diameter) and favour their transport to the underlying lymphoid tissues [154]. Finally, 3) TLR ligands present on both OMPs and LPS can improve the efficiency of uptake into the mucosa by promoting the adherence of the vaccine antigens to the mucosal surface [155].

1.4.2. Targetting innate immunity

1.4.2.1. Toll-like receptor pathways

The control of adaptive immune responses to infectious diseases is mediated by the initial recognition of specific molecular patterns, or PAMPs by TLRs [156]. The TLR family is currently comprised of eleven known members (TLR1-11), all of which act in response to specific natural or synthetic PAMPs (Table 1). The TLR family can be divided in two groups based on their sub-cellular location. The first group, comprising TLR1, 2, 4, 5 and 6 are embedded within the plasma membrane, while the second group (TLR3, 7, 8 and 9) are intracellular and may signal via acidic endosomes [157] (Figure 3). TLR signalling can therefore recognize surface and intracytoplasmic PAMPs. Moreover, TLRs activate different downstream signalling pathways by selective use of adaptor proteins. [158,159].

For extracellular TLRs, the signal transduction cascade is initiated with the cytoplamic receptor, the Toll/IL-1 receptor (TIR) domain, followed by the phosphatidylinositol 4,5-biphosphate (PIP2) targeting to the plasma membrane of TIRAP [157,160] (Figure 3). TLR4 ligation can also trigger the plasma membrane targeting of the TRIF-related adaptor molecule (TRAM) via N-terminal myristoylation (Myr) [161], providing the

TLR4 signal transduction pathway with greater versatility to induce downstream activation of nuclear factors. TIRAP, in turn, recruits the myeloid differentiation factor 88 (MyD88) to activate the downstream cascade via TRAF6, leading to early phase NF-κB activation and inflammatory cytokine production such as TNFα [158]. TRAM, on the other hand, recruits the adaptor protein TIR-domain-containing adaptor inducing interferon-β (TRIF) which has the potential to signal either through TBK1 or TRAF-6. TRAM consequently offers the possibility of not only activating late phase NF-κB resulting in TNFα production without MyD88 involvement [159,162,163], but also activates IRF-3, leading to IFN-β production. IFN-β and TNF-α are complemented by several other pro-inflammatory cytokines or chemokines resulting from TLR ligation. IFN-α, IFN-γ, IL-12-p40/IL-12-p70, IL-1β, IL-2, MCP-1, MIP-1α, IL-8 and RANTES for example, have all been identified as being produced in response to different TLR ligation in humans [164].

Proteosome-based adjuvants, with their *N. meningitides* outer membrane proteins (OMP) and gram-negative S.*flexneri* lipopolysaccharide (LPS) [131] in the case of Protollin, possess major TLR2 and TLR4 ligands. As a result, they are thought to trigger the TLR innate immunity cascade, although the implications of these interactions for proteosome-based vaccine efficacy have not been fully characterized. Porin B (PorB) has been demonstrated to interact specifically with TLR1/TLR2 heterodimers [165], and this interaction is known to promote the production of IL-6 [166]. An extensive body of literature demonstrates that gram-negative bacteria LPS is the most important ligand for TLR4 [162,167], causing the homodimerization of the receptor in the presence of MD-2

and CD14 [168], leading to the subsequent release of a multitude of effectors, including IL-1, IL-6, TNF-α and IFN-β [169].

The efficient priming of an adaptive immune response following TLR ligation requires a number of immunological events to take place. As described above, TLR ligation promotes the expression of genes leading to inflammatory cytokine production, but also to up-regulation of co-stimulatory molecule expression such as CD40, CD80 (B7.1), CD86 (B7.2) and CD70 on APCs [170]. Phagocytosis-mediated degradation of vaccine components and subsequent presentation of peptide antigens via MHC class II molecules, together with TLR-dependent gene expression ultimately direct the development of antigen-specific acquired immunity [171] (Figure 3). The polarization of Th0 cells into Th1 cells following contact with loaded MHC II molecules on DCs is a MyD88-dependent process [172]. For example, T cells from MyD88-/- mice fail to proliferate or elicit IFNγ in response to antigen stimulation. However, type-2 cytokine signals (IL-4 and IL-13) appear unaffected by the absence of MyD88 in OVA/CFA or alum immunized wt and MyD88-/- mice. Moreover, these mice are not capable of eliciting Th1-dependent IgG2a, consistent with reduced IFNγ levels [156]. Indeed, it has recently been demonstrated that TLR-activated DCs favour the development of Th1 cells, possibly due to the inflammatory cytokines elicited following TLR ligation [170].

Interestingly, the impact of MyD88 on the upregulation of co-stimulatory molecules on DCs appears to be stimulus-dependent. For example, in a heat-killed *M. tuberculosis*/CFA immunization model, MyD88-deficient DCs fail to express up-regulated CD80, CD86 or MHC class II. However, TNF-α or CD40 cross-linking promote the upregulation of CD86 in these mice [156]. Therefore, immunization of

MyD88-/- animals with stimuli that elicit inflammatory signals such as TNF-α may promote the upregulation of co-stimulatory molecules on DCs.

Although the body of literature demonstrating the role of TLRs in triggering adaptive immune responses to vaccine adjuvants that bear their ligands is extensive, recent studies in mice genetically deficient in both MyD88 and TRIF suggest that TLRs may not be the only link between innate and antigen-specific immune responses [173]. Even though these $MyD88^{-/-}$; $TRIF^{Lps2/Lps2}$ mice are incapable of TLR signalling [167,174], they mount robust antibody responses to T-cell dependent antigens when formulated with four different adjuvants; alum, Freund's complete and incomplete adjuvants or monophosphoryl-lipid A (MPLA)/trehalose dicorynomycolate adjuvant (together known as Ribi). However, $MyD88^{-/-}$; $TRIF^{Lps2/Lps2}$ mice fail to elicit isotype class switching compared to wild-type mice following immunization with the Ribi vaccine, the only adjuvant bearing a TLR ligand (MPLA) [173]. These results suggest that although TLR responses may be critical for early pathogen suppression and the promotion of Th1-dependent antibody-class switching, the magnitude of the overall antibody response is controlled by other mechanisms. One such mechanism may be the CD1d-NKT cell pathway.

1.4.2.2. CD1d and iNKT cells pathway

The role of the CD1d-invariant NKT (iNKT) cell pathway as a significant innate contributor to the regulation of adaptive immune responses has been confirmed in recent years. CD1 molecules are β2-microglobulin-associated MHC I-like molecules specialized for lipid presentation to T cells, but specifically to iNKT cells [175]. Unlike MHC molecules, the capacity of the almost invariant antigen-binding groove of CD1 to interact with a wide range of structurally variable lipids is not due to genetically encoded variations of its surface-binding amino acids [176]. Instead, the presentation of diverse (lipid) antigens is accomplished by the expression of a number of CD1 isoforms that display structurally different binding grooves, consisting mainly of narrow and deep hydrophobic clefts [177]. In humans, T cells that recognize different CD1 molecule isoforms can be divided in two groups. The first group has many characteristics similar to those of peptide-specific T cells and recognizes CD1a, CD1b, CD1c, and CD1e present on stimulated myeloid DC precursors. The second group, the iNKT cells, recognizes only CD1d, which is required for their development in mice and humans and is constitutively expressed on APCs [178]. The iNKT cell subset is a conserved, innate-like lymphocyte lineage involved in various infectious, allergic, autoimmune and tumor responses, via the recognition of exogenous or endogenous lipids [179-181]. CD1d-restricted iNKT cells are phenotypically characterized as CD161 ($NK1.1^{+/-}$), NKG2D+ and Ly49+ [182,183] and express a constant TCRα chain in humans (Vα24-Jα18) and mice (Vα14-Jα18), paired with a narrow set of TCRβ chains (Vβ11 and Vβ2. -7, -8, respectively) [184-186]. iNKT cells, present at a frequency of approximately 2% in the mouse spleen, can contribute to the expression of IL-17 in concert with αβ CD4+ T cells

and γδ T cells, collectively called neutrophil-regulatory T cells (Tn) [187]. Tn cells expression of IL-17 promotes the release of TNFα, IL-1β, IL-6 and IL-8 as well as granulocyte colony-stimulating factor (G-CSF) [188,189]. IL-17 production is itself triggered by the early stimulatory signal IL-23 produced by macrophage/DC after exposure to foreign effectors [190]. This escalating cycle is downregulated by the phagocytosis of apoptotic neutrophils in tissues, causing the abrogation of IL-23 production [191].

It was initially thought that NKT cells elicited only Th2 responses, characterized by the rapid production of IL-4 [192]. However, it was subsequently demonstrated that CD1-deficient mice that also lack NKT cells, can elicit normal amounts of IgE [193]. Recent publications have demonstrated the potential of NKT cells to skew immune responses to Th1 responses and contribute to DC-mediated priming of MHC class-I restricted CD8+ T cells [194,195].

The role of the CD1d-NKT cell pathway in mediating adjuvant activity has not been fully investigated. Most of the literature on CD1d-NKT cells describes studies performed using the CD1d ligand α-GalCer, a shingolipid derived from the marine sponge *Agelas mauritianus* or the *Shingomonas* bacteria that colonize this sponge [196]. For example, mice immunized with proteins and α-GalCer develop higher antibody titers higher than those injected with proteins alone [197]. Moreover, activation of iNKT cells prior to influenza vaccination enhances protection against influenza combined with higher frequencies of memory B cells and higher antibody responses [197]. In the same study,

immunization with α-GalCer was also found to elicit IgG responses in mice lacking MHC class II molecules, suggesting that iNKT cells can, in some circumstances, replace CD4(+) T cell help to B cells. Moreover, it was also demonstrated that the decay of circulating antibodies is faster in mice lacking iNKT cells [197]. These observations suggest that the CD1d-iNKT cells pathway may provide an alternative mechanism for bolstering antibody responses and B cell memory.

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1.6. Table 1

TLR	Natural ligands	Synthetic ligands	Host-derived ligands
TLR1/TLR2	Triacyl lipopeptides	Pam3Cys	
TLR2/TLR6	Diacyl lipopeptides	Pam2Cys (or MALP-2)	
TLR2	Peptidoglycans and bacterial lipopeptides Neisserial porins, CFA, BCG	(See directly above)	
TLR3	Double stranded RNA	Poly I:C	
TLR4 (with CD14 andMD-2)	LPS, MPLA	RC-529	Heat shock proteins (HSP) and β-defensin-2
TLR5	Flagellin		
TLR7/8	Viral single stranded RNA	Imiquimod and Resquimod (R848)	
TLR9	DNA CpG motifs	CpG ODN	

Note:

Tabulation of TLR ligands based on information available in reviews [161,194]. TLR10 and TLR11 are poorly characterized. TLR10 is thought to be non-functional in mice [169] and TLR11 is not produced in humans due to a stop codon on the *TLR11* gene [195].

1.7. Figure Legends

Figure 1

RSV gene map. Genes are identified by the proteins which they encode: NS1 and NS2, non-structural proteins 1 and 2; N, nucleocapsid protein; P, phosphoprotein; M, matrix protein; SH, small hydrophobic protein; G, attachment protein; F, fusion protein; M2-1, transcription anti-termination factor; M2-2, RNA synthesis factor (M2-1 and M2-2 are encoded by two overlapping open reading frames in the same M2 mRNA); L, large polymerase protein.

Figure 2

Antigen sampling at the upper airways. a) At the nasal passage, DCs may assemble under the epithelia and extend their dendrites directly into the lumen [139]. b) At the follicular-associated epithelium (FAE) overlying the NALT (depicted within the adenoid tissues [152]), luminal antigens are transported via the M cells contained within the FAE [145]. Immature DCs, T and B cells migrating to the NALT via high endothelial venules (HEVs) follow chemokine signals and may migrate to the FAE. Following contact with M cells, DCs capture antigens transported by these cells. This process and other signals promote DC migration to the underlying interfollicular B-cell zones and T cell areas (depicted as B and T, respectively), where they process and present antigen to naïve T cells [139]. DC activated T-helper cells in turn activate IgA-committed B cells [145]. Activated effector cells migrate via the efferent lymphatics, thoracic duct and blood circulation to the nasal passage. There, cytokine producing T-helper cells promote the maturation of B cells into IgA-producing plasma cells [147].

Figure 3

TLR signaling pathway, innate and adaptive immunity. TLR signaling is initiated following PIP2 recruitment of TIRAP which acts as a sorting adaptor for the TIR-domain containing adaptor MyD88 [155]. MyD88 engages IRAK and TRAF-6 resulting in nuclear translocation of NF-κB and cytokine induction. Alternatively, TRAM can be recruited to the plasma membrane following N-myristoylation and colocalize with TLR4, acting as a sorting adaptor for TRIF [159]. TRIF engages TBK1 leading to IRF-3 activation and cytokine production. Cross-talk between the TIRAP/MyD88 and TRAM/TRIF pathways enables the latter to upregulate NF-κB-mediated cytokine production. Cellular sampling of antigen following TLR recognition leads to the induction of gene expression enabling antigen presentation, the upregulation of co-stimulatory molecules and pro-inflammatory cytokine production. Together these events instruct the development of antigen-specific immunity.

1.8. Figures

Figure 1

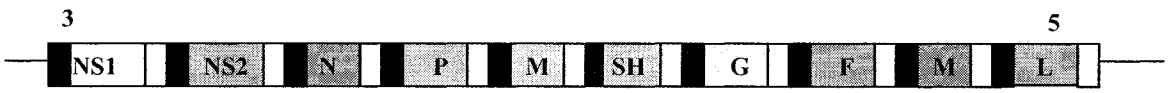


Figure 2

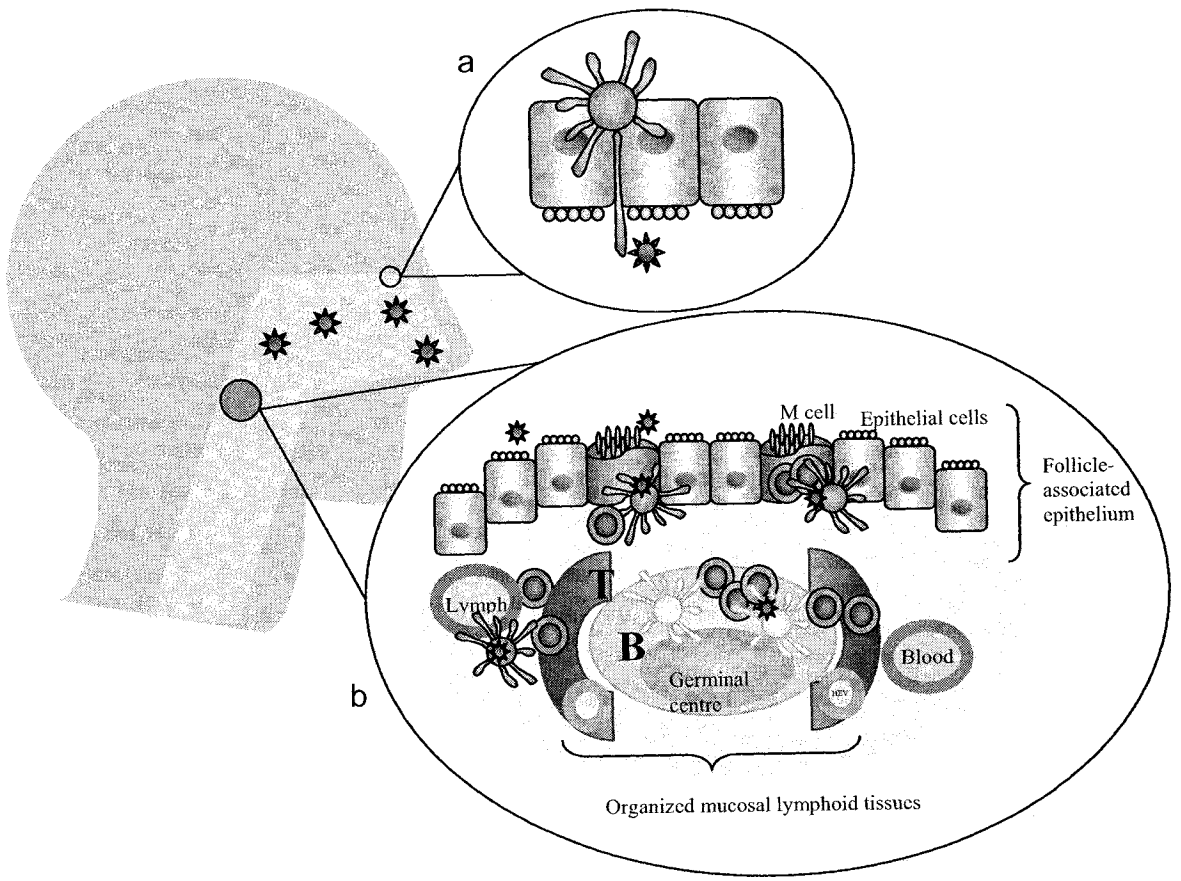
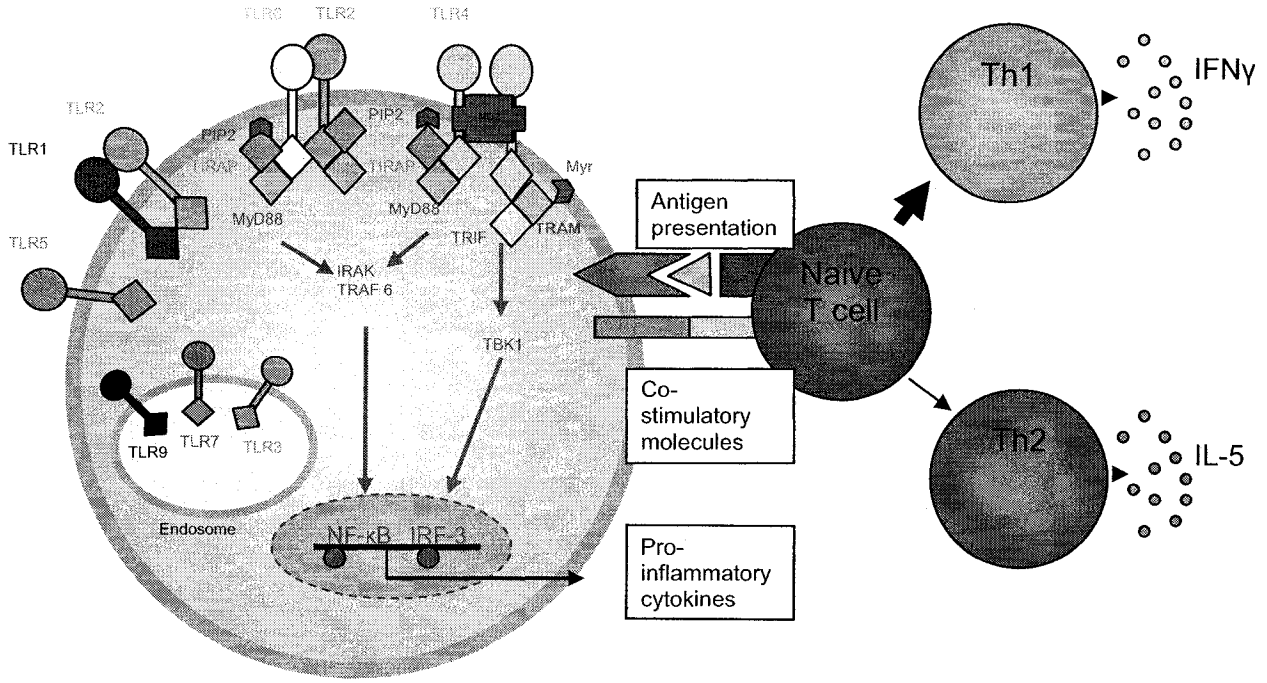

a
b
M cell
Epithelial cells
Follicle-
associated
epithelium
T
B
Lymph
Blood
Germinal
centre
Organized mucosal lymphoid tissues

Figure 3


TLR2
TLR4
TLR2
TLR1
PIP2
TIRAP
PIP2
TIRAP
Myr
MyD88
MyD88
TRIF
TRAM
TLR5
IRAK
TRAF 6
TBK1
TLR9
TLR7
TLR3
Endosome
NF-κB
IRF-3
Antigen presentation
Co-stimulatory molecules
Pro-inflammatory cytokines
Naive T cell
Th1
IFNγ
Th2
IL-5

Chapter 2

Intranasal proteosome-based respiratory syncytial virus (RSV) vaccines protect BALB/c mice against challenge without eosinophilia or enhanced pathology[‡]

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2.1 Preface

The work presented in this chapter was the first demonstration that intranasal RSV vaccines, formulated with either proteosomes or Protollin could elicit protective immunity when instilled intranasally in BALB/c mice. Although it only represents a small portion of this chapter, the first year of my thesis was devoted to developing the chromatography protocol to enrich the RSV antigens. This was essential to improve the poor immunogenicity obtained following mouse immunization with a non-enriched split RSV antigen. A particularly interesting aspect of this work is the observation that RSV antigen-specific CD8+ T cells expressing IFNγ can be detected following Protollin-eRSV immunization. Until recently, non-living antigens were believed to be presented via the MHC class II pathway only, and therefore thought to elicit almost exclusively CD4+ T cell immunity. This observation shows that the novel adjuvant Protollin can promote MHC class I presentation of subunit antigens.

2.2. Abstract

A safe and effective vaccine against respiratory syncytial virus (RSV) is still unavailable. Proteosome-based adjuvants are derived from the outer membrane proteins (OMP) of Neisseria species and are potent inducers of both mucosal and systemic immunity in humans and animals. Candidate RSV subunit vaccines comprising enriched RSV proteins (eRSV) formulated with proteosomes alone or with LPS (Protollin) were produced. Administered intranasally in BALB/c mice, both vaccines elicited long-lasting systemic and mucosal RSV-specific antibodies and fully protected against challenge. *In vitro* restimulation of lymphocytes from the Protollin-eRSV immunized mice with F (MHC-I) and G (MHC-II) peptides elicited F peptide-specific CD8+ T cells and supernatant IFNγ, TNFα, IL-2 and IL-10 while the formalin-inactivated RSV (FI-RSV) vaccine elicited predominantly IL-5. Pulmonary eosinophilia did not develop following immunization with either proteosome-based vaccine following challenge compared to mice immunized with FI-RSV. Proteosome-based eRSV vaccines can therefore protect against RSV challenge in mice without increasing the risk of pulmonary immunopathologic responses.

2.3. Introduction

Respiratory syncytial virus (RSV) is a major cause of serious lower respiratory tract infections in infants and young children in both the developed and the developing world. Fifty years of sustained effort by academic and industrial groups has failed to generate an effective vaccine that is free from serious adverse events. A major hurdle has been our limited understanding of host immune responses to RSV antigens that can lead to immunopathology [1-4]. Children immunized with the early formalin inactivated (FI)-RSV vaccine were at markedly elevated risk for severe respiratory disease when subsequently exposed to wild-type virus [5]. Since very limited data is available from these patients, our understanding of this pathology is based mainly on cotton rat and mouse models, which exhibit exacerbated pulmonary granulocytic infiltrates, including neutrophils [6] and eosinophils [7-9]. The pathophysiologic mechanisms underlying this severe adverse outcome have been partially elucidated in the last decade with better understanding of the nature of the RSV antigens themselves [10-12], the alum-based adjuvant [13;14] and the formalin inactivation process [15]. All of these factors can result in strongly Th2-biased responses that leave vaccinees susceptible to enhanced pathology upon exposure to natural infection. Another significant hurdle facing RSV vaccine development is the incomplete protection conferred even by natural infection [16;17]. Any RSV vaccine development program therefore has the challenging goals of inducing an immune response that is qualitatively better than that elicited by RSV infection itself while avoiding both natural and vaccine-exaggerated Th2-deviated lung inflammation.

To address this dual challenge, novel adjuvant and antigen delivery systems that promote balanced Th1/Th2 responses could theoretically be used [18-20]. Vaccine formulations that target the respiratory tract with a mucosal adjuvant and induce balanced immune responses may be particularly effective against RSV [21-23]. Proteosome-based adjuvants are potent inducers of both mucosal and systemic immunity in humans and animals and consistently induce a balanced Th1/Th2 immune phenotype [24-29]. Proteosome-based adjuvants [30] including Protollin [31], consist mainly of meningococcal outer membrane proteins (OMP). Protollin is formulated with additional lipopolysaccharides (LPS) from *Shigella flexneri*. Safe and efficacious proteosome-based vaccines have been developed for respiratory viruses [24-27] and other mucosal pathogens [28;29]. The potential for this strategy in RSV vaccine development has recently been demonstrated by Etchart et al who combined inactivated RSV (iRSV) with *N. meningitidis* outer membrane vesicles (OMV) to immunize mice [32] .

Overall, we demonstrate the successful use of proteosome-based adjuvants for intranasal RSV vaccination. Complete protection was conferred following IN immunization of BALB/c mice with a subunit RSV antigen enriched in F and G proteins (eRSV) formulated with either proteosomes or Protollin. Strong RSV-specific mucosal (IgA) and systemic (IgG) RSV-specific responses were generated by both formulations. The anti-RSV immune response induced by the Protollin vaccine was durable, lasting for over 3 months, and was characterized by release of IFNγ, TNFα, IL-2 and IL-10 following restimulation of mouse T cells with MHC-I and MHC-II RSV peptide epitopes. Moreover, RSV F peptide-specific CD8+ T cells were detected in splenocytes from

Protollin-eRSV immunized mice. In contrast to animals exposed to FI-RSV immunization, the protection induced by proteosome-based vaccines was not associated with pulmonary eosinophil infiltration or the production of RSV peptide-specific IL-5 by splenocytes or lung cell suspensions. These results demonstrate the potential of both proteosome-based formulations to fulfill the requirements for a safe and effective IN vaccine against RSV.

2.4. Materials and methods

2.4.1. Tissue and virus culture

RSV Long strain (ATCC# VR-26) was propagated (m.o.i. of 0.005-0.01) in Vero cells (ATCC# CCL-81) in 10-layer Corning CellSTACKs (Fisher, Nepean, ON) or on microcarrier beads in a 5L Bioreactor (NewBrunswick Scientific, BioFlo3000) in RSV media; D- MEM with 50% 199-H media, 0.5% fetal bovine serum , 2mM glutamine and 50µg/mL gentamicin (all Invitrogen, Burlington, ON) and harvested following one freeze/thaw cycle. RSV quantitation was performed by serial dilution on Vero cell monolayers in 96-well plates and incubation for 6 days. The 50% tissue culture infective dose ($TCID_{50}$) calculations were performed using the Reed-Muench method [33].

2.4.2. Enriched RSV proteins (eRSV) and vaccine formulation

The infected cell lysate was solubilized with 20mM Tris-Cl pH 8.0 containing 0,05% (w/v) CHAPS (EMD Biosciences, San Diego, CA) for 1 hour at 4°C. Clarified supernatants were filtered through Millistack+ CE Mini capsule grade 45 and 0.45µm filters (both Millipore, Mississauga, ON). Surface glycoproteins were enriched by affinity chromatography on Lentil Lectin Sepharose 4B [34] (GE Healthcare, Baie d'Urfe, QC), equilibrated in 20mM Tris-Cl, 0.05% CHAPS, 0.5mM $CaCl_2$, 0.5mM $MgCl_2$ and 0.15M NaCl, pH 8.0. Supernatants were circulated overnight and washed with the same buffer containing 0.5M NaCl. Bound proteins were eluted with 1M NH_4SCN (Sigma, Oakville ON) [35], concentrated using a 200mL Amicon stirred ultrafiltration

cell with 10K MWCO nitrocellulose ultrafiltration discs (both Millipore, Mississauga, ON) and dialyzed against PBS.

Proteosome vaccine formulation: Chemically-extracted, detergent solubilized (Empigen BB, Calbiochem, San Diego, CA) proteosomes [30] (GlaxoSmithKline Biologicals), consisting mainly of *Neisseria meningitidis* OMP were added to eRSV (3:1 starting ratio w/w of total proteins, respectively) in a stirred ultrafiltration cell, mixed briefly and diluted to below the critical micelle concentration (CMC) of empigen (1.6-2.1mM) and concentrated. Formulated monovalent Proteosome-HA (HA antigen from Influenza A (H_3N_2) Wyoming strain – GlaxoSmithKline Biologicals) was used as negative control.
Protollin vaccine formulation: The adjuvant Protollin, consisting of proteosomes formulated with *S.flexneri* LPS (1:1) was prepared as described [31] (GlaxoSmithKline Biologicals). eRSV was mixed with Protollin immediately prior to immunization. The FI-RSV vaccine was formulated as previously described [7].

2.4.3. Mouse immunizations and challenge

Female 6-8 week old BALB/c mice were purchased from Charles River Laboratories (St-Constant, QC) and maintained according to the Canadian Council on Animal Care guidelines. Eight to ten mice per group were lightly anesthetized by isoflurane inhalation and immunized IN in a total volume of 16µl (8µl/nare) twice (days 0, 21) with either Proteosome-eRSV (0.6, 1.7 or 5µg, based on eRSV concentration) or Protollin-eRSV (3µg of Protollin mixed with 2.5, 5.0 or 10 µg of eRSV). Protollin (3µg)-eRSV (5µg) was also given three times on days 0, 21 and 42. Control mice received equivalent doses

in the same volume of eRSV alone, Proteosome-HA, Protollin alone or PBS IN. Mice immunized IM with FI-RSV received one dose (50 µl) on day 0. Two weeks post-boost, five mice per group were challenged IN with 10^7 $TCID_{50}$ of Long strain RSV in 50 µl following anesthesia as above. Mice were weighed before and every day during challenge. Based on protection from RSV challenge (see below and Figure 5), the 5 µg dose was selected for comparisons between vaccine formulations. All immunologic outcomes presented are from animals that received this dose. When relevant, comments on outcomes at other vaccine doses are included in the text. Serum, bronchoalveolar lavage (BAL), the spleens and lungs were collected from the remaining mice at this time (serum samples from the same animals were used for ELISAs and neutralization assays). Challenged mice were euthanized 4 days post-challenge and BAL and lungs were collected for cytospin and histopathology, respectively. To assess the duration of the systemic and mucosal responses, groups of 4 mice that had received 2 doses of Protollin-eRSV (5 µg) were sacrificed on days 31, 59, 91 or 119 for analysis of antibody titers. For intracellular cytokine staining (ICS) and pentamer experiments, naïve mice were infected with the challenge dose described above 8 days prior to *in vivo* stimulation.

2.4.4. Vaccine characterization

Characterization was performed by SDS-PAGE and Western Blot using Mabs anti-RSV F (clone 1E3) and anti RSV G (clone 1C2) provided by G.Toms, University of Newcastle-upon-Tyne, UK and mouse anti-F2 antiserum obtained by immunizing mice with purified recombinant RSV Long strain F2 (GlaxoSmithKline Biologicals, QC). Quantification of total proteins was performed by Pierce bicinchoninic acid (BCA) Assay

(MJS Biolynx, Brockville, ON) according to manufacturer's instructions. RSV F and G protein concentrations were estimated by densitometry of Coommassie stained gels. Transmission electron microscopy (Hitachi 7100, Japan) with immunogold labelling of RSV proteins was performed as previously described [27]. Briefly, centrifuged vaccines on Nickel grids (120,000 x g) were labeled with goat anti-RSV (Biodesign International, Brockville ON) and gold-labelled (10nm) anti-goat antibodies (Sigma) followed by negative staining with 3% phosphotungstic acid (M.E.C.A. Ltee, Montreal, QC).

2.4.5. Antibody titer determination

IgG and IgA antibody titers were determined on individual serum and BAL samples by ELISA as previously described [25]. Briefly, 96-well plates were coated with 7.5μg/ml of PEG-concentrated [33], UV-inactivated RSV or 2μg/ml of purified inactivated RSV (Biodesign International) and incubated overnight at 4°C. Serum and BAL samples were serially diluted in blocking buffer starting at 1:50 and 1:2 dilution, respectively, along with purified mouse IgG and IgA (Sigma) at starting concentrations of 200ng/ml and 1μg/ml, respectively. Bound antibody was detected with HRP-conjugated anti-mouse IgG or IgA (Sigma). Unless otherwise indicated, titers are expressed as geometric mean concentrations with 95% confidence intervals (GMC [X:Y]).

2.4.6. Neutralization Assay

Pooled sera from immunized animals were serially diluted from a starting dilution of 1:8 in RSV media in 96-well plates (20 μl/well). Control wells contained RSV media only or goat polyclonal anti-RSV antibody at 1:50 (Biodesign international). 500-1000 infectious

doses of RSV Long strain were added, the plates were incubated for 20 minutes at 33°C and the mixture was transferred to 96-well flat-bottomed plates previously seeded with $1x10^5$ cells/mL Vero cells. After 5-6 days at the same temperature, supernatants were removed; plates were washed with PBS and adhering cells fixed with 1% paraformaldehyde in PBS for 1 hour, followed by indirect immunofluorescence (IFA).

2.4.7. Lung viral titration

Lungs from immunized animals were washed, weighed and homogenized individually in RSV media with an automated Potter homogenizer (Fisher, Nepean ON), then centrifuged at 2655 x g for 2 minutes at 4°C. The supernatants were titered on Vero cells as above starting at a 1:10 dilution.

2.4.8. Indirect immunofluorescence assay (IFA).

Briefly, after blocking with PBS + 2% skim milk, plates were incubated at RT for 2 hours with a 1:400 dilution of goat anti-RSV antibody (Biodesign international) and washed with PBS. Anti-goat IgG FITC conjugate (Sigma) was used at a 1:400 dilution for detection. Wells were considered positive when ≥1 fluorescent syncytium was detected. Infectious titers were calculated as above.

2.4.9. BAL leukocyte differential counts and histopathology

BAL fluids were collected and centrifuged as previously described [26] and the pellets were resuspended in 500µl of PBS. 100µl were used for the cytospin (Thermoelectron Co, by Fisher, Nepean ON). Cells were fixed and stained with hematoxilin and eosin (H

& E) [33], enumerated using a hemocytometer (40X) and a manual differential count was performed on 300 cells. Lungs were stored in Glyo-Fixx (Thermoelectron Co, by Fisher, Nepean ON) and embedded in paraffin 1 – 2 weeks later. Tissues sections 5μm thick were cut and stained with H & E [33].

2.4.10. Peptide-specific T cell analysis

2.4.10.1. Cytokine ELISA

Organs were processed for *in vitro* re-stimulation as previously described [25]. Briefly, washed spleens were passaged through a 100μm mesh (BD Biosciences, Oakville ON) using sterile 3mL syringe plungers. Following lysis of RBCs and washing, the cells were resuspended in RPMI supplemented with 4% FBS, 2mM glutamine, 50μg/mL gentamicin (all Invitrogen) and 50mM β-mercaptoethanol (Sigma) (cRPMI) and counted. Washed lungs were minced before processing as above. Cells were seeded at a concentration of $2X10^6$ cells/mL (splenocytes) or $1X10^6$ cells/mL (lungs) and incubated in cRPMI alone, with increasing doses (0.1, 1.0, 10 μg/mL) of RSV F_{85-93} CD8+ T cell-restricted peptide KYKNAVTEL [36] or RSV $G_{183-197}$ CD4+ T cell-restricted peptide WAICKRIPNKKPGKK [37] or supplemented with 0.5μg/ml of Concavalin (Sigma) for 72 hours. The plates were then centrifuged, and the supernatants harvested and stored at -80°C until assayed for IFNγ, IL-2, IL-5, IL-10 by ELISA (BD Biosciences kit, including purified standards). TNFα was measured (except for FI-RSV immunized animals) by cytokine bead array (CBA) (BD Biosciences), as per the manufacturers' instructions.

2.4.10.2. FACS analysis

For FACS analysis, immunized mice were restimulated *in vivo* for 20h by intra-venous injection of F-peptide pulsed (1μg/ml for 1 hour) splenocytes and lymph node

lymphocytes (mixed population, $2x10^7$cells/mouse) isolated from naïve mice. The splenocytes from immunized mice were then collected and restimulated with 10μg/ml of F-peptide (pentamer analysis) or purified inactivated RSV (Biodesign International, intracellular IFNγ analysis) in cRPMI overnight. For intracellular cytokine staining (ICS), BD GolgiPlug™ (BD Pharmingen) was added 4h prior to harvest as per manufacturers instructions. Cells were washed and surface stained with allophycocyanin (APC)-conjugated rat anti-mouse CD8a monoclonal antibody (BD Pharmingen) and incubated for 20 minutes at room temperature. Cells were then washed, permeabilized and fixed with BD Cytofix/Cytoperm (BD Pharmingen), stained intracellularly with fluorescein isothyocyanate (FITC) –conjugated rat anti-mouse IFNγ monoclonal antibody, washed in Perm/Wash buffer (BD Pharmingen) and kept at 4°C until analyzed. To determine the frequency of F peptide-specific T cells, R-Phycoerythrin (R-PE)-labelled Pro5™ recombinant MHC Pentamer ($H\text{-}2K^d$) conjugated to the F peptide KYKNAVTEL (ProImmune Ltd, Oxford, England) was used to surface stain splenocytes immediately prior to the CD8 staining described above, as per manufacturer's instructions.

2.5. Results

2.5.1. Vaccine characterization

The lentil affinity column enriched viral protein concentrations from the detergent-lyzed RSV-infected preparations at least 5-fold: hence enriched RSV antigen (eRSV) (Figure 1). eRSV contained both RSV major surface glycoproteins F (F1 and F2, 48 and 23 Kda, respectively) and G (55 and 95Kda) that together accounted for approximately 7.5% of the total protein concentration based on densitometry (data not shown). Similarly, the formulated vaccine contained F1, F2, the G protein and major proteosome proteins, PorA, PorB and class 4 OMP. To evaluate possible associations between proteosomes and eRSV antigen following the formulation process, the formulated vaccine soluble and insoluble fractions were characterized by SDS-PAGE (Lanes 4-6) and Western Blot (Lanes 7-9) showing both components in both fractions. EM characterization of the vaccines showed aggregation of eRSV independent of the presence of either proteosomes or Protollin. Immunogold labeling revealed that virtually all RSV reactivity co-localized with these aggregates in both the Protollin-eRSV and Proteosome-eRSV formulations (top and bottom panels, respectively, Figure 1). Both antigen alone and vaccines had the appearance of a colloidal suspension.

2.5.2. Immunogenicity

RSV-specific serum IgG and BAL IgA titers following two IN doses of Proteosome-eRSV were 62 μg/mL [34-111] and 36 ng/mL [23-71] respectively; both significantly greater than titers elicited by antigen alone (Figure 2A and 2B). Mice that received two doses of Protollin-eRSV developed comparable serum IgG (50 μg/mL [35-72]) and BAL IgA titers (43 ng/mL [14-88]), but the IgG titer was not statistically different from that elicited by immunization with eRSV alone. However, serum IgG (150 μg/mL [129-

180]) and BAL IgA (285 ng/ml [176-461]) were significantly increased after a third dose of Protollin-eRSV vaccine (Figure 2C and D). To assess the longevity of the antibody response, serum and BAL RSV-specific antibodies were measured over a period of three months in animals that received 2 doses of Protollin-eRSV vaccine. RSV specific IgG and IgA titers did not show signs of waning at 98 days after the last boost (data not shown).

Two doses of IN-administered Proteosome-eRSV or Protollin-eRSV elicited good serum neutralization titers (log_2 5.09±1.42 and $log_2$5.09±0.83 in repeat studies) (Table 1). The three-dose regimen of Protollin-eRSV significantly increased the neutralization titers (log_2 8.00±0.50: $p<0.05$ vs two-doses). Although neutralizing titers were generally higher in animals that received proteosome-based vaccines compared to those exposed to eRSV antigen alone (log_2 4.75±1.75 and 5.13±2.13 for two or three doses respectively), these differences did not reach statistical significance. Neither Proteosome-HA nor Protollin alone control vaccines elicited neutralizing antibody responses above detection limits but neutralizing titers of log_2 6.69 ±1.38 were induced by a single dose of FI-RSV.

2.5.3. Cytokine profiles

Cytokine profiles were measured in splenocyte supernatants (SS) and lung cell preparation supernatants (LS) restimulated with either RSV F (MHC-I epitope) or G (MHC-II epitope) peptides. The cytokine profile elicited following peptide-restimulation of splenocytes was strongly suggestive of a type-1 biased response in animals immunized with Protollin-eRSV. All measured cytokines (IFNγ, IL-2, IL-10 and TNFα except for IL-5 were elevated in this group following restimulation with either the RSV G protein epitope that targeted $CD4^+$ T cells or the F protein epitope that targeted $CD8^+$ T cells (Figure 3A-F). IL-5 production in SS was detected only in the group immunized with FI-RSV following any restimulation (Figure 3E). Similarly in LS, IL-5 was detected

mainly in groups immunized with eRSV and FI-RSV (Figure 3F). Other cytokines in LS were present only at very low levels (IFNγ IL-2 and IL-10, data not shown).

Despite the relatively strong serum IgG and neutralization responses seen following immunization with the Proteosome-eRSV formulation, neither splenocytes nor lung cells isolated from animals in this group responded to F or G peptide epitope re-stimulation with the production of significant quantities of any of the cytokines measured, including IL-5. These results suggest that qualitative differences exist in the immune responses generated by our candidate Protollin-eRSV versus Proteosome-eRSV vaccines: the former eliciting a balanced Th1/Th2 RSV-specific response both systemically and mucosally while the latter also generated good mucosal and systemic antibodies and restrained IL-5 production but did not support the production of detectable Th1 cytokine production in response to the RSV peptides tested.

2.5.4. Peptide-specific CD8+ T cells

Intracellular cytokine analysis was performed to confirm the presence of IFNγ-producing peptide-specific CD8+T cells following immunization with Protollin-eRSV. Mice immunized with Protollin-eRSV had a significantly greater percentage of RSV F peptide-specific CD8+ T cells producing IFNγ (0.53 ±0.03%) compared with animals given eRSV antigen alone (0.34 ±0.02%), adjuvant alone (0.37 ±0.02%) or even live RSV (0.28 ±0.02%) (Figure 4A). To determine the frequency of F peptide-specific CD8+T cells, pentamer staining was performed (Figure 4B). Similar to the findings for intracellular IFNγ production, the percentage of CD8+T cells specific to the MHC-I RSV F peptide was greater in splenocytes from Protollin-eRSV immunized mice (1.15 ±0.17%) than in mice immunized with control vaccines (eRSV: 0.62 ±0.08%, Protollin: 0.79 ±0.18%), or infected with RSV (0.86 ±0.08%). However, the differences only reached statistical significance for the group immunized with eRSV alone ($p<0.05$). These results

demonstrate that Protollin-eRSV promoted a strong CD8+ T cell response with the production of antigen-specific IFNγ following RSV F-peptide restimulation.

2.5.5. Protection studies

Two doses of either Proteosome-eRSV or Protollin-eRSV vaccine (both 5μg eRSV) completely abrogated RSV proliferation in the lungs of immunized BALB/c mice following challenge (Figure 5), without causing any weight loss (data not shown). The extent of protection provided by these vaccines appeared to correlate with viral antigen dose since higher viral titers were detected in the lungs of mice given lower vaccine doses. The partial protection elicited by eRSV alone is consistent with the antibody data. Moreover, in experiments using a higher dose of eRSV (10 μg/dose), the difference in protection between animals immunized with the antigen alone or combined with Protollin was not significant, suggesting that eRSV alone can be immunogenic via the intranasal route at high doses. Not surprisingly, all mice immunized with FI-RSV were also protected.

2.5.6. Lung pathology following RSV challenge

The lungs of mice immunized with Proteosome-eRSV or Protollin-eRSV vaccines responded to RSV challenge with similar limited peribronchiolar infiltrates and mild alveolitis as the non-protective infiltrates observed in the lungs of PBS immunized mice (Figure 6A-C). The post-challenge lung pathology observed in animals that received eRSV alone was similar to those immunized with the vaccines (data not shown). In contrast, the lungs of mice that had received a single IM dose of FI-RSV had dense peribronchiolar and perivascular inflammation after challenge as well as substantial alveolitis (Figure 7D). Analysis of the BAL revealed either absence or very small numbers and proportions of eosinophils in the groups immunized with Proteosome-eRSV (0.05% [0.01-0.3]) and Protollin-eRSV (0%) (Figure 6E). The group immunized with

eRSV alone had increased eosinophil numbers in the BAL (1% [0.3-3]) compared with either proteosome-based vaccine group, but this difference was not statistically significant. As reported previously [9;38;39], animals immunized with FI-RSV had evidence of significant eosinophil infiltration following challenge (20% [15-26] vs <1% in all other groups). Neutrophils were observed in BAL following challenge in all of the groups whether they had been immunized with eRSV alone, eRSV formulated with an adjuvant or a control vaccine (data not shown).

2.7. Discussion

We have shown that the IN administration of two doses of proteosome-adjuvanted vaccines containing virion-derived RSV surface antigens can be highly immunogenic and fully protect BALB/c mice from RSV challenge without enhanced lung pathology. This study both confirms and extends the observations of Etchart et al who recently demonstrated the potential of *N. menigitidis* OMP-based adjuvants in RSV vaccine development [32]. In both studies, virus-specific mucosal and systemic immune responses as well as protection against challenge were significantly improved with the addition of the OMP-based adjuvants compared to animals given RSV antigen alone. Moreover, no pulmonary eosinophilia was detected after RSV challenge when OMP-based adjuvants were used.

Our comparison of Proteosome-eRSV and Protollin-eRSV IN immunization demonstrated that both formulations can elicit equivalent levels of antigen-specific mucosal IgA responses, unlike the findings of Etchart et al, [32]. Based on observations of higher mucosal IgA following immunization with outer membrane vesicles (OMV) depleted of LPS (DOMV) compared to native OMV (NOMV), these authors suggest that 'excess LPS' present in native OMV may interfere with the development of IgA plasma cells in an IL-10 dependent fashion [32]. In contrast, our results suggest that the presence of LPS within Protollin-eRSV not only contributed to elevated mucosal IgA responses, but also favoured the production of IL-10-producing helper T cells. These differences may be explained in part by structural differences between the native *N. meningitidis* membrane-bound endotoxin which is in the form of lipooligosaccharide (LOS) in their work and the lipopolysaccharide (LPS) of *S. flexneri* in our work. Although not yet fully understood, structural differences have recently been implicated in differential induction of the TLR4-MD2-MyD88-dependent and –independent pathways

[40]. Furthermore, the precise composition of the OMP that we used to formulate our Proteosome-based vaccine candidates is unlikely to be identical to the material used by Etchart et al following their depletion of 'native LPS' to produce DOMV.

The RSV-specific, peptide-stimulated cytokine production profiles differed both qualitatively and quantitatively among the vaccine groups in our studies. The most striking of these differences were the broad Th1-predominant, anti-RSV response seen only in animals immunized with Protollin-eRSV, the suppression of peptide-specific IL-5 production in animals exposed to either of the adjuvanted vaccines and the nearly exclusive Th2 cytokine-dominated response in animals exposed to FI-RSV. The first observation of Th1 cytokine production primarily in the Protollin-eRSV group is also at odds with the findings of Etchart et al who saw no differences in IFNγ-production by ELISPOT between their OMV vaccinated groups (with or without 'native LPS' depletion) [32]. However, these authors used RSV-infected cells to stimulate splenocytes and measured only IFNγ responses. In our hands and using RSV F or G-specific peptides to re-stimulate splenocytes, immune cell preparations from Protollin-eRSV vaccinated animals produced a number of Th1-type cytokines including abundant secreted IFNγ. These animals also responded to F peptide re-stimulation with increased numbers of F-specific CD8+ T cells, many of which were shown to be producing intracellular IFNγ. Both the magnitude and the range of the cytokine response elicited by Proteosome-eRSV immunization were attenuated compared to animals exposed to the Protollin-eRSV vaccine. These observations suggest that the Protollin formulation may have significant advantages in the induction of virus-specific cellular responses. The *S. flexneri* LPS contained as an integral part of the Protollin formulation is an obvious candidate to explain this difference. It has recently been demonstrated that the expansion of antigen-specific $CD4^+$ and $CD8^+$ T cells via up-regulation of co-stimulatory molecules requires LPS-mediated TLR4 signaling via the MyD88-independent TRIF/TICAM pathway in

some circumstances [41]. Furthermore, RSV antigens themselves may up-regulate TLR4 expression and enhance responses to LPS [42-44]. Alternatively, since discreet $CD4^+$ and $CD8^+$ T cell epitopes were used for re-stimulation in our studies, it is possible that the relative inability of Proteosome-eRSV to induce cytokine responses to these specific peptides was due to differences in processing of eRSV G and F proteins when formulated with Protollin versus proteosomes. Finally, it is tempting to speculate that the detection of RSV F peptide-specific CD8+ T cells in mice immunized with Protollin-eRSV is indicative of antigen cross-presentation. A role for TLRs in such cross-presentation has recently been proposed [45] and could occur via internalization of particulate antigens into phagosomes followed by fusion with the endoplasmic reticulum [46].

Immune cells isolated from the spleens and lungs of animals immunized with FI-RSV responded in a narrow way to both the F ($CD8^+$) and G ($CD4^+$) peptide re-stimulation by eliciting predominantly IL-5, an archetypal Th2 cytokine [9;47]. In response to the G peptide, animals immunized with eRSV alone also produced relatively large amounts of IL-5. In sharp contrast, vaccination with either Proteosome-eRSV or Protollin-eRSV completely abrogated this response; IL-5 was either undetectable or barely detectable in RSV peptide-restimulated splenocyte and lung cell culture supernatants from these animals.

The unfortunate history of RSV vaccine development is such that the avoidance of an aberrant immune response is at least as important as the demonstration of an appropriate response. Our results suggest that IN proteosome-based eRSV vaccines can be administered repeatedly without either short-term adverse effects (eg. weight loss) or risk of enhanced pulmonary pathology and eosinophil infiltration. Neither of the proteosome-containing candidate vaccines elicited eosinophil infiltration following RSV challenge. These conclusions, however, are limited to a relatively short interval between

immunization and challenge. It was previously reported that pulmonary pathology was enhanced in cotton rats challenged 6 months after immunization [48]. Further experimentation is in progress to confirm that protection without exacerbated responses still occur if the challenge is delayed. It is interesting that immunization with our eRSV alone did not result in the profuse pulmonary eosinophilic exudates like those observed in animals immunized with FI-RSV suggesting that the Th2-biased response to RSV antigens and the immunopathology associated with inactivated RSV vaccines can be dissociated to some degree. This observation is supported by the recent demonstration that formalin-induced protein carbonylation of RSV antigens is an important factor in the enhanced pathology associated with inactivated RSV vaccines [15].

Although both proteosome-adjuvanted vaccines were more effective that antigen alone, immunization with eRSV at higher doses elicited detectable antibody titers and partial protection. Etchart et al also noted reductions in RSV titers in mice immunized with their inactivated RSV formulation following challenge but reported full protection only after three doses [32]. The surprising efficacy of IN eRSV antigen may be the result of its particulate nature. Such particles may benefit from facilitated uptake by M cells in the follicle-associated epithelium of NALT [49-51]. However, both the systemic and mucosal RSV-specific antibody responses were higher following immunization with either of the proteosome-based formulations and the Protollin adjuvanted vaccine also elicited more vigorous cellular (cytokine) responses. Although the correlates of protection against RSV are not yet completely understood [52], both antibody [53] and cellular responses [54;55] are likely to have important and complementary roles in humans [20]. It therefore seems logical that an RSV vaccine candidate that can safely induce several layers of immune defense (eg: mucosal and serum antibodies, systemic T cell responses) will provide superior protection [56-58]. Since an over-vigorous cellular

response against a viral pathogen may be detrimental in the lung [20], our work to date does not permit the selection of a lead candidate between the proteosome and Protollin formulations. Both have been administered safely in Phase I/II human trials either alone (Protollin as a candidate shigellosis vaccine [28]) or as proteosomes formulated with influenza antigens [24]). In the case of Protollin, it is not yet fully understood how formulation of LPS within the proteosome complex quenches the reactogenic lipid A signal, but this portion of the LPS molecule is likely to interact with the hydrophobic moieties of the OMP [31].

In conclusion, we have demonstrated that the IN administration of proteosome-based RSV vaccine candidates can protect BALB/c mice against RSV challenge without risk of enhanced Th2-driven pulmonary pathology. This protection is characterized by the production of durable systemic and mucosal RSV-specific antibodies. Protollin-eRSV was also shown to induce a broad T cell cytokine response to RSV surface glycoprotein epitopes as well as antigen-specific CD8+T cells. Studies are on-going to determine the precise mechanism of action of the proteosome-based adjuvants in these candidate RSV vaccines.

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2.8. Table 1

Table 1	***Serum Neutralization titers $log_2 \pm SEM$***[a]		
Number of doses[b]	1 dose	2 doses	3 doses
Collection day[c]	35	35	56
Protollin-eRSV	NA	5.09 ±0.59	8.00 ±0.50[d]
eRSV	NA	4.75 ±1.75	5.13 ±2.13
Protollin	NA	3.00 ±0.00[e]	3.34 ±0.36
Proteosome-eRSV	NA	5.09 ±1.42	NA
Proteosome-HA	NA	3.00 ±0.00	NA
FI-RSV	6.69 ±1.38	NA	NA

a.Average and SEM from at least two repeat studies (pooled N=5 for each group)
b.Mice immunized with the proteosome-based vaccines were not bled after the first dose. Mice were given only two doses of the Proteosome-eRSV vaccines and only one dose of the control FI-RSV vaccine.
c.Number of days post-prime
d.Significant difference compared to two-dose regimen ($P<0.05$).
e. Detection limit of the assay is 3.00+/-0.00

2.9. Figure Legends

Figure 1

eRSV and formulated vaccine characterization. Lanes 1-3, eRSV characterization: lane 1, enriched RSV (eRSV) following lectin chromatography on Commassie stained gel, and lane 2, crude RSV extract before lectin chromatography. Lane 3, western Blot with immunodetection cocktail of anti-F and anti-G Mabs. On the Western blot, G proteins are found at 95 and 55 kDa (broad, diffuse bands) and F_1 is found at 48kDa (sharp band). Lanes 4-9, Proteosome-eRSV vaccine characterization post-formulation: lanes 4-6, commassie stained gel and lanes 7-9, western blot with anti-G and anti-F Mabs cocktail. Lanes 4 and 7 show the whole vaccine, lanes 5 and 8 show the soluble fractions following a short centrifugation at 14 000 X g and lanes 6 and 9 represent the insoluble fractions. Porins A and B can be seen at 42 and 40 kDa, respectively and class 4 outer membrane protein (Rmp) at 21 kDa. Lanes 10 and 11, western blot with anti-F2 mouse antiserum detection demonstrating the presence of F2 in eRSV alone and the Proteosome-eRSV formulated vaccine, respectively (equivalent total protein weight). Scanning electron microscopy by negative coloration following PTA staining and immunodetection with polyclonal goat anti-RSV and gold-labelled anti-goat antibodies. Protollin-eRSV (46 000X) above and Proteosome-eRSV (44 000X) below.

Figure 2

Proteosome-eRSV (Pro-eRSV) and Protollin-eRSV (Prl-eRSV) elicited significant RSV-specific titers. Following two IN immunizations, RSV-specific serum IgG (A) and BAL

IgA (B) were quantified by ELISA. Similarly, after 3 doses, RSV-specific serum IgG (C) and BAL IgA (D) were measured. Results are shown as geometric means of 5 animals per group with upper and lower 95% confidence intervals. The data are representative of at least two separate experiments. Statistical analyses were performed by one-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test. For the three dose-regimens, unpaired t test was performed. A P-value of ≤ 0.05 (two-tail) was considered significant. $P < 0.05$,*; $P < 0.01$, **; $P < 0.001$,***.

Figure 3

Restimulation of cells from Proteosome-eRSV (Pro-eRSV) and Protollin-eRSV (Prl-eRSV) immunized mice with CD4+T cell-specific RSV G and CD8+T cell-specific RSV F peptides. Splenocyte and lung cell preparations from immunized animals (2 dose-regimen) were re-stimulated with increasing doses of F or G peptides (0.1, 1.0, 10.0 μg/mL) and supernatants were assayed for cytokines IFNγ (A), IL-2 (B), IL-10 (C), TNFα (D) and IL-5 (E,F). SS: splenocyte supernatants; LS: Lung cells preparation supernatants. The data presented were normalized against non-restimulated cell supernatants and represent the highest peptide-specific response, typically observed at 10 μg/ml. The data is representative of at least two separate experiments.

Figure 4

Percentage of CD8+ T cells positive for intracellular IFNγ determined by FACS in purified inactivated RSV restimulated splenocytes from Protollin (Prl)-eRSV, eRSV and Protollin (Prl) immunized as well as live RSV infected BALB/c mice (A). Percentage of

CD8+ T cells specific to the H-2K^d-restricted RSV F peptide (KYKNAVTEL)-conjugated pentamer in F-peptide restimulated splenocytes from immunized BALB/c mice (B). Results are shown as mean ± SEM. Statistical analyses were performed by one-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test. A *P*-value of ≤ 0.05 (two-tail) was considered significant. $P < 0.05$,* $P < 0.01$, **; $P < 0.001$,***.

Figure 5

Mice immunized with 2 doses of proteosomes (Pro) or Protollin (Prl) formulated with eRSV (≥5 μg) were fully protected against RSV challenge as shown by RSV lung titers. Lungs of mice immunized with decreasing doses of eRSV vaccine and antigen (μg dose in parentheses on X-axis) were homogenized four days after challenge with 10^7 $TCID_{50}$ Long strain RSV and titered on Vero cells using indirect immunofluorescence. The bar represents the geometric mean virus titers in the lungs of 5 mice per group and is representative of ≥two separate experiments. One-way Analysis of Variance (ANOVA) was performed with Tukey-Kramer Multiple Comparisons Test. A *P*-value of ≤ 0.05 (two-tail) was considered significant; $P < 0.01$, **.

Figure 6

Mice immunized with e-RSV formulated with proteosomes (Pro) or Protollin (Prl) do not develop exacerbated lung pathology or pulmonary eosinophilia following challenge. Representative sections from immunized and challenged mice show mild peribronchiolar infiltration (thin arrow) and mild alveolar septal thickening (short arrow) in B) Prl-eRSV

and C) Pro-eRSV (both 2 doses of 5 μg, IN). Similar, but non-protective peribronchiolar infiltration and alveolar septal thickening is observed in A) PBS immunized mice. Important cellular infiltrates, both peribronchial (thin arrow) and perivascular (open arrow) including the alveolar septa and lumen (parallel double arrows), are observed in D) FI-RSV (one dose, IM). E) Enumerations of eosinophils in individual mouse BAL were performed and the data expressed as percentages of eosinophils over all leukocytes. Bars represent the geometric mean of 5 animals per group with upper and lower 95% confidence intervals (data are representative of ≥ two separate experiments). proteosome-based vaccines and antigen alone comparison with FI-RSV: One-way Analysis of Variance (ANOVA) was performed with Tukey-Kramer Multiple Comparisons Test. A P-value of ≤ 0.05 (two-tail) was considered significant; $P < 0.001$, ***.

2.10 Figures

Figure 1

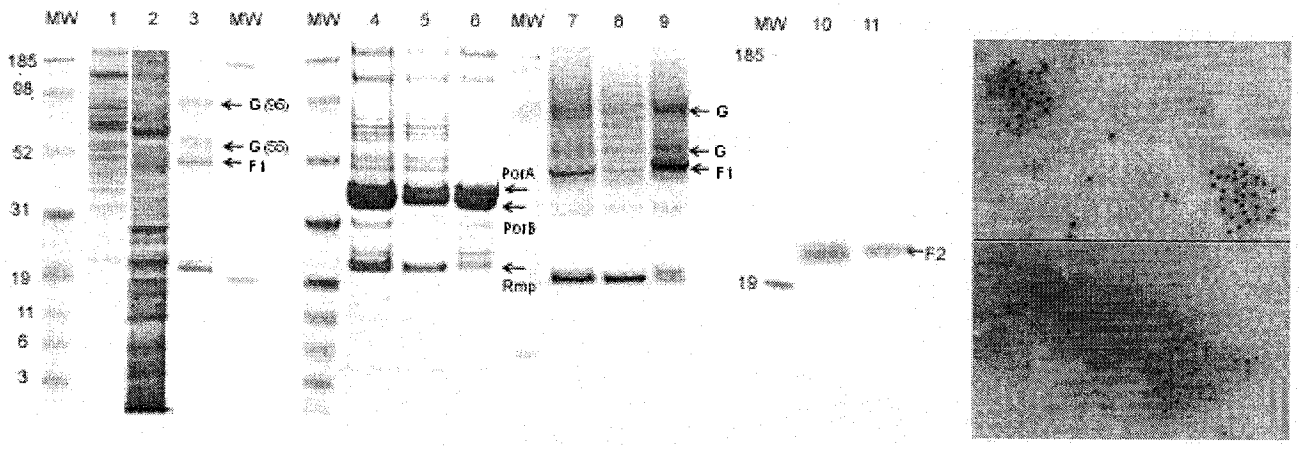
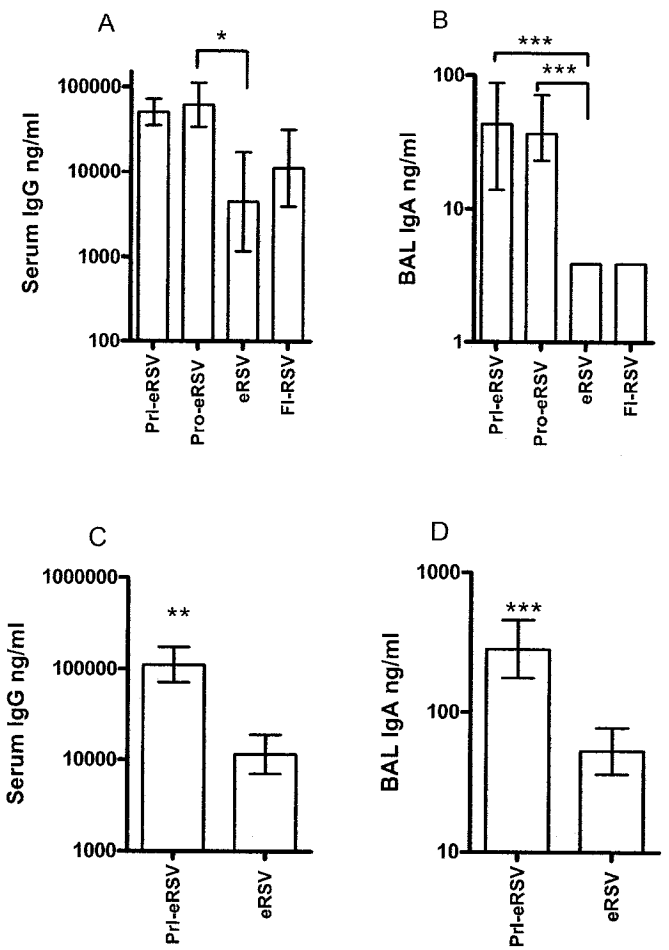


Figure 2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*
100000
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100
Serum IgG ng/ml
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Pro-eRSV
eRSV
Fl-RSV
B
***
***
100
10
1
BAL IgA ng/ml
Prl-eRSV
Pro-eRSV
eRSV
Fl-RSV
C
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1000000
100000
10000
1000
Serum IgG ng/ml
Prl-eRSV
eRSV
D
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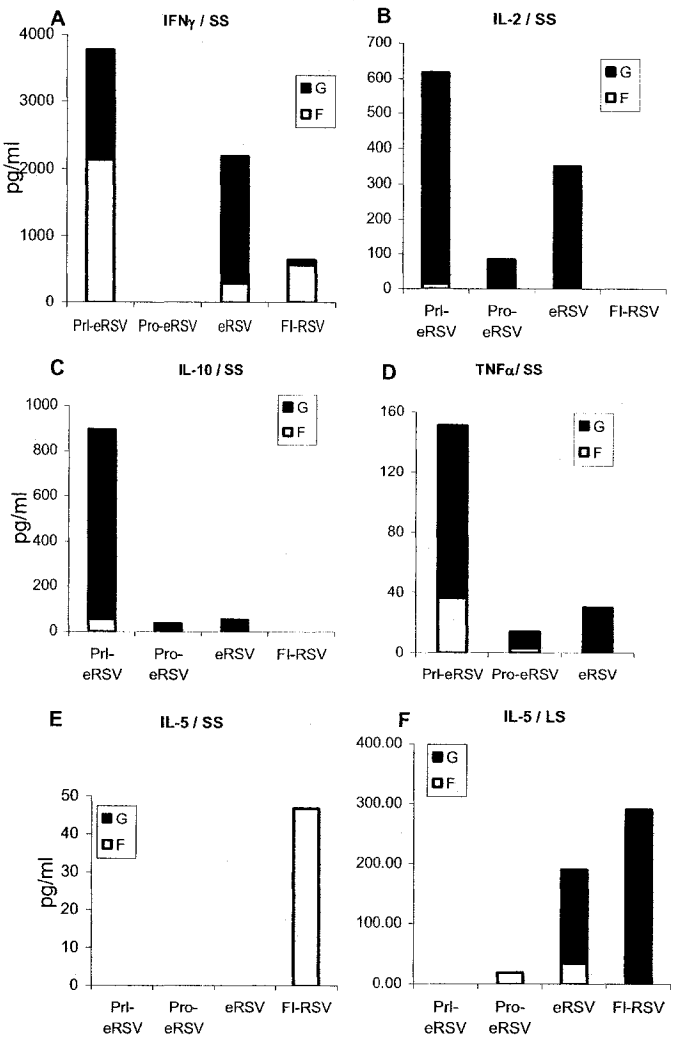
BAL IgA ng/ml
Prl-eRSV
eRSV

Figure 3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IFNγ / SS
pg/ml
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3000
2000
1000
0
G
F
Prl-eRSV
Pro-eRSV
eRSV
Fl-RSV
B
IL-2 / SS
700
600
500
400
300
200
100
0
Prl-eRSV
Pro-eRSV
C
IL-10 / SS
1000
800
600
400
200
D
TNFα/ SS
160
120
80
40
E
IL-5 / SS
50
40
30
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10
F
IL-5 / LS
400.00
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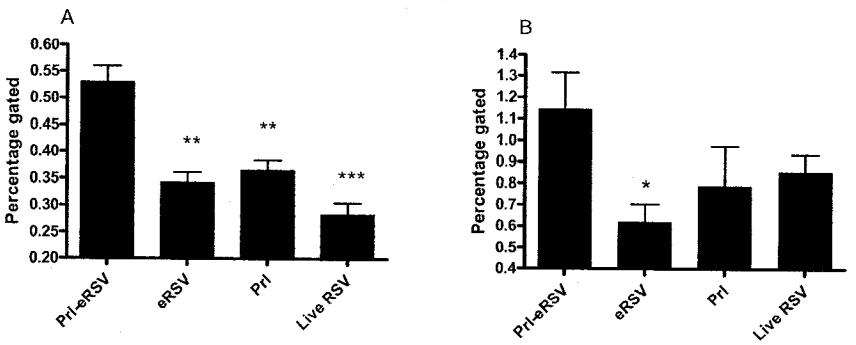
Figure 4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Percentage gated
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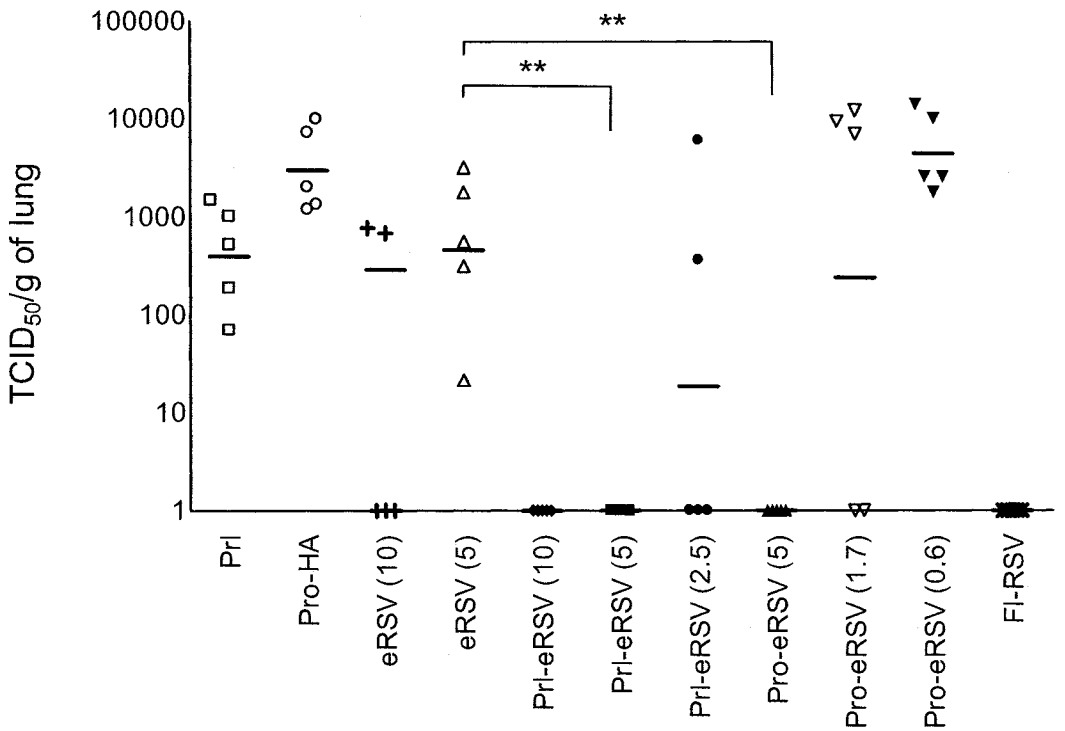
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
**
**
***
Prl-eRSV
eRSV
Prl
Live RSV
B
Percentage gated
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
*
Prl-eRSV
eRSV
Prl
Live RSV

Figure 5


100000
10000
1000
100
10
1
TCID50/g of lung
**
**
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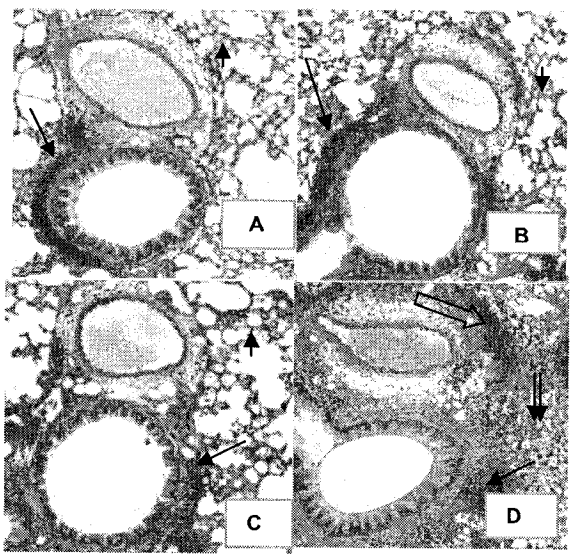
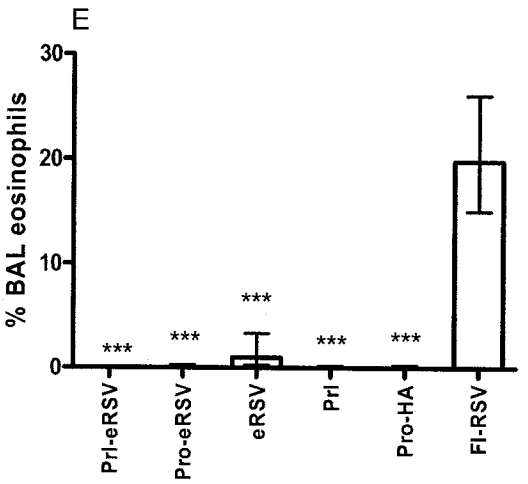
Pro-HA
eRSV (10)
eRSV (5)
Prl-eRSV (10)
Prl-eRSV (5)
Prl-eRSV (2.5)
Pro-eRSV (5)
Pro-eRSV (1.7)
Pro-eRSV (0.6)
Fl-RSV

Figure 6


A
B
C
D


E
% BAL eosinophils
30
20
10
0
***
***
***
***
***
Prl-eRSV
Pro-eRSV
eRSV
Prl
Pro-HA
Fl-RSV

Chapter 3

C57Bl/6 mice are protected from respiratory syncytial virus (RSV) challenge and IL-5 associated pulmonary eosinophilic infiltrates following intranasal immunization with Protollin-eRSV vaccine[‡].

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3.1. Preface

Following the successful demonstration of protective efficacy in the absence of Th2-driven pathology in BALB/c mice, and in order to take advantage of TLR knock-out models available on the C57Bl/6 background, both Proteosome- and Protollin-eRSV vaccines were evaluated in C57Bl/6 mice. Successful immunization resulting in protection was eventually achieved in C57Bl/6 mice by immunizing with equivalent eRSV antigen doses but increased doses of the Protollin adjuvant (compared to BALB/c mice). Proteosome-eRSV immunization did not elicit full protection in this strain at any of the doses tested. Interestingly, this work demonstrated that, like the Th2-biased BALB/c animals, enhanced pulmonary disease could also be elicited in this intrinsically Th-1 biased strain by prior formalin-inactivated RSV vaccination.

3.2. Abstract

The protective efficacy of an intranasal (IN) Protollin-eRSV vaccine has recently been demonstrated in the RSV-susceptible BALB/c mouse model. Here, we report the safety, immunogenicity and efficacy of Protollin-eRSV vaccine in the relatively resistant C57Bl/6 mouse model. C57Bl/6 mice immunized IN with either 2 or 3 doses of Protollin-eRSV produced significant systemic and mucosal RSV-specific antibodies. Mice immunized with the Protollin vaccine displayed polarized Th1 responses with augmented IFNγ/IL-5 ratios in RSV-restimulated lung and spleen cell preparations compared with animals that received antigen alone. The Protollin-eRSV immunized C57Bl/6 mice were fully protected against challenge without eosinophilic pulmonary pathology observed in the animals immunized with the formalin-inactivated RSV vaccine. This new model will permit us to dissect the respective roles of the TLR2 and TLR4 ligands contained in the vaccine using TLR knock-out animals established on the C57Bl/6 background.

3.3. Introduction

Respiratory syncytial virus (RSV) is the main viral cause of lower respiratory tract infections in young children, causing symptoms ranging from rhinitis to bronchitis. No RSV vaccine is currently available. Protollin-eRSV (Prl-eRSV) is an intranasal (IN) subunit candidate vaccine composed of an enriched RSV (eRSV) antigen mixed with the proteosome-based adjuvant Protollin (Prl) (*N. meningitides* outer membrane proteins formulated with *S. flexneri* lipopolysaccharides (LPS)) [1,2]. Proteosome-based vaccines administered intranasally (IN) in different animal models and humans have been demonstrated to be safe and efficacious against several respiratory [3,4] and gastrointestinal pathogens [5].

Safety is of the utmost importance for an RSV vaccine due to the early experiences with formalin-inactivated (FI)-RSV vaccines that caused exacerbated pulmonary disease following natural infection. This enhanced pathology was characterized by pulmonary eosinophilic inflammatory infiltrates and T-helper 2 (Th2) cytokine production (CMI) [6]. Recent observations that have contributed to our understanding of the pathology associated with the FI-RSV vaccines include Th2-deviated cytokine production in response to both the RSV G protein itself [7] and the carbonylation of formalin-exposed vaccine antigens [8]. BALB/c mice are particularly susceptible to FI-RSV enhanced pathology following challenge compared to other mouse strains due to their natural propensity for Th2-biased responses and their permissiveness to RSV replication [9,10]. C57Bl/6 mice on the other hand, are relatively resistant to RSV proliferation and are

generally predisposed to produce T-helper (Th1)-biased responses to microbial challenge, often with only modest antibody responses [11,12].

In order to take advantage of the existing knock-out models for innate immune signaling available primarily on a C57Bl/6 background, Prl-eRSV was first tested for safety and efficacy in C57Bl/6 animals. We report herein that Prl-eRSV vaccine given IN in two or three doses to C57Bl/6 mice elicited significant virus-specific systemic and mucosal antibody responses that could protect against subsequent challenge. The vaccine response was characterized by Th1- polarization compared with mice given antigen alone and did not result in enhanced eosinophilia on subsequent challenge.

3.4. Materials and Methods

3.4.1. Mouse immunizations and challenge

Female 6-8 week old C57Bl/6 mice were purchased from Charles River Laboratories (St-Constant, QC) and maintained according to the Canadian Council on Animal Care guidelines. Eight mice per group were immunized IN with a constant dose of 5μg of eRSV combined with either 3μg or 10μg of Prl adjuvant (two-dose regimen, days 0, 21) or 3μg of Prl (three-dose regimen, days 0, 21 and 42). Control mice received Prl (negative data, not shown) or eRSV alone IN. Two weeks post-boost, four mice per group were challenged IN with 10^7 $TCID_{50}$ of Long strain RSV (ATCC# VR-26) in 50μl. The remaining mice were sacrificed at this time: serum, bronchoalveolar lavage (BAL), the spleens and lungs were collected. Challenged mice were euthanized 4 days post-challenge and BAL and lungs were collected. Mice immunized IM with FI-RSV received one dose on day 0 as previously described [13] and followed the same challenge schedule as Prl immunized mice.

3.4.2. Antibody titer determination

IgG and IgA antibody titers were determined on individual serum and BAL samples by ELISA as previously described [4]. Briefly, 96-well plates were coated with 7.5μg/ml of PEG-concentrated [14], UV-inactivated RSV and incubated overnight at 4°C. Plates were washed, blocked and serum and BAL samples were serially diluted starting at 1:50 and 1:2 dilution, respectively. Bound antibody was detected with HRP-conjugated anti-mouse IgG or IgA (Sigma, Oakville, ON).

3.4.3. Titration assays

Neutralization assay and lung viral titrations were performed as previously described [13]. Briefly, pooled sera were serially diluted, mixed with RSV and incubated for 20 minutes at 33°C. The mixture was then transferred to Vero cell monolayers for titer determination. Lung viral titration was performed on weighed and washed lungs that were homogenized individually then centrifuged at 2655 x g for 2 minutes at 4°C. Supernatants were also titered on Vero cell monolayers. In each case, monolayers were incubated for 5-6 days and fixed (1% paraformaldehyde, Sigma). Plaques were detected using 1:400 dilution of goat anti-RSV antibody (Biodesign international) followed by anti-goat IgG FITC (1:400) conjugate (Sigma) for detection. Wells were considered positive when ≥1 fluorescent syncytium was detected. Viral titers (50% tissue culture infective dose or $TCID_{50}$) were calculated using the Reed-Muench method [14].

3.4.4. BAL leukocyte differential counts

BAL fluids were collected and centrifuged as previously described (22) and the pellets were resuspended in 500µl of PBS. Individual BAL (100µl) fluid were cytospun (Thermoelectron Co, by Fisher, Nepean ON) and cells were fixed and stained with hematoxilin and eosin (H & E)(Sigma)[14]. Total cells were enumerated using a haemocytometer (40X) and a manual differential count was performed on 300 cells.

3.4.5. In vitro restimulation of splenocytes and lung cell preparations

Organs were processed for *in vitro* re-stimulation as previously described [4]. Briefly,

washed spleens were passed through a 100μm mesh, RBCs were lysed and the cells were washed and counted. Washed lungs were minced before processing as above. Cells were incubated with increasing doses (1, 3 and 9μg/mL) of purified inactivated RSV (Biodesign International, Brockville ON) for 72 hours. The harvested supernatants were frozen until assayed for IL-5 and IFNγ by ELISA (BD Biosciences, Oakville ON), as per the manufacturers' instructions.

3.4.6. Statistics

Statistical analyses were performed by one-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test. A p-value of ≤ 0.05 (two-tail) was considered significant.

3.5. Results

3.5.1. Systemic and mucosal RSV-specific humoral immunity following IN immunization with Prl-eRSV.

C57Bl/6 mice tolerated well the Protollin-eRSV vaccine and produced good RSV-specific systemic and mucosal humoral responses following IN immunization with Prl-eRSV. At constant antigen dose (5μg), both the two-dose regimen of the Prl (10μg)-eRSV vaccine and the three-dose regimen of the Prl (3μg)-eRSV elicited RSV-specific serum IgG levels (Fig. 1A) that were significantly higher than those elicited by immunization with 5μg of eRSV antigen alone. Good serum neutralization titers ($log_2$7.75) were elicited after the administration of three doses of Prl (3μg)-eRSV only (data not shown). RSV-specific IgA titers in BAL were increased in vaccine-immunized mice compared to mice immunized with the antigen alone following the two-dose regimen, but these differences did not reach statistical significance. On the other hand, significant differences in BAL titers were observed between vaccine and eRSV immunized mice following the three-dose regimen of the Prl (3μg)-eRSV (Fig. 1B).

3.5.2. Cell mediated immunity profile following IN immunization with Prl-eRSV.

As observed with BALB/c mice, C57Bl/6 mice immunized with Protollin-eRSV displayed a polarized Th1 cellular immune profile following *in vitro* restimulation, with elevated levels of Th1-marker IFNγ and decreased levels of Th2-marker IL-5 (Fig. 2) compared to mice immunized with antigen alone. Interestingly, this Th1 phenotype shift

was observed despite the inherent C57Bl/6 Th1 background, both mucosally and systemically.

3.5.3. Protection against RSV challenge and vaccine-enhanced pulmonary eosinophil infiltration following IN immunization with Prl-eRSV

Compared to our BALB/c model, the C57Bl/6 animals were not as permissive to lung RSV replication. The viral titers in the lungs of unvaccinated and challenged animals were only 10^3 $TCID_{50}$/g of lung compared to 10^4 $TCID_{50}$/g of lung seen in the BALB/c model [13]. Nonetheless, titers were high enough that we were able to demonstrate statistically significant protection in mice immunized with the Protollin-adjuvanted vaccines. Following two-doses, mice immunized with Prl (3μg)-eRSV were partially protected (3/4: 75%) while mice immunized with Prl (10μg)-eRSV were completely protected from pulmonary viral replication (4/4: 100%) compared with mice that received eRSV antigen alone (Figure 3A). In contrast, there were no significant differences between protection levels in mice given the three-dose regimen of Prl-eRSV or antigen alone. As expected, all mice given FI-RSV were protected. Although two doses of Prl (10μg)-eRSV and three doses of either Prl(3μg)-eRSV elicited the same levels of protection as FI-RSV, mice given the Prl vaccines had significantly less pulmonary eosinophil infiltration (Figure 3B). These results suggest that the Prl-RSV vaccine can not only protect against challenge, but can do so without risk of enhanced pulmonary pathology.

3.6. Discussion

Prl-eRSV given IN elicited good systemic and mucosal RSV-specific titers, including serum neutralizing titers. Interestingly, although vaccination with two or three doses of eRSV antigen alone was able to elicit RSV-specific serum IgG, no RSV-specific IgA was detected in the BAL from any of these groups. This observation suggests that Protollin may be required to elicit mucosal antibody responses, in addition to promoting strong systemic immunogenicity. Immune stimulation by Protollin at the mucosa may occur as a result of TLR-mediated uptake by M cells (Chabot *et al*, submitted) or dendritic cell extension past the epithelial barrier to sample the particulate vaccine [15].

Despite exhibiting a strong Th1 background, IN immunization of C57Bl/6 mice with Prl-eRSV elicited a further polarization of the CMI. Immunization with Prl-eRSV elicited a Th1-bias with elevated IFNγ/IL-5 ratios in both restimulated lung and spleen cell preparations compared with mice given the antigen alone. The levels of IL-5 detected in the antigen re-stimulated cell preparations were surprisingly elevated, suggesting RSV antigens can elicit strong Th2 responses, even in the relatively resistant C57Bl/6 model. These findings differ from previous work by Hussell *et al* where C57Bl/6 mice infected with vaccinia-virus expressing the RSV G protein (rVV-G) did not elicit IL-5 or eosinophils following challenge, unlike other mouse strains [16]. These observations suggest two things; 1- Although the G protein favours Th2 responses and contributes to vaccine enhanced-pathology, other components of the RSV antigen may also contribute to Th2-driven pulmonary inflammation including host cell proteins (even without formaldehyde treatment [8]); 2- The G protein given as an infectious entity may have

elicited Th1-enhancing anti-viral immunity, and avoided the exacerbated Th2 responses observed here in the C57Bl/6 mouse.

Complete protection against challenge was conferred with two doses of Prl-eRSV as well as partial protection with antigen alone following three doses. Interestingly, although only one dose of FI-RSV elicited an impressive amount of pulmonary eosinophil infiltrates, all immunization regimens with the Prl-eRSV vaccine and the antigen alone (two-dose regimen only) reduced the eosinophils to background levels. Again this observation of high eosinophilia in the C57Bl/6 model differs from the results reported by Hussell *et al*, most probably due to the difference in vaccine used as discussed above.

Overall, we found the Prl-eRSV vaccine was safe, immunogenic and efficacious in the C57Bl/6 mouse model. These observations were surprisingly similar to our observations in the more Th2-biased BALB/c model [13]. Similar to the BALB/c model, the Prl-adjuvanted vaccines given IN in two or three doses to C57Bl/6 mice elicited excellent immunogencity and complete protection in some groups without enhanced pulmonary pathology following challenge. An important difference between the two models is an apparent requirement for a higher dose of Prl (10μg instead of 3μg) to elicit protection after a two-dose regimen. This observation is consistent with the generally lesser capacity of C57Bl/6 mice to generate humoral responses. Nevertheless, intranasal Prl-eRSV was successfully demonstrated to protect this relatively recalcitrant model and therefore, TLR knock-out models on this background can be exploited for the study of the

innate immune mechanisms elicited by the vaccine and their role in promoting safe and effective adaptive immunity.

3.7. Reference List

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3.8. Figure Legends

Figure 1

RSV-specific serum IgG (A) and BAL IgA (B) following IN immunization with Prl-eRSV compared to eRSV alone determined by ELISA. Mice were immunized IN with a constant dose of 5μg of eRSV combined with either 3μg or 10μg of Prl adjuvant (two-dose regimen, days 0, 21) or 3μg of Prl (three-dose regimen, days 0, 21 and 42). Control mice received 5μg of eRSV alone IN. Results are expressed as geometric mean (ng/ml) with upper and lower 95% confidence limits. The data are representative of at least two separate experiments. Detection limits are 39ng/ml (serum IgG) and 4ng/ml (BAL IgA). Vaccine comparisons with antigen alone: $p < 0.01$, **; $p < 0.001$, ***.

Figure 2

RSV-specific cytokine response ratio in lung and spleen cell preparations following IN immunization with Prl (3μg)-eRSV and eRSV immunized mice at constant antigen dose (5μg, 3 dose regimen). Lung and spleen cell preparations were restimulated *in vitro* with increasing concentrations of purified inactivated RSV (1, 3 and 9μg/ml). Cytokines IFNγ and IL-5 in cell supernatants were measured by ELISA. The data represent cytokine ratios following restimulation with the highest stimulant dose (9μg/ml) only if a dose response was observed. The data are representative of at least two separate experiments.

Figure 3

Protection from challenge and pulmonary eosinophilic infiltrates following immunization with Prl-eRSV. A) Homogenized lung titers following immunization and challenge were

determined by titration on Vero cells with detection by immunofluorescence. Briefly, after blocking with PBS + 2% skim milk, plates were incubated at RT for 2 hours with a 1:400 dilution of goat anti-RSV antibody (Biodesign international) and washed with PBS. Anti-goat IgG FITC conjugate (Sigma) was used at a 1:400 dilution for detection. Wells were considered positive when ≥1 fluorescent syncytium was detected. Results are expressed in $TCID_{50}$ per gram of lung. B) BAL leukocytes were collected from immunized mice 4 days following challenge, fixed on microscope slides and stained with H&E. Eosinophils were enumerated by morphological characteristics by three independent analysts. Geometric mean of $TCID_{50}$ per gram of lung or percentages (compared with FI-RSV): $p < 0.05$,*; $p < 0.01$, **.

3.9 Figures

Figure 1.

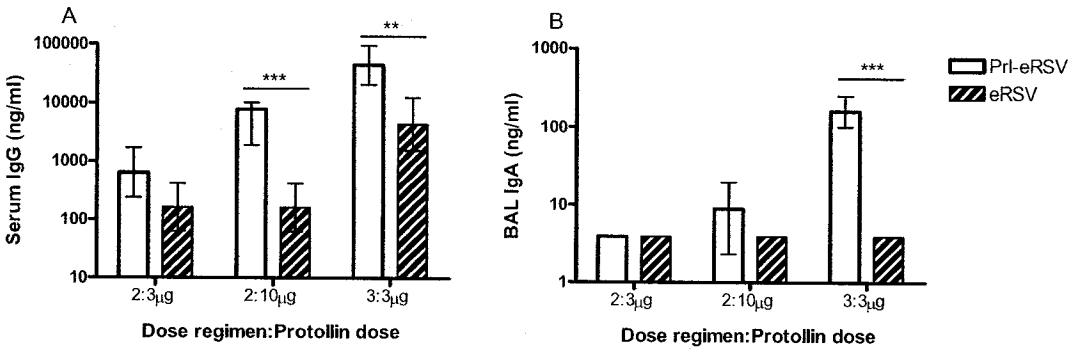


Figure 2

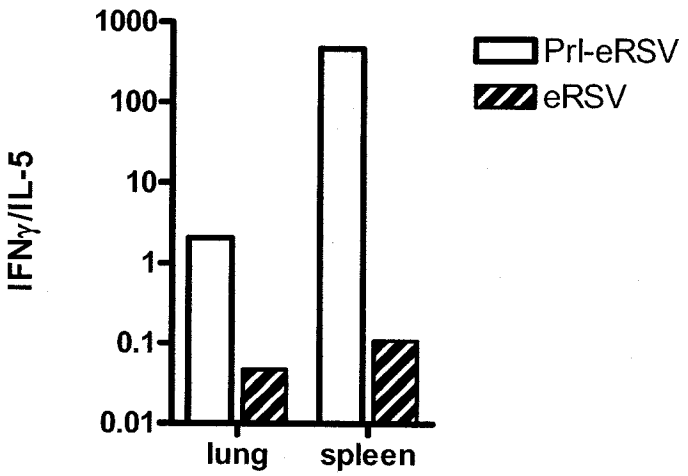
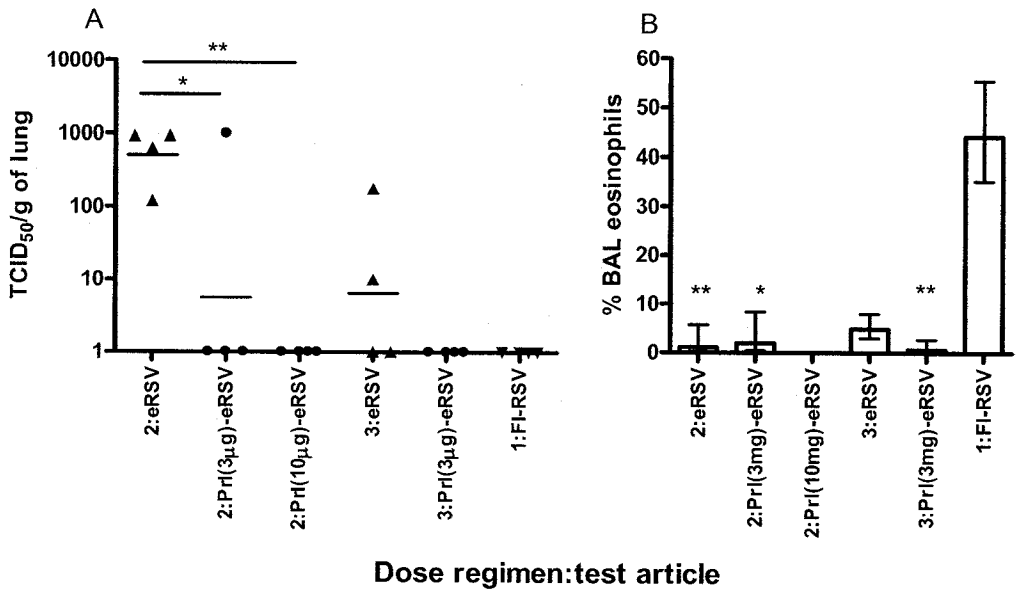


Figure 3


A
B
TCID50/g of lung
10000
1000
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1
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*
% BAL eosinophils
60
50
40
30
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10
0
2:eRSV
2:PrI(3µg)-eRSV
2:PrI(10µg)-eRSV
3:eRSV
3:PrI(3µg)-eRSV
1:FI-RSV
2:PrI(3mg)-eRSV
2:PrI(10mg)-eRSV
3:PrI(3mg)-eRSV
Dose regimen:test article

Chapter 4

Intranasal, Protollin-based RSV vaccination in mice: TLR4/MyD88 signaling is required both for protection and the control of pulmonary granulocyte recruitment following challenge.

Submitted for publication.

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4.1. Preface

Following the demonstration of protective efficacy of the Protollin-eRSV vaccine in C57Bl/6 mice, we proceeded with the evaluation of vaccine immunogenicity and efficacy in TLR2, TLR4 and MyD88-/- mice on the same background. Surprisingly, TLR2 ligation did not appear to contribute to any aspect of immunity or protection following immunization with Protollin-eRSV. In contrast, TLR4 appeared to be critical for the stimulation and maturation of antigen presenting cells and the MyD88 adaptor protein contributed significantly to driving immunity away from a Th2-biased response. These results suggest, for the first time, that the presence of *Shigella* LPS in Protollin may supercede the theoretical contribution of *Neisseria* porins to innate immune regulation for this family of adjuvants.

4.2. Abstract

Protollin-eRSV, an intranasal vaccine composed of TLR2 ligand *N. meningitidis* outer membrane proteins and TLR4 ligand *S. flexneri* LPS (Protollin) and enriched RSV proteins (eRSV) has been demonstrated to promote balanced Th1/Th2 responses without eosinophil recruitment and protect against challenge in mouse models. We exposed splenocytes of naïve C57Bl/6 mice (wild-type and TLR2-/-, TLR4-/- and MyD88-/- knockouts) to the individual components of the Protollin-eRSV vaccine and demonstrated that rapid IFN-β and TNFα production was mediated by the LPS component acting via the TLR4/MyD88-independent pathway. Up-regulation of the co-stimulatory molecules CD40 and CD86 on splenic CD11c+ cells isolated from Protollin-eRSV immunized mice also required the TLR4/MyD88-independent signaling pathway. However, MyD88-dependant signaling was crucial to elicit Th1-biased adaptive immunity following immunization, characterized by increased production of antigen-specific IFNγ by splenocytes with concomitant reduction of IL5, IgG2a isotype switching and abrogation of pulmonary eosinophil recruitment following challenge. Neutrophil recruitment to the lungs following challenge was also mediated via MyD88-dependant signaling. Our findings indicate that adjuvant actions of Protollin including early release of inflammatory cytokines and upregulation of co-stimulatory molecules occur via TLR4/MyD88-independent pathway while protection without risk of enhanced pulmonary pathology requires intact TLR4/MyD88-dependent signaling.

4.3. Introduction

The development of RSV vaccines has been complicated by the potential for serious adverse events. In an early and pivotal study, children who had received a formalin-inactivated (FI)-RSV vaccine were at markedly elevated risk for enhanced disease following natural infection [1]. The propensity for FI-RSV and other subunit RSV vaccines to prime for enhanced pulmonary inflammation, characterized by Th2-biased responses and neutrophilic or eosinophilic infiltrations after challenge was reproduced by several groups in different animal models [2-6]. The induction of a balanced cellular immune response is therefore critical for any candidate RSV vaccine. Adjuvants with the capacity to steer away from Th2-biased immune responses have recently been demonstrated to abrogate the aberrant pulmonary priming induced by subunit RSV vaccines [7-10]. In particular, Toll-like receptor (TLR) adjuvants have the potential to prime for more balanced, antigen-specific Th1-Th2 responses [11,12].

The innate inflammatory cytokines elicited by the TLR signal transduction cascade initiate the up-regulation of a series of events leading to phagocytosis-mediated antigen-presentation which can lead to antigen-specific Th1 lymphocyte activation [13,14]. These cytokines link innate and adaptive immunity through regulation of the antigen-presentation machinery and co-stimulatory molecule expression leading to dendritic cell (DC) maturation and migration from the site of infection or immunization to regional lymphoid organs [15-17]. TLR signaling can direct T cell help via activated DCs [18,19] and B cell activation and the promotion of immunoglobulin class switching are also modulated by TLR signals [20,21]. Two key inflammatory cytokines, IFN-β and TNFα,

are known to restrict RSV replication in human respiratory tract epithelium in vitro [22]. The former can be elicited following TLR4- or TLR3-mediated, MyD88-independent IRF-3 up-regulation via the adapter proteins TRAM (TLR4) or TRIF (TLR3) [23]. The latter can be produced in response to ligation of either TLR2 or TLR4 via MyD88-dependent signaling pathways or TLR4 ligation with MyD88-independent signaling [24,25]. Another proinflammatory cytokine, IL-12p40, is elicited following the ligation of various TLRs, including TLR2 and TLR4 [26]. IL-12 is implicated in the downstream production of IFNγ and is central to the differentiation of Th1 cells [27,28]. In RSV infection, IL-12 likely plays an important role in modulating pulmonary inflammation by favoring the development of Th1 responses [29].

Protollin is a potent adjuvant composed of hydrophobic complexes of *Neisseria meningitidis* outer membrane protein (OMP) and gram-negative *Shigella flexneri* lipopolysaccharide (LPS) [30], Neisseria spp OMP porin B (PorB) interacts specifically with a TLR2/TLR1 heterodimer [31] and bacterial LPS is the most important ligand for TLR4, causing the homodimerization of this receptor in the presence of MD-2 [32]. Intranasal (IN) immunization studies combining Protollin with subunit antigens from various bacterial and viral sources have demonstrated the capacity of this adjuvant to elicit strong mucosal and systemic antibodies in the context of a balanced Th1/Th2 response [33-35]. We [8,9] and others [10] have recently shown that RSV subunit antigens given with OMP/LPS can elicit complete protection without IL-5-driven eosinophil recruitment in mice. It is interesting that RSV antigens given alone tend to elicit Th2-biased responses [8-10] and fail to provide complete protection despite the fact

that the RSV F protein has recently been recognized as a TLR4 ligand [36,37]. In our studies using enhanced RSV antigen (eRSV, [8]), Protollin-eRSV elicits sharp increases in the ratio of antigen-specific IFNγ:IL5 production as well as increased IL-10 production. Among its many known functions, IL-10 is important in the maintenance of lung homeostasis [38] and the promotion of B-cell development [39]. In light of these observations, we reasoned that full protection and the prevention of Th2-driven aberrant responses following subunit RSV vaccination were mediated by individual or combined OMP or LPS ligation of their respective innate receptors (ie: TLR2 or TLR4). The recent finding that default Th2-cell development occurs in the absence of intact MyD88 signaling [40] strengthened our hypothesis that TLR2 or TLR4 mediated MyD88-dependant signaling was likely to be pivotal in the prevention of enhanced pulmonary pathology in Protollin-eRSV immunized and challenged mice.

We used TLR2, TLR4, MyD88 knock-out (-/-) mice and their wild-type (wt) littermates, to demonstrate that the LPS component of Protollin up-regulates early innate immune events such as TNFα, IFN-β and co-stimulatory molecule expression via the TLR4/MyD88-independent signaling pathway. We further demonstrate that the absence of Th2-skewed, antigen-specific cytokine responses and protection were mediated by a TLR4/MyD88-dependant mechanism. Finally, we show that pulmonary granulocyte recruitment following immunization and challenge is controlled primarily by MyD88-dependent signaling. These results suggest that Protollin-TLR4 interactions are crucial for Protollin-eRSV mediated protection and that MyD88 signaling is required for the

prevention of enhanced Th2-responses and eosinophilic pulmonary pathology in the murine model of subunit RSV immunization.

4.4. Materials and Methods

4.4.1. Mouse models

The knockout mouse strains (TLR2-/-, TLR4-/- and MyD88-/-) were generated by Dr. S. Akira, Osaka University, Japan and were backcrossed onto the C57BL/6 background for ≥8 generations before use. They were maintained and bred according to the Canadian Council on Animal Care guidelines. MyD88 +/+ or +/- wt littermates were used as controls for all studies.

4.4.2. Stimulation of innate cytokine production by Protollin-eRSV components

To study the capacity of the components of our Protollin-eRSV vaccine to elicit early, proinflammatory cytokine production, spleens from three naïve TLR2-/-, TLR4-/-, MyD88-/- and wt mice were collected following euthanasia and processed individually to isolate splenocytes as previously described [41]. Briefly, washed spleens were passaged through a 100μm mesh (BD Biosciences) using sterile 3mL syringe plungers. Following lysis of RBCs and washing, the cells were resuspended in RPMI supplemented with 4% FBS, 2mM glutamine, 50μg/mL gentamicin (all Invitrogen) and 50mM β-mercaptoethanol (Sigma, Oakville ON) (cRPMI) and then counted. Fresh splenocytes were seeded at a final concentration of $2x10^6$ cells/mL with Protollin (0.01 μg/ml), eRSV (2μg/ml) or purified *S. flexneri* LPS (0.01 μg/ml) and incubated for 16 hours at 37°C with 5% CO_2. Supernatants were analyzed by ELISA for IFN-β (PBL InterferonSource, Piscataway, NJ) and TNFα and IL-12p40 (BD Biosciences, Oakville, ON) according to

manufacturer's instructions. Unless otherwise stated, all cytokine results are expressed as mean ± SEM of three individual mouse results per group.

4.4.3. Immunization schedule and RSV challenge

Equal numbers of males and females aged 6-12 week old were chosen from each strain plus wt controls for a total of six to ten mice per group. The mice were lightly anesthetized by isoflurane inhalation and immunized IN in a total volume of 16-20μl (8-10 μl/nare) twice on days 0 and 21 with Protollin-eRSV (10 and 5 μg respectively), prepared as previously described [8]. Two weeks post-boost, half the mice per group were challenged IN with 10^7 $TCID_{50}$ of Long strain RSV (ATCC# VR-26, Bethesda, MD) in 50 μl following anesthesia as above. Serum, bronchoalveolar lavage (BAL), spleens and lungs (histopathology) were collected from the remaining mice at this time. Challenged mice were euthanized 4 days post-challenge and broncheoalveolar lavage (BAL) and lungs were collected for leukocyte differential counts or virus titration and lung histopathology, respectively. All samples were processed individually.

4.4.4. Determination of co-stimulatory molecule up-regulation

Immunized mouse spleens were collected as above and splenocytes were prepared as for the naïve mice splenocytes and frozen at -150°C. Thawed splenocytes were placed in culture overnight in cRPMI and surface-labeled with allophycocyanin (APC)-conjugated hamster anti-mouse CD11c and either R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD40 or fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD86 (B7-2) monoclonal antibodies (all BD Biosciences, Oakville, ON) and analyzed using a

FACSCalibur flow cytometer. The percentage of gated CD11c+CD40+ and CD11c+CD86+ cells were compared between the groups. Data were analyzed using the CellQuest software (BD Biosciences).

4.4.5. Antibody titer determination

Antigen-specific IgG, IgG1,IgG2a and IgA antibody titers were determined on individual serum (IgGs) and BAL (IgA) samples by ELISA as previously described [42]. Briefly, 96-well plates were coated with 7.5μg/ml of PEG-concentrated, UV-inactivated RSV [43] and incubated overnight at 4°C. Serum and BAL samples were serially diluted in blocking buffer starting at 1:50 (IgG) or 1:2 (IgA). Bound antibody was detected with HRP-conjugated anti-mouse IgG, IgA (Sigma) or IgG1, IgG2a (Southern Biotech, Birmingham, AL). Results are reported as mean ± SEM based on extrapolation of immunoglobulin concentrations from standard curves. Statistical analyses of normalized data were performed by one-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test.

4.4.6. RSV antigen-specific T-helper cell cytokine production

Spleens from Protollin-eRSV immunized mice were processed for *in vitro* re-stimulation as above. Fresh cells were seeded at a concentration of $2x10^6$ cells/mL and incubated in cRPMI alone, with purified inactivated RSV (1 μg/mL for IL-5 and IL-10 and 9μg/mL for IFNγ: Biodesign International, Brockville, ON) or 0.5μg/mL of Concavalin (Sigma) for 72 hours. The plates were then centrifuged, and the supernatants harvested and stored at -80°C until assayed for IFNγ, IL-5 and IL-10 by ELISA (BD Biosciences) as per the

manufacturers' instructions. For intracellular IFNγ analysis, frozen and thawed splenocytes from immunized mice were restimulated with 1μg/mL of purified inactivated RSV (Hytest, Turku, Finland) in cRPMI for 48h. BD GolgiPlug™ (BD Pharmingen) was added 4h prior to harvest as per manufacturers instructions. Cells were washed and surface stained with allophycocyanin (APC)-conjugated rat anti-mouse CD8a and PerCP-CY5.5 conjugated rat anti-mouse CD4 (L3T4) monoclonal antibodies (BD Pharmingen) and incubated for 20 minutes at room temperature. Cells were then washed, permeabilized and fixed with BD Cytofix/Cytoperm (BD Pharmingen), stained intracellularly with fluorescein isothyocyanate (FITC) -conjugated rat anti-mouse IFNγ monoclonal antibody, washed in Perm/Wash buffer (BD Pharmingen) and kept at 4°C until flow cytometer analysis. The percentage of gated CD8+IFNγ+ and CD4+IFNγ+ cells were compared between the groups. Data were analyzed using the CellQuest software (BD Biosciences).

4.4.7.Lung viral titration

Lungs from immunized animals were washed, weighed and homogenized individually in RSV media (D- MEM with 50% 199-H media, 0.5% fetal bovine serum , 2mM glutamine and 50μg/mL gentamicin (all Invitrogen, Burlington, ON)) with an automated Potter homogenizer (Fisher, Nepean ON), then centrifuged at 2655 x g for 2 minutes at 4°C. The supernatants were titered as previously described [8]. Briefly, lung homogenates were serially diluted in eight replicates starting at a 1:10 on a previously seeded Vero cell (ATCC# CCL-81) monolayer in 96-well plates and incubated for 6 days. RSV was detected by indirect immunofluorescence using a 1:400 dilution of goat anti-RSV antibody (Biodesign International, Brockville, ON) followed by a 1:400 dilution of anti-

goat IgG FITC conjugate (Sigma). A well was considered positive when ≥1 fluorescent syncytium was detected. The 50% tissue culture infective dose ($TCID_{50}$) calculations were performed using the Reed-Muench method [43] and normalized per gram of lung tissue for each individual mice.

4.4.8. BAL leukocyte differential counts

BAL fluids were collected and processed as previously described [8] and the pellets were resuspended in 500µl of PBS. 100µL of the resuspension was used for the cytospin (Thermoelectron Co, by Fisher, Nepean ON). Cells were fixed and stained with hematoxilin and eosin (H & E) (Sigma) [43], enumerated using a hemocytometer (40X) and a manual differential count was performed on 300 cells. Eosinophils, neutrophils, macrophages/monocytes and lymphocytes were differentiated based on their characteristic morphologies. Percentages of eosinophils and neutrophils were calculated for individual mice.

4.5. Results

4.5.1. Stimulation of innate cytokine production by Protollin-eRSV components.

Early innate cytokine production in response to vaccine components has the potential to efficiently influence subsequent adaptive responses. We investigated the ability of the isolated vaccine components (eg: Protollin, eRSV or LPS alone) to elicit inflammatory cytokine production by splenocytes of naïve TLR2-/-, TLR4-/-, MyD88-/- and wt animals to better understand events occurring immediately following immunization. Splenocytes from all groups except in the TLR4-/- animals significantly increased IFN-β production following both Protollin and LPS stimulation (Figure 1). IFN-β levels were also reduced in TLR4-/- splenocytes following eRSV stimulation compared to wt, possibly due to the absence of a direct interaction between RSV and TLR4 , but this difference did not reach statistical significance. These data suggest that the LPS component of Protollin can induce early IFN-β production via the MyD88-independent pathway (Figure 1a).

The induction of TNFα was also decreased in the TLR4-/- animals following Protollin stimulation compared to the wt group (eg: 74 ± 18ng/ml and 443 ± 65ng/mL respectively, $p \leq 0.05$). Although the difference compared to the wt group was not significant, Protollin-induced TNFα production was also decreased in the TLR2-/- group (174 ± 7ng/mL) suggesting the OMP ligation of TLR2 can also contribute to the production of this cytokine (Figure 1b). TNFα production in response to LPS was decreased in TLR4-/- splenocytes compared to both TLR2-/- and wt groups (TLR4-/-, 48 ± 26 ng/mL, TLR2-/-, 270 ± 17ng/mL; wt, 284 ± 64 ng/mL; ($p \leq 0.05$ for both)), providing further evidence for the hypothesis that Protollin interacts with both TLR2 and TLR4. Perhaps not surprisingly as eRSV is relatively crude, it elicited the highest levels of TNFα of all the

vaccine components and there were no significant differences between wt splenocyte production and the knock-out groups. Unexpectedly, however, TNFα levels were not significantly reduced in the MyD88-/- group following stimulation with any vaccine components. Although most of the TNFα in other systems is MyD88-dependent [13], recent findings have demonstrated that MyD88-independent expression of TNFα and time-delayed NF-kappaB upregulation required IRF-3 signaling [24]. Our results corroborate these findings and suggest that contrary to TLR4, MyD88 is not required for LPS or Protollin-mediated TNFα production.

The pattern of splenocyte IL-12p40 production following Protollin, eRSV or LPS stimulation was reduced in all knock-out groups compared to wt, but differences between the groups did not reach statistical significance (Figure 1c). Once again, the eRSV component consistently elicited the greatest IL-12p40 response compared to Protollin and LPS in all groups. Together, these results suggest that the early release of IFN-β and much of the initial TNFα response elicited by Protollin-eRSV vaccine are attributable to the LPS component and are mediated by the TLR4/MyD88-independent pathway.

4.5.2. Expression of co-stimulatory molecules following Protollin-eRSV vaccination. Inflammatory cytokines promote the up-regulation of co-stimulatory molecules on APCs. [44,45]. APCs were identified within the splenic cell populations by the CD11c marker present on a number of different leukocytes, mainly DCs, macrophage, granulocytes and NK cells. Consistent with the IFN-β and TNFα production profiles, the percentages of CD11c+ cells expressing both CD40 and CD86 cells were reduced vs. the wt group on

TLR4-/- mice splenocytes (Figure 2). For example, CD40 expression on CD11c+ splenocytes was almost halved in the immunized TLR4-/- mice compared to the wt group (4.5 ± 0.6 % vs 7.1 ± 0.9 % respectively: $p \leq 0.05$). Here again, CD11c+ splenocytes from MyD88-/- mice displayed similar expression of CD40 (7.5 ± 0.2 %) and CD86 (6.3 ± 0.3 %) than wt mice splenocytes (CD86: 6.3 ± 0.8 %) and this expression was statistically higher than those of the TLR4-/- group (CD40: $p \leq 0.01$ and CD86 (TLR4; 3.9 ± 0.5 %):$p \leq 0.05$). These results suggest that the TLR4/MyD88-independent pathway is important for up-regulation of co-stimulatory molecule expression on splenocytes following Protollin-eRSV vaccination.

4.5.3. Influence of TLR on Adaptive Immunity following Protollin-eRSV vaccination.

4.5.3.1. Helper T cell cytokine production pattern

Helper T-cell stimulation by APCs activated by the presence of type-1 inflammatory cytokines favours the development of committed Th1 cells [46]. We have previously shown that splenocytes isolated from BALBc mice immunized with eRSV alone produce relatively low levels of IFNγ and much larger amounts of IL-5 in response to re-stimulation with viral antigens compared to immunization with Protollin-eRSV [8]. In the current work, we replicated our prior observation that formulation of the eRSV antigen with Protollin was sufficient to enhance splenocyte IFNγ production and completely eliminate IL-5 production in response to RSV antigen restimulation (Figure 3). This Th1 immunomodulatory effect of Protollin was unchanged in the TLR2-/- mice but was markedly reduced in splenocytes isolated from immunized TLR4-/- and MyD88-/-

groups. Levels of RSV antigen-specific IFNγ production were significantly decreased in both TLR4-/- (0.48 ± 0.16 μg/mL) and MyD88-/- groups (0.37 ± 0.026μg/mL) compared to both TLR2-/- and wt mice (6.1 ± 3.0μg/mL and 6.2 ± 2.4 μg/mL respectively: Figure 3a. RSV antigen-specific IL-5 production, on the other hand, was generally increased in the TLR4-/- (32 ± 11 ng/mL) and MyD88-/- groups (99 ± 57ng/mL) although these differences only reached statistical significance for the MyD88 vs wt comparison (detection limit, 16 ± 0 ng/mL, $p \leq 0.05$), (Figure 3b). The production of IL-10 following RSV antigen restimulation was significantly reduced only in the splenocytes isolated from MyD88-/- animals (31 ± 0 ng/mL vs 199 ± 58 ng/mL in wt: $p \leq 0.01$)(Figure 3c).

To further assess the possible requirement for intact TLR4 and MyD88 signaling pathways to promote CD4+ T-helper cell polarization towards IFNγ production and the type-1 phenotype, FACS analysis of RSV antigen-restimulated splenocytes was performed. The percentage of CD4+IFNγ+ T cells (Figure 3d), but not CD8+IFNγ+ T cells were reduced only in MyD88-/- mice compared to the wt group (2.5 ± 0.1% vs 4.4 ± 0.9%, respectively, $p \leq 0.05$: data not shown). These results suggest that preferential commitment of RSV-specific helper T-cells to a Th1 phenotype following Protollin-eRSV immunization occurs via the pivotal MyD88 adaptor protein that can prevent Th2 cell development [40].

4.5.3.2. Antigen-specific antibody responses

Protollin-eRSV vaccine elicited similar total RSV-specific serum IgG responses in the wt and TLR2-/- groups (Figure 4a). The immunized TLR4-/- group had significantly

reduced titers (4.2 ± 2.7μg/mL) compared to wt (33 ± 11μg/mL, $p \leq 0.05$). The MyD88-/- mice also had lower titers overall compared to wt (7.7 ± 3.5μg/ml), but this difference did not reach statistical significance. Although the absence of the MyD88 pathway did not have a significant effect on total IgG production, the ratio of RSV-specific IgG1/IgG2a was dramatically different in the MyD88-/- mice compared to all other groups (Figure 4b). The MyD88-/- group had much higher IgG1/IgG2a ratios (255 compared vs 5-15 in other groups figure 4b), mainly due to at least 10-fold higher concentrations of IgG1 and background levels of IgG2a following Protollin-eRSV immunization compared to other groups (data not shown). These results corroborate the recent observation that MyD88 is crucial to antibody isotype switching from Th2-type pattern (IgG1) to a Th1-type pattern (IgG2a) [47]. RSV antigen-specific IgA responses in the BAL elicited by Protollin-eRSV were low after two immunizations and there were no significant differences between the groups (data not shown).

4.5.4. Protection from challenge and lung pathology

As we have recently demonstrated [8], two doses of Protollin-eRSV were sufficient to protect 100% of the wt animals from challenge (Figure 5). The TLR2-/- animals were also almost completely protected (90%: Figure 5a). In contrast, the protection conferred by vaccination was significantly reduced in both the TLR4-/- and MyD88-/- groups ($p \leq$ 0.001 and 0.05 respectively vs wt). Specifically, the majority of the TLR4-/- (70%) and MyD88-/- (56%) mice immunized with Protollin-eRSV were incapable of clearing RSV. The leukocyte differential counts in the immunized and challenged animals provided striking evidence for a pivotal role of MyD88 signaling in the induction of a balanced

Th1:Th2 response to RSV antigens. In our own studies [8,9] and those of those of others [10,48,49], mice exposed to subunit RSV antigen alone and subsequently challenged typically elicit a strong Th2-biased response with eosinophilic infiltrates in the lungs. We have recently shown that this effect can be completely abrogated by combining eRSV antigens with Protollin [8,9]. This latter observation was replicated in the current work and wt mice had very few eosinophils in the BAL (0.30 ± 0.09%). In sharp contrast, the BAL of both MyD88-/- and, to a lesser extent, TLR4-/- mice contained much higher percentages of eosinophils than the wt group (31 ± 6.8%: $p \leq 0.001$ and 1.30 ± 0.56%: $p \leq 0.05$ respectively). Surprisingly, the levels of neutrophils, recruited to the lungs following RSV challenge were markedly reduced in the MyD88-/- mice to levels comparable as those observed in unvaccinated and challenged mice (both < 2% compared to the wt group in our study, 18 ± 2.7%, $p \leq 0.001$).

4.6. Discussion

TLR ligands are among the most promising new tools for vaccine development, including molecules long recognized as powerful adjuvants without full understanding (eg: LPS) as well as newly-discovered molecules with adjuvant properties (eg: Por B). To date, most attempts to harness the potential of TLR ligands to create better vaccines have focused on the development of systemic immunity. Several recent reports describe the adjuvant-mediated activation of TLR via the intranasal route and their efficacy against various pathogens [50-52]. We hereby provide evidence that intranasal adjuvants that target TLRs can successfully elicit balanced antibody and cell-mediated immunity and effectively protect against RSV challenge. We have recently demonstrated that a subunit RSV antigen can protect against challenge when combined with Protollin [8,9], an intranasal adjuvant containing both PorB (TLR2) and LPS (TLR4). In the current work, we exploited defined TLR knock-out (-/-) mice to show that both this protection and avoidance of Th2-biased responses that have historically plagued RSV vaccine development are mediated by the TLR4/MyD88-dependent signalling pathway.

Although optimal protection following Protollin-eRSV vaccination was mediated by the TLR4/MyD88-dependant pathway, it is likely that some of the earliest effects of Protollin use other signalling pathways as well. IFN-β production by splenocytes isolated from RSV antigen-naïve mice was completely abrogated only in the TLR4-/- mice but TNFα production was reduced in both TLR2 and TLR4-/- animals. The observation that TNFα reductions only reached statistical significance in TLR4-/- mice may reflect either the greater versatility of the pathways downstream of TLR4, greater accessibility of Protollin

LPS to its receptor compared to Protollin OMP or different intrinsic potencies of these pathways to influence TNFα production. Our findings are consistent with the observation that TLR4 can lead to NF-κB up-regulation in the absence of MyD88 [24,53]. It is possible that the apparent lack of requirement for MyD88 in early innate events following Protollin or LPS stimulation (vs later events) is a refection of the kinetics or efficacy of the recruitment of the sorting adaptor proteins TIRAP and TRAM [54]. The requirement for PIP2 or Myr targeting to the plasma membrane may occur with dissimilar efficiency rates in different cell types or at different stages during immune cell maturation [55]. The reduction in splenocyte IL-12p40 production observed in all knock-out models following Protollin or eRSV stimulation may give insight into another aspect of the mechanism of Protollin-eRSV action. In addition to promoting Th1 cells following TLR ligation, IL-12 has been linked to the control of RSV-induced airway inflammation as distinct from viral clearance [29]. More specifically, IL-12p40 and IL-18 can limit lung inflammation and Th2 effector molecule production following murine RSV infection [29]. Our results are consistent with this observation in that no correlation was observed between the levels of IL-12p40 elicited and the degree of protection observed following vaccination and challenge in the knock-out mice.

Cytokine-driven dendritic cell maturation, co-stimulatory and MHC molecule up-regulation and migration to the lymph nodes are critical to the integrated function of the innate and acquired immune systems. Our results suggest that co-stimulatory molecule up-regulation on APCs, including DCs, following Protollin-eRSV immunization occurs via TLR4/MyD88-independent pathways. This observation is consistent with the

previous report that direct stimulation of DCs isolated from MyD88-/- mice with TNFα can promote CD86 surface molecule expression [56]. Although TNFα levels in response to the Protollin-eRSV vaccine components were also reduced in the TLR2-/- mice in our study, the primary up-regulation of TNFα production required intact TLR4 signalling using either of the pathways downstream from this receptor. It is interesting that up-regulation of antigen presentation via the MHC-II pathway leading to antigen-specific CD4+ T cell priming was recently demonstrated to occur only when the TLR agonist and antigen are present on the same particle and internalized in the same endocytic compartment [44]. This observation suggests that even though Protollin and eRSV were mixed together immediately prior to vaccination, they may associate by electrostatic or hydrophobic forces or be in close enough proximity to facilitate their simultaneous uptake into the same phagocytic compartment. Such a close physical association is suggested by immunogold localization studies for both RSV [8] and other viral surface glycoproteins combined with Protosome-based adjuvants [33]. The contribution of the particulate nature of Protollin-based vaccines to their efficacy and the nature and degree of physical association between Protollin and viral antigens require further study.

Our observations with Protollin vaccines indicate that the priming of antigen-specific type-1 T-helper cells is optimal only in the presence of the MyD88 adaptor protein, consistent with the findings of other groups [40,56]. The shift in IFNγ to IL-5 cytokine ratios towards Th2 responses observed with the Protollin-eRSV vaccine in MyD88-deficient mice suggests that this adaptor is crucial to the up-regulation of Th1 responses. Although restimulated splenocytes from immunized TLR4-/- and MyD88-/- mice

produced significantly less supernatant IFNγ than wt in response to RSV antigens, only the immunized MyD88-/- mice had lower percentages of restimulated CD4+ IFNγ+ T cells compared to wt, further suggesting the pivotal role of this adaptor protein in contributing to Th1 responses. Future immunophenotyping studies on cells isolated from draining lymph nodes following RSV challenge will likely provide a more accurate picture of the precise role of the TLR4 and TLR2 signalling pathways in promoting the balanced Th1/Th2 response that we observe.

The impact of removing TLR signaling pathways on the magnitude and pattern of the antibody response following Protollin-eRSV vaccination was most striking in TLR4 and MyD88-/- mice, respectively. While TLR4-deficient animals were the only group in which significantly lower antigen-specific total serum IgG responses were elicited, MyD88-deficient mice generated much higher levels of IgG1, contributing to a strikingly elevated IgG1/IgG2a ratio. These results are consistent with previous reports suggesting that MyD88 is important for antibody class switching recombination events [47,57]. It is interesting that Protollin-eRSV immunized MyD88-deficient mice were the only group which elicited significantly less IL-10 in restimulated splenocytes supernatants. The role of IL-10 in promoting activated or memory B lymphocyte differentiation and growth has clearly been established [39,58,59]. However, it remains unclear whether or not IL-10 is related to antigen-specific IgG2a isotype switching. Although it is likely that the impaired IgG2a production in the MyD88-/- group was related to lower IFNγ production [60,61], there may be a combined negative effect of simultaneous reductions of both IFNγ and IL-

10 production. Nevertheless, the high IgG1/IgG2a ratios observed in MyD88-/- mice further indicates the requirement of this adaptor protein for Th1-commitment.

The protection results obtained in Protollin-eRSV immunized TLR4-/- and MyD88-/- mice correlate well with our in vitro findings. The TLR4-/- group had incomplete protection, consistent with deficient up-regulation of TNFα, IFN-β and co-stimulatory molecules as well as impaired downstream promotion of antigen-specific T-helper 1 cell activation and antibody production. Although significantly impaired, protection was affected to a lesser extent in MyD88-/- mice, but the impact of the strong Th2 bias in these mice had major influence on granulocytic pulmonary infiltrations. Eosinophilia was prominent in MyD88-/- mice following challenge, suggesting the pivotal effect of this adaptor protein on controlling not only adaptive immunity but also lung infiltration following immunization and RSV challenge. These results demonstrate for the first time the impact of the MyD88-related signals on RSV vaccine-associated eosinophilic pulmonary inflammation in mice. Closely related to RSV immunopathology, whether in mediating inflammation in RSV bronchiolitis [62-66] or as a possible marker for enhanced pathology following FI-RSV immunization and natural infection [2], neutrophil recruitment was also drastically altered in MyD88-/- mice. To our knowledge, this is the first report indicating the role of the MyD88 adaptor protein in regulating neutrophil pulmonary recruitment following immunization and challenge. The neutrophil recruitment observed in mice immunized with Th2-preventing Protollin-eRSV and challenged cannot be attributed to the vaccine alone, since interstitial pneumonitis and alveolitis (both associated primarily with neutrophils) also occur after a secondary RSV

infection in the BALB/c model [67]. Moreover, neutrophil recruitment after challenge is not likely to be a unique effect of the Protollin adjuvant since it was also seen following challenge in animals immunized with the antigen alone (unpublished observations). Possible explanations for neutrophil recruitment following experimental challenge are the large volume and impurity of the challenge virus (concentrated infected-cell lysate) upon second live or subunit RSV contact. Although it is assumed that neutrophils are recruited as a result of the inflammatory cytokines released by infected cells, further studies are required to determine the mechanism underlying the priming for neutrophil activation in the context of RSV vaccination. The relationship that we have identified between the MyD88 adaptor protein and pulmonary neutrophil recruitment suggests several interesting avenues of research that may eventually clarify this question.

We have demonstrated that the initial response to intranasal Protollin-eRSV vaccine such as pro-inflammatory cytokine production and up-regulation of co-stimulatory molecule expression is driven primarily by an interaction between the LPS component and TLR4 via MyD88-independent signalling pathways. However, the induction of effective and Th1/Th2 balanced RSV-specific immunity following Prolollin-eRSV vaccination required intact TLR4/MyD88-dependant signalling. This latter pathway was also shown to be pivotal to avoid priming for Th2-driven pulmonary eosinophilic immunopathology that has historically been associated with subunit RSV vaccines. We also show, for the first time, that intact MyD88-dependant signalling is central to pulmonary neutrophil infiltration in the mouse model of RSV infection.

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4.8. Figure legends

Figure 1

Production of cytokines by splenocytes isolated from naïve TLR2-/-, TLR4-/-, MyD88-/- and wt mice and restimulated with Protollin-eRSV vaccine components: 0.01μg/mL of Protollin, 0.01μg/mL *Shigella flexneri* LPS or 2μg/mL of eRSV alone. Supernatants were collected after 16h culture and cytokine levels IFN-β (a), TNFα (b) and IL-12p40 (c) were measured by ELISA. Results are shown as mean + SEM. Statistical analyses were performed by one-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test. A P-value of ≤ 0.05 (two-tail) was considered significant. Stars indicate difference with wt ($P < 0.05$,* $P < 0.01$, **)

Figure 2

Upregulation of co-stimulatory molecules by Protollin-eRSV. Splenocytes from immunized mice were cultured in complete RPMI media overnight, labeled with anti-CD11c, CD40 and CD86 and analyzed by FACS. Results are shown as mean of three individual mice per group ± SEM. Statistical analyses were performed by unpaired t test, comparing TLR4-/- with wt, A P-value of ≤ 0.05 (two-tail) was considered significant. These data are representative of at least two separate experiments.

Figure 3

Splenocytes from Protollin-eRSV immunized mice were restimulated *in vitro* with purified inactivated RSV. Cytokines IL-5 (a), IFNγ (b) and IL-10 (c) were quantified in

the supernatants by ELISA following a 72h incubation. (d) CD4+ T-cell intracellular IFNγ staining. Percentage of CD4+IFNγ+ gated following 48h incubation with purified inactivated RSV. These data are representative of at least two separate experiments. Results are reported as mean of three individual mice per group ± SEM. Statistical analyses were performed by unpaired t test, comparing the individual groups with wt. A *P*-value of ≤ 0.05 (two-tail) was considered significant. $P < 0.05$,* $P < 0.01$, **.

Figure 4

RSV-specific serum IgG titers (a) and IgG1/IgG2a ratios (b) following Protollin-eRSV immunization. RSV-specific serum IgG, IgG1 and IgG2a were quantified by ELISA. IgG results are shown as the mean ± SEM. Statistical analyses were performed by one-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test. A *P*-value of ≤ 0.05 (two-tail) was considered significant. $P < 0.05$,*. IgG1/IgG2a ratios were calculated from geometric mean values for each group. These results are representative of at least two separate experiments.

Figure 5

Protection and pulmonary granulocytic infiltrates following Protollin-eRSV immunization and challenge in TLR2-/-, TLR4-/-, MyD88-/- and wt mice. a) Lung viral titers from Protollin-eRSV immunized and RSV challenged mice were performed on Vero cells and detected by immunofluorescence (reported as number per gram of lung homogenate). Percentages of granulocytes in individual mouse BAL following enumeration of eosinophils (b) or neutrophils (c). BAL enumerations are expressed as

percentages of all leukocytes. The bars represent the geometric mean of each group (data are representative of at least two separate experiments). Statistical analyses were performed by one-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test. A P-value of ≤ 0.05 (two-tail) was considered significant. $P < 0.05$,*; $P < 0.001$,***.

4.9. Figures

Figure 1

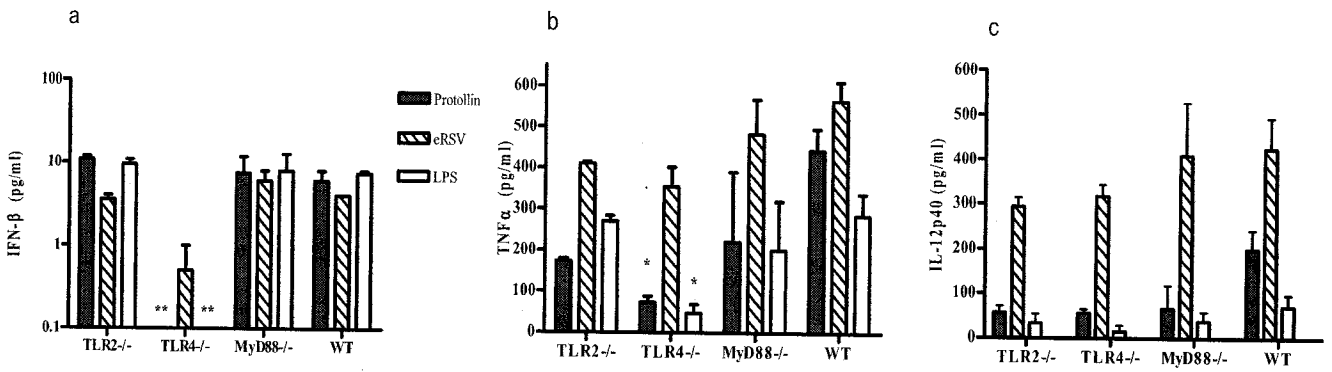
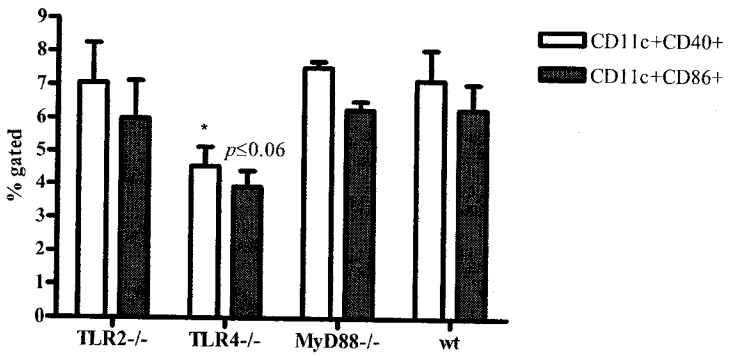


Figure 2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CD11c+CD86+
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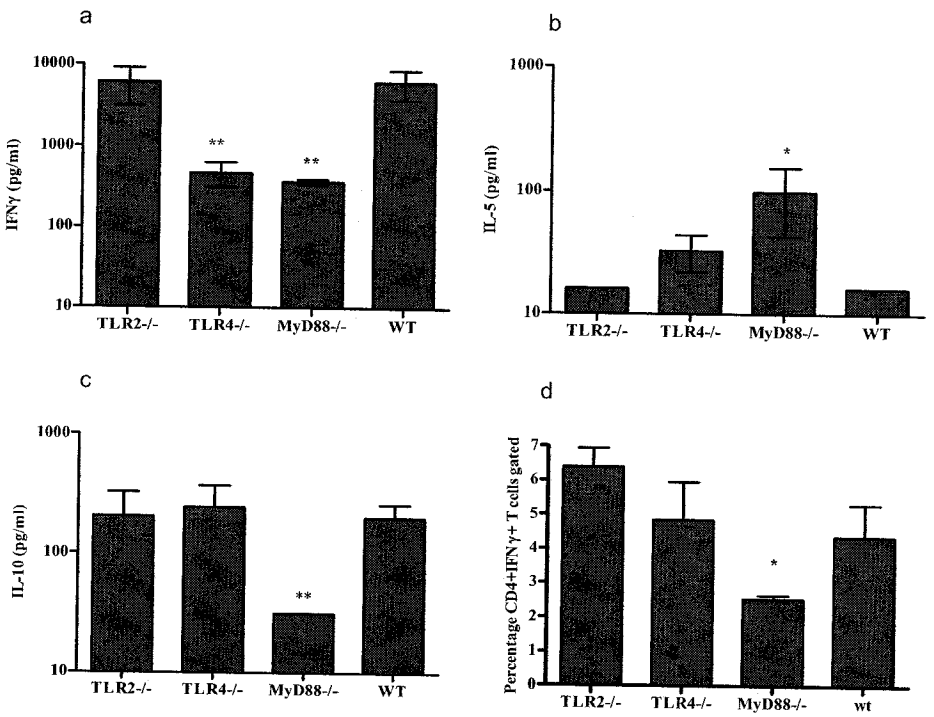
TLR2-/-
TLR4-/-
MyD88-/-
wt

Figure 3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TLR2-/-
TLR4-/-
MyD88-/-
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IL-10 (pg/ml)
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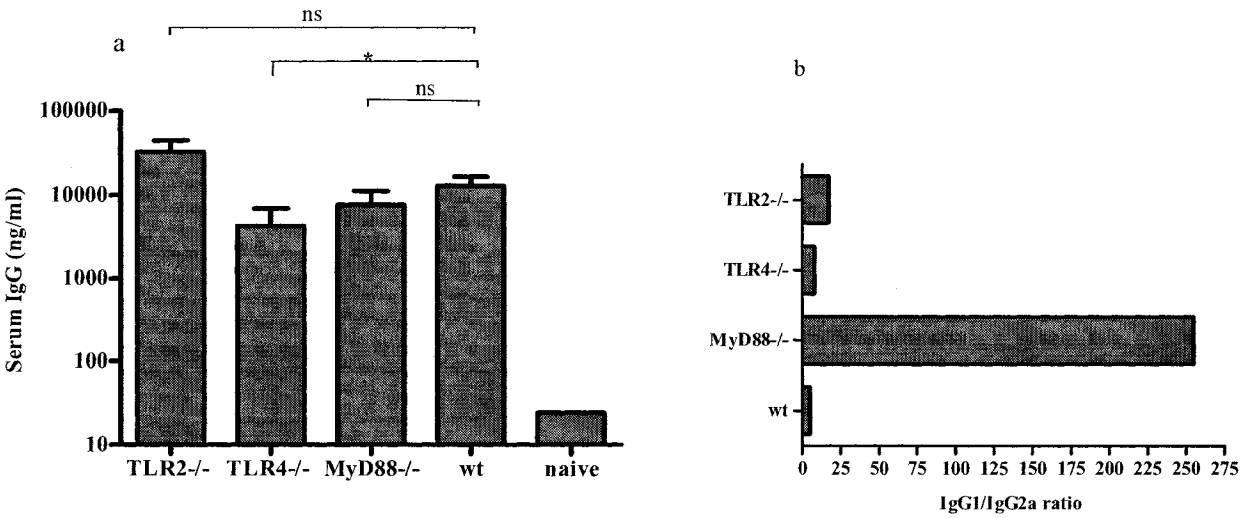
MyD88-/-
wt

Figure 4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TLR4-/-
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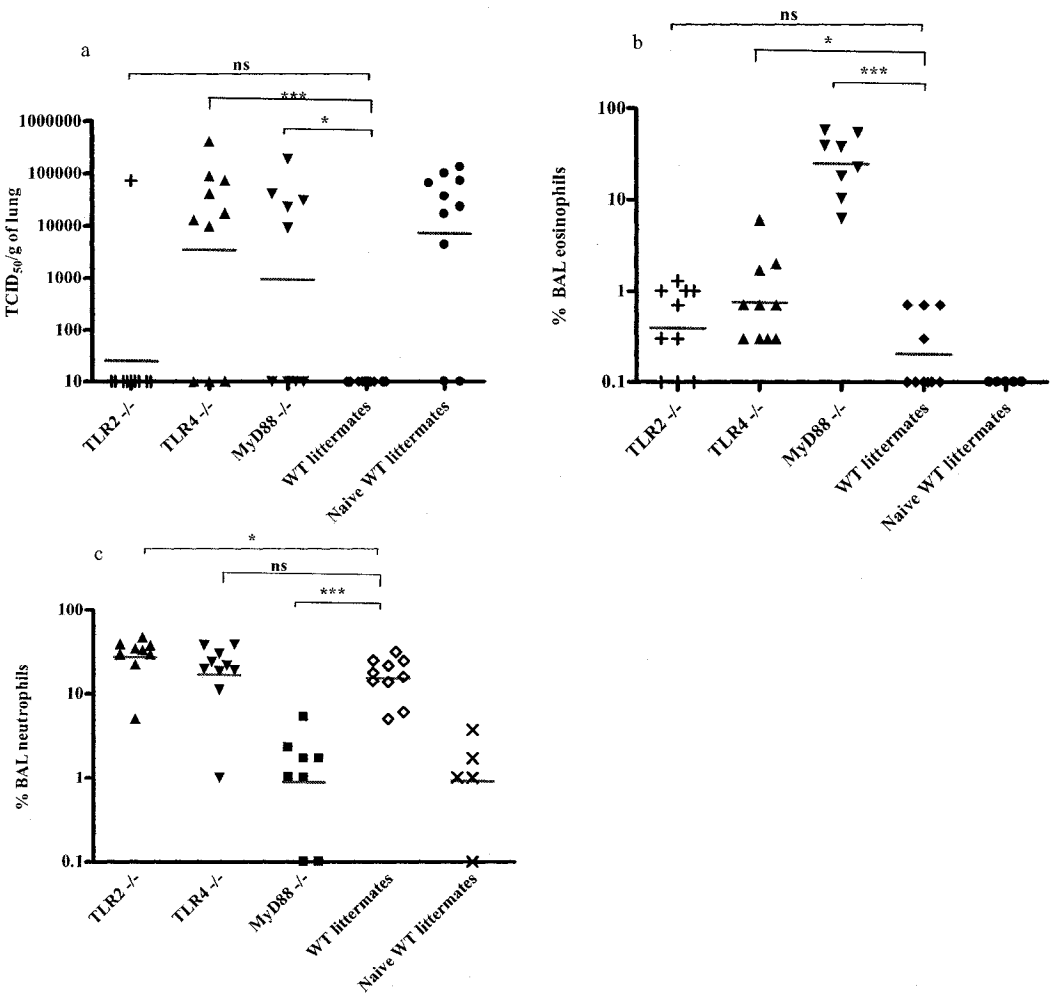
IgG1/IgG2a ratio

Figure 5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TLR4 -/-
MyD88 -/-
WT littermates
Naive WT littermates

Chapter 5

CD1d signaling and natural killer T (NKT) cell activation may contribute to the immunogenicity of proteosome-based Respiratory Syncytial Virus (RSV) vaccines in mice.

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5.1. Preface

The demonstration of the major contribution of TLR4 and MyD88 to the protective efficacy of the Protollin-eRSV vaccine in chapter 4 identified LPS as the key immune modulator in the Protollin-based vaccine. Although these results suggested that antigen presentation and T-helper cell polarization are highly dependent on the TLR4-MyD88 signalling pathway, antigen-specific antibody levels were not dramatically altered, especially in MyD88-/- mice. This observation suggested that another mechanism may contribute to immunogenicity. Recent findings had suggested that lipids, via CD1 presentation to T cells, can also play a pivotal role in inducing immunity. Combined with the confirmation that lipids co-purified with proteosome OMPs (personal communication with colleagues), we investigated the immunogenicity of both Proteosome- and Protollin-eRSV vaccines in CD1d-/- mice. Although these results demonstrate the possible role of the innate CD1d-NKT cells pathway on the mechanism of action of proteosome-based adjuvants, a repeat study with age-matched mice has not yet been performed. Reproduction in our CD1-/- breeding colony stopped abruptly and the number of animals available for study dwindled rapidly in the last months of the thesis work. A confirmatory study will be performed with age-matched mice in the coming months. However, the preliminary observation that proteosome-based adjuvants appear to utilize the CD1d-NKT cell pathway in a TLR-optimized manner to promote significantly stronger immune responses will certainly encourage the further development of this area of research for all adjuvants that include lipids.

5.2. Abstract

CD1d-associated lipid presentation to T cells has recently been recognized for its contribution to host-defense mechanisms and as a major pathway for rapid, MHC-unrestricted antibody responses to pathogens. Little is known about the potential role of CD1 ligation in vaccine adjuvants. Recent studies have shown that proteosomes (Pro) or Protollin (Prl) based-adjuvants, made of *N. meningitidis* outer membrane proteins, LPS (Prl only) and co-purified lipids were safe and effective in mice following intranasal (IN) immunization and challenge when formulated with enriched RSV (eRSV). In this study, we used Pro and Prl-based vaccines to immunize wildtype and BALB/c backcrossed C.129S2-*Cd1*$^{tm1/Gru}$ mice (CD1-/-), deficient in CD1d and CD1d-resticted natural killer T (NKT) cells. Although CD1-/- and BALB/c mice were protected from RSV challenge, CD1-/- mice displayed lower levels of antigen-specific serum IgG following Pro-eRSV immunization ($p \leq 0.01$) and bronchoalveolar lavage (BAL) IgA following both Pro-eRSV ($p \leq 0.05$) and Prl-eRSV ($p \leq 0.01$) immunization. IgG1 and IgG2a levels were also reduced in CD1-/- mice compared to wt following immunization with Pro-eRSV ($p \leq 0.001$ and $p \leq 0.01$, respectively) and Prl-eRSV ($p \leq 0.05$, IgG1 only). Moreover, levels of antigen-specific pro-inflammatory cytokines IFNγ and IL-17 as well as the B lymphocyte stimulating cytokine IL-10 were markedly reduced in the CD1-/- mice, but BAL neutrophil percentages were significantly higher following challenge (Prl-eRSV only, $p \leq 0.05$ vs. wt). Overall, these data suggest that CD1d-dependent NKT cells vigorously influence the activation of humoral and cell mediated immune responses elicited following immunization with proteosome-based eRSV vaccines as well as the regulation of pulmonary neutrophil recruitment after RSV challenge.

5.3. Introduction

The CD1 pathway is newly recognized as a lipid-recognition system that can identify modifications in host cell lipid content and promote consequential immune signals which influence the outcome of infectious, allergic, autoimmune and neoplastic conditions [1]. Five CD1 isoforms are present in humans (CD1a-e). All but CD1d require cell activation for expression and lipid presentation to T cells [2]. The constitutively expressed CD1d is also the only isoform found in mice and is required for the maturation of CD1d-restricted natural killer T (NKT) cells in the presence of DC-derived IL-12 [3]. The CD1d-NKT cell pathway may constitute an important innate mechanism contributing to the upregulation of antibody and cellular responses to diverse pathogens [4]. For example, NKT cells are recognized for their role in modulating NK cell maturation as well as T and B cell activation by secreting abundant quantities of IL-4, IL-10, IFNγ [5,6] and IL-17A (IL-17) [7,8]. Together with neutrophil-regulatory T cells (Tn: αβ CD4+ and γδ T cells) [9], a major subset of NKT cells called invariant NKT (iNKT) contribute to neutrophil regulation through IL-17 expression and the promotion of TNFα, IL-1β, IL-6, IL-8 and granulocyte colony-stimulating factor (G-CSF) release. [10,11]. The CD1d-NKT cell pathway has been shown to regulate immunogobulin G (IgG) responses to lipid-anchored surface antigens of Plasmodium and Trypanosoma species [4]. Despite evidence that the CD1d-NKT cell pathway may play a pivotal role in the modulation of both innate and adaptive immune responses to various immunomodulators, very little is known as to how CD1d ligation might contribute to the efficacy of vaccines that include lipids (eg: liposomes, proteosomes). Indications that α-galactosylceramide (α-GalCer),

the best known ligand to CD1, can be successfully used as an adjuvant have recently emerged. For example, α-GalCer was shown to increase the protective immunity induced by malaria [12] and influenza antigens given IN [13], as well as acting synergistically with monophosphoryl lipid A (MPLA) and TLR to increase immune cell proliferation kinetics and effector function [14].

Proteosome-based adjuvants are derived from chemically extracted *Neisseria meningitidis* outer membrane proteins (OMP) [15] and contain co-purified lipids, mainly phophatidylethanolamine (PE), known to be presented by CD1d [16], and phosphatidylglycerol (PG) (unpublished observations). The hydrophobic OMP extract can be formulated with a variety of antigens, including *Shigella flexneri* LPS and a range of viral antigens including enriched RSV antigens (eRSV)[17]. The former combination (OMP and LPS) results in the soluble adjuvant Protollin [18]. Intranasal vaccines composed of eRSV formulated with proteosomes or mixed with Protollin were recently demonstrated to be safe and effective in both BALB/c and C57Bl/6 mice. Furthermore, these vaccines elicited balanced Th1/Th2 immune responses without the risk of pulmonary eosinophilia or enhanced pathology [19]. Excess granulocytes, including neutrophils, are associated with RSV pulmonary disease [20] as well as formalin-inactivated RSV (FI-RSV) vaccine-enhanced disease histopathology following infection [21-23]. Animal vaccination models suggest that the enhanced pathology arose mainly as the result of intense neutrophilic and eosinophilic infiltrations and exacerbated Th2 responses, with contributions from RSV G protein itself, the formalin-inactivation process and the Th2-inducing alum-based adjuvant [24-26]. Using C57Bl/6 TLR knock-

out mice, we have recently demonstrated that Protollin-eRSV prevents Th2-biased immune pathologic responses and eosinophilia by a LPS/TLR4/MyD88-dependent mechanism [17] (Cyr et al, manuscript submitted). Interestingly, pulmonary neutrophil infiltration was found to be upregulated by a MyD88-dependant process following immunization and RSV challenge in this model (Cyr et al, manuscript submitted).

The role of lipids in proteosome-based adjuvant and the potential impact of CD1d-restricted NKT cells in mediating enhanced immune responses remain unexplored. In this study using wildtype (wt) and BALB/c backcrossed CD1d-deficient mice, we demonstrate that the CD1d-NKT-cells pathway contributes to increased levels of antigen-specific serum IgG, IgG1, IgG2a and BAL IgA responses following Protollin-eRSV immunization, but more markedly following Proteosome-eRSV immunization. The CD1d-NKT cell pathway may also contribute to the cellular immune responses elicited by both vaccines, through lower production of antigen-specific cytokines. Neutrophil recruitment to the lungs following challenge was augmented in Protollin-eRSV immunized CD1-/- mice.

5.4. Materials and Methods

5.4.1. Mouse models

Two breeding pairs of mice homozygous for the *Cd1*$^{tm1/Gru}$ targeted mutation, C.129S2-*Cd1*$^{tm1/Gru}$ (CD1-/-) mice, were obtained from The Jackson Laboratory (stock number 003814) and were maintained by homozygous sibling matings. CD1-/- mice were generated by targeted deletion of CD1.1 and CD1.2 genes encoding for CD1d1 and CD1d2 molecules, respectively, and backcrossed to BALB/c mice (N11) [27]. BALB/c control mice were purchased from Charles River Laboratory. All mice were maintained and bred (CD1-/- only) according to the Canadian Council on Animal Care guidelines. Mice bred well at a younger age and were otherwise healthy but appeared to display early signs of aging compared to wt mice (abnormal gait, hunched posture, excessive sibling fighting between males and failure to breed over time).

5.4.2. Immunizations and challenge

An equivalent number of males and females aged 6-12 week old were chosen from the CD1-/- strain. Aged-matched female BALB/c mice were used as controls for a total of seven mice per group. A recent study showed that the number of NKT cell levels may be more elevated in human females than males [28]. Therefore, as CD1-/- lack the NKT cell subset altogether, restraining the control group to females only may allow for less variability of responses within this group. The mice were lightly anesthetized by isoflurane inhalation and immunized IN in a total volume of 25µl (12.5 µl/nare) twice on days 0 and 21 with Protollin-eRSV (3 and 5 µg respectively) and formulated proteosome

(5μg, based on eRSV), prepared as previously described [19]. Two weeks post-boost, 4 mice per group were challenged IN with 10^7 $TCID_{50}$ of Long strain RSV (ATCC# VR-26) in 50 μl following anesthesia as above. Serum, bronchoalveolar lavage (BAL) and spleens were collected from the remaining mice at this time. Challenged mice were euthanized 4 days post-challenge and BAL and lungs were collected for leukocyte differentials and virus titration, respectively.

5.4.3. Antibody titer determination

Antigen-specific IgG, IgG1,IgG2a and IgA antibody titers were determined on individual serum (IgGs) and BAL (IgA) samples by ELISA as previously described [29]. Briefly, 96-well plates were coated with 7.5μg/ml of PEG-concentrated, UV-inactivated RSV [30] and incubated overnight at 4°C. Serum and BAL samples were serially diluted in blocking buffer starting at 1:50 and 1:2 (IgA) dilution. Bound antibody was detected with HRP-conjugated anti-mouse IgG, IgA (Sigma) or IgG1, IgG2a (Southern Biotech, Birmingham, AL). Unless otherwise indicated, titers are expressed as geometric mean concentrations with 95% confidence intervals (GMC [X:Y]).

5.4.4. Neutralization assay

Pooled sera from immunized animals were serially diluted from a starting dilution of 1:8 in RSV media in 96-well plates (20 μl/well). Control wells contained RSV media only or goat polyclonal anti-RSV antibody at 1:50 (Biodesign international). 500-1000 infectious doses of RSV Long strain were added, the plates were incubated for 20 minutes at 33°C and the mixture was transferred to 96-well flat-bottomed plates previously seeded with

$1x10^5$ cells/mL Vero cells. After 5-6 days at the same temperature, supernatants were removed; plates were washed with PBS and adhering cells fixed with 1% paraformaldehyde in PBS for 1 hour, followed by indirect immunofluorescence (see lung titration method).

5.4.5. T-helper cell cytokine production

Spleens from immunized mice were pooled per group and processed for *in vitro* re-stimulation as previously described [31]. Briefly, washed spleens were passaged through a 100μm mesh (BD Biosciences) using sterile 3mL syringe plungers. Following lysis of RBCs and washing, the cells were resuspended in RPMI supplemented with 4% FBS, 2mM glutamine, 50μg/mL gentamicin (all Invitrogen) and 50mM β-mercaptoethanol (Sigma) (cRPMI) and then counted. Fresh splenocytes were seeded at a final concentration of $2X10^6$ cells/mL in cRPMI alone or supplemented with increasing doses (1, 3 and 9μg/ml) of purified inactivated RSV (Biodesign International) or with increasing doses (0.1, 1.0, 10 μg/mL) of RSV F_{85-93} CD8+ T cell-restricted peptide KYKNAVTEL [32] or RSV $G_{183-197}$ CD4+ T cell-restricted peptide WAICKRIPNKKPGKK [33] and incubated for 72h hours at 37°C with 5% CO_2. The plates were then centrifuged, and the supernatants harvested and stored at -80°C until assayed for IFNγ, IL-5 and IL-10 by ELISA (BD Biosciences, Oakville, ON) and IL-17 (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

5.4.6. Lung viral titration

Lungs from immunized animals were washed, weighed and homogenized individually in RSV media with an automated Potter homogenizer (Fisher, Nepean ON), then

centrifuged at 2655 x g for 2 minutes at 4°C. The supernatants were titered as previously described [19]. Briefly, lung homogenates were serially diluted starting at a 1:10 dilution on a previously seeded Vero cell (ATCC# CCL-81) monolayer in 96-well plates and incubated for 6 days. RSV was detected by indirect immunofluorescence with goat anti-RSV antibody (Biodesign international) followed by anti-goat IgG FITC conjugate (Sigma). Wells were considered positive when ≥1 fluorescent syncytium was detected. The 50% tissue culture infective dose ($TCID_{50}$) calculations were performed using the Reed-Muench method [30].

5.4.7. BAL leukocyte differential counts

BAL fluids were collected and centrifuged as previously described [19] and the pellets were resuspended in 500μl of PBS. Briefly, BAL collection is performed by the canulation of the trachea100μl were used for the cytospin (Thermoelectron Co, by Fisher, Nepean ON). Cells were fixed and stained with hematoxilin and eosin (H & E) [30], enumerated using a hemocytometer (40X) and a manual differential count was performed on 300 cells. Eosinophils, neutrophils, macrophages/monocytes and lymphocytes were differentiated based on their characteristic morphologies. Percentages of leukocytes were calculated for individual mice and averaged for each group.

5.5. Results

5.5.1. RSV-specific Immunoglobulin titers

RSV-specific serum IgG, IgG1, IgG2a and BAL IgA levels were overall reduced in CD1-/- mice compared to wt following IN immunization with Proteosome-eRSV or Protollin-eRSV (Figure 1). Significant differences between CD1-/- and wt mice were observed for all parameters measured following Proteosome-eRSV immunizations. RSV-specific serum IgG and BAL IgA levels were reduced to 44 [36-53] μg/ml and 35 [24-51] ng/ml respectively, in CD1-/- compared to 151 [135-170] μg/ml ($p \leq 0.01$) and 183 [93-360] ng/ml ($p \leq 0.05$), respectively, in wt mice. Similarly, serum isotypes IgG1 and IgG2a were reduced significantly in CD1-/- mice immunized with Proteosome-eRSV compared to wt mice; 11 [10-12] vs. 105 [92-119] μg/ml (IgG1, $p \leq 0.001$) and 15 [8-27] vs. 79 [57-110] μg/ml (IgG2a, $p \leq 0.05$). IgG1/IgG2a ratios did not differ significantly between CD1-/- and wt groups, but ratios from CD1-/- mice tended to be slightly lower following immunization with either vaccine (data not shown). Although all Ig isotypes/isoforms were lower in CD1-/- than wt mice following immunizations with Protollin-eRSV, significant differences were observed only in serum IgG1 (25 [13-46] μg/ml) and BAL IgA (5 [3-8] ng/ml) compared to wt (85 [54-135] μg/ml, $p \leq 0.05$) and (36 [18-75] ng/ml $p \leq 0.01$, respectively). Neutralization titers were measured, but responses were low overall, and no differences were observed between the groups (data not shown).

5.5.2. RSV-specific cytokine titers

Distinctive splenocyte cytokine patterns were observed between CD1-/- and wt mice immunized with either Proteosome- or Protollin-eRSV following restimulation with inactivated RSV or individual RSV G or F peptides. In general, levels of IFNγ, IL-17 and IL-10 were reduced in CD1-/- mice in a dose-response manner following restimulation with inactivated RSV, regardless of the vaccine given (Figure 2A-C). Responses from F-restimulated splenocytes (Figure 2D-F) differed considerably from those elicited by RSV restimulation. All targeted cytokines were elicited only in wt mice given Protollin-eRSV, suggesting that only the T-helper cells of Protollin-eRSV immunized wt mice respond to *in vitro* F restimulation and that this response depends on *in vivo* priming by CD1 and NKT cells. Following G peptide restimulation, rapid increases in all three cytokine levels were observed in restimulated splenocytes from wt mice immunized with either vaccine (Figure 2G-I). Although IFNγ responses appear erratic after G restimulation (figure 2G), the responses were highest at the lowest dose of stimulant in the splenocytes supernatants of wt mice, and dropped quickly with increasing doses of stimulant, possibly due cellular "exhaustion" at the higher stimulant doses by the 72h collection time point. Higher levels of IL-17 were released from the G-restimulated splenocytes of wt animals than those of CD1-/- animals, but an important difference was only observed in Protollin-eRSV immunized animals (Figure 2H). Similarly, IL-10 production was abrogated in the splenocytes of CD1-/- mice immunized with either vaccines compared to the levels detected in the G-restimulated splenocytes of wt animals (Figure 2I). No IL-5 was detected in any supernatant (data not shown).

5.5.3. Protection

Despite having elicited lower levels of serum IgG and BAL IgA, CD1-/- mice were fully protected against RSV challenge, equivalent to wt mice (Figure 3). No viable RSV was detected in the lungs of any immunized group 4 days post-challenge. In contrast, naïve mice were entirely vulnerable to pulmonary RSV proliferation. These results suggest that CD1 and NKT cells, despite their impact on immunoglobulin production and Th-cytokine levels, are not absolutely required for protection.

5.5.4. Leukocytic pulmonary exudates

BAL leukocyte differentials following challenge were similar in all groups except for the CD1-/- group immunized with Protollin-eRSV (Figure 4). In that group, neutrophils were detected in significantly larger numbers than in wt mice immunized with the same vaccine ($p \leq 0.05$). As BAL differentials are calculated as percentages, the relative increase in neutrophils was accompanied by a decrease in both monocytes/macrophages and lymphocytes in that same group, although these differences were not significantly different. These results suggest that CD1-/- mice may be more susceptible to pulmonary neutrophil infiltrations following Protollin-eRSV but not Proteosome-eRSV immunization and challenge. Consistent with the absence of IL-5 production, no eosinophils were detected in any of the groups following challenge.

5.6. Discussion

Although the CD1d-NKT cell pathway can potentially contribute to the activation of innate responses and the regulation of subsequent adaptive responses [3,4,34], the role of this pathway in the actions of lipid-containing vaccine adjuvants has never been assessed. We have recently demonstrated the safety and efficacy of the intranasal proteosome-based eRSV vaccines in C57Bl/6 and BALB/c mice [17,19]. This model offered the opportunity to explore the role of the CD1d-NKT cell pathway in the immunogenicity and protection provided by our adjuvanted RSV vaccines. We hereby report that the CD1d-NKT cells pathway may contribute to systemic and mucosal antigen-specific antibody responses as well as T-helper cell pro-inflammatory cytokine responses following immunization with proteosome-based vaccines. Although systemic and mucosal antigen-specific antibodies as well as T-helper cell responses were reduced in CD1-/- animals immunized with either the proteosome-formulated or the Protollin-mixed eRSV vaccines, statistically significant reductions of all Ig isotypes was observed in mice given Proteosome-eRSV only. In light of the recent demonstration that Protollin-eRSV elicits protective efficacy via the TLR4/MyD88-dependant pathway in a C57Bl/6 mouse model (Cyr et al, manuscript submitted), it is interesting to hypothesize that the LPS/TLR4 pathway can either supersede or somehow compensate for the CD1d-NKT cell pathway following immunization. Consequently, in the absence of LPS, as is the case when immunizing with Protosome-eRSV, the impact of the CD1d-NKT cell pathway may be more prominent. Alternatively, some of the CD1d effects may have been due to lipid material present in the eRSV preparation. Levels of RSV-derived membrane lipid could be different in the proteosome vaccine compared to the Protollin

vaccine. Surprisingly, the CD1d-NKT cell pathway appeared to have no impact on the overall protection elicited by either vaccine. However, even the reduced antigen-specific IgG antibody titers in Proteosome-eRSV immunized mice were quite high. It is also possible that differences in protection between the wt and CD1d-/- animals may have been observed if sub-optimal doses of the vaccine had been tested.

It is evident from the restimulation of splenocytes from Pro- and Prl-eRSV vaccines immunized animals that the CD1d-NKT cell pathway makes a major contribution to the T-helper cell cytokine responses observed. Alternatively, the missing contribution of NKT cells to cytokine production in the splenocyte populations of CD1-/- animals may lead to the reductions observed in vitro. This latter cell type, in addition to conventional T-helper cells, should henceforth be considered for its potentially important contribution to cytokine production in murine vaccine studies. Interestingly, the detailed analysis of cytokine responses following individual RSV protein stimulation (MHC-I restricted F and MHC-II restricted G peptides) suggests that immunization with either the Pro- or Prl-eRSV vaccine can influence the phenotype of T cells elicited. Although all cytokines were markedly reduced in the RSV-restimulated splenocytes of CD1-/- mice immunized with either vaccine, only splenocytes from wt mice immunized with Protollin-eRSV responded to F restimulation. This observation suggests that the additional stimulation provided by the LPS/TLR4 pathway may favour the activation of F-specific CD8+ T cells [19], and/or that the CD1-NKT cell pathway is required to elicit optimal expansion of these cells [35]. Similarly, the important difference in G-specific IL-17 levels in wt mice compared to CD1-/- mice following Protollin-eRSV immunization was not

observed in mice immunized with Proteosome-eRSV. Once again, this might indicate that the LPS/TLR4 and CD1/NKT cell pathways act conjointly [14,35] to promote maximal G-specific CD4+T cell production of IL-17. In Proteosome-eRSV immunized mice, lower IL-17 responses by G-specific CD4+ T cells appear unrelated to the CD1d-NKT cell pathway. These observations correlate well with the increased neutrophil infiltrates in the lungs of Protollin-eRSV immunized CD1-/- mice after challenge, not observed in those given Proteosome-eRSV. These findings could indicate that the CD1d-NKT cell pathway contributes to the regulation of neutrophil infiltration in the lungs following RSV challenge [9,36], only when RSV antigen priming has been mediated by the LPS/TLR4/MyD88-dependent pathway (Cyr et al, submitted). The absence of IL-5 or pulmonary eosinophilia in any of the groups, combined with the IgG1/IgG2a ratios suggest that the innate CD1d-NKT system is not central to the regulation of Th1/Th2 responses following immunization. Overall, the most striking impact of a deficient CD1d-NKT cell pathway may well be impaired immunoglobulin production, which was correlated with reduced IL-10 production [37,38] observed in all *in vitro* restimulation experiments following immunization with either vaccine. The observed impact of the CD1d-NKT cell pathway on Ig production is consistent with previous work indicating that the regulation of IgG responses is strongly influenced by lipid-anchored surface antigens [4]. These observations suggest that proteosome-based adjuvants appear to utilize the CD1d-NKT cell pathway in a TLR-influenced manner to promote both mucosal and systemic antibody responses as well as cell-mediated immune responses. Overall, these data suggest that CD1d-NKT influence should be considered in animal and human studies involving lipid containing antigens or adjuvants.

5.7. Reference List

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5.8. Figure Legend

Figure 1

RSV-specific serum IgG, IgG1, IgG2a and BAL IgA in CD1-/- and wt mice following IN immunizations with Proteosome-eRSV (Pro-eRSV) and Protollin-eRSV (Prl-eRSV) were quantified by ELISA. 96-well plates were coated with 7.5μg/ml of PEG-concentrated, UV-inactivated RSV and incubated overnight at 4°C. Serum and BAL samples were serially diluted in blocking buffer starting at 1:50 (IgGs) and 1:2 (IgA) dilution along with purified mouse IgG (Sigma), IgG1, IgG2a (both Southern Biotech, Birmingham, AL) and IgA (Sigma) at starting concentrations of 200ng/ml (IgG), 31μg/ml (IgG1, IgG2a) and 1μg/ml (IgA) and incubated for 2h at room temperature. Bound antibody was detected with HRP-conjugated anti-mouse IgG, IgA (Sigma) or IgG1, IgG2a (Southern Biotech, Birmingham, AL) for 30 min at 37°C. Plates were read in a UV spectrophotometer at OD450 following detection with 3,3,5,5-tetramethylbenzidine (TMB) substrate. Data extrapolation from standard curve was performed using an assymetric sigmoid non-linear regression. Serum IgG (A), BAL IgA (B), IgG1 (C) and IgG2a (D) are shown as mean ± SEM. Statistical analyses were performed by one-way Analysis of Variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test. A *P*-value of ≤ 0.05 (two-tail) was considered significant. $P < 0.05$,*; $P < 0.01$, **; $P < 0.001$, *** show the difference between CD1-/- and wt mice immunized with the same vaccine.

Figure 2

Restimulation of Proteosome-eRSV (Pro-eRSV) and Protollin-eRSV (Prl-eRSV) immunized CD1-/- and wt mice splenocytes with purified inactivated RSV (A-C),

CD8+T cell-specific RSV F (D-F) or CD4+T cell-specific RSV G peptides (G-I). Splenocytes from immunized animals were re-stimulated with increasing doses of RSV (1, 3 and 9μg/ml) or F or G peptides (0.1, 1.0, 10.0 μg/mL) and supernatants assayed for cytokines IFNγ (A, D, G), IL-17 (B, E, H) and IL-10 (C, F, I) by cytokine ELISA kits (BD Biosciences) according to manufacturer's instructions. The data presented were extrapolated from the standard curve using linear regression and were normalized against non-restimulated (cRPMI alone) cell supernatants.

Figure 3

All mice immunized with proteosomes (Pro) or Protollin (Prl) formulated with eRSV were fully protected against RSV challenge as shown by RSV lung titers. Lungs from immunized and challenged animals were washed, weighed and homogenized individually in RSV media, then centrifuged at 2655 x g for 2 minutes at 4°C. RSV quantitation in the supernatants was performed by serial dilution on Vero cell monolayers in 96-well plates starting at a 1:10 dilution and incubation for 6 days at 33°C. Supernatants were then removed; plates were washed with PBS and adhering cells fixed with 1% paraformaldehyde in PBS for 1 hour, followed by indirect immunofluorescence (IFA). After blocking with PBS + 2% skim milk, plates were incubated at RT for 2 hours with a 1:400 dilution of goat anti-RSV antibody (Biodesign international) and washed with PBS. Anti-goat IgG FITC conjugate (Sigma) was used at a 1:400 dilution for detection. Wells were considered positive when ≥1 fluorescent syncytium was detected. Infectious titers were calculated using the Reed-Muench Method [30] . The bar represents the geometric mean virus titers in the lungs of 4 mice per group.

Figure 4

BAL leukocytes of mice immunized with proteosomes (Pro) or Protollin (Prl) formulated with eRSV following challenge were isolated by centrifugation of the BAL and resuspension of the pellet in 500µl of 0.01% BSA in PBS. 100µl of the cell suspension was used to centrifuge on microscope slides using a cytospin. Cells were fixed and stained with hematoxilin and eosin (H & E) [30], enumerated using a hemocytometer (40X) and a manual differential count was performed on 300 cells. Enumeration of leukocytes in individual mouse BALs was based on morphological characteristics and the data are expressed as percentages. Bars represent the mean value of 4 animals per group ± SEM. Statistical analyses were performed by unpaired t test, comparing similarly immunized CD1-/- with wt mice. A P-value of ≤ 0.05 (two-tail) was considered significant. $P < 0.05$,*.

5.9. Figures

Figure 1

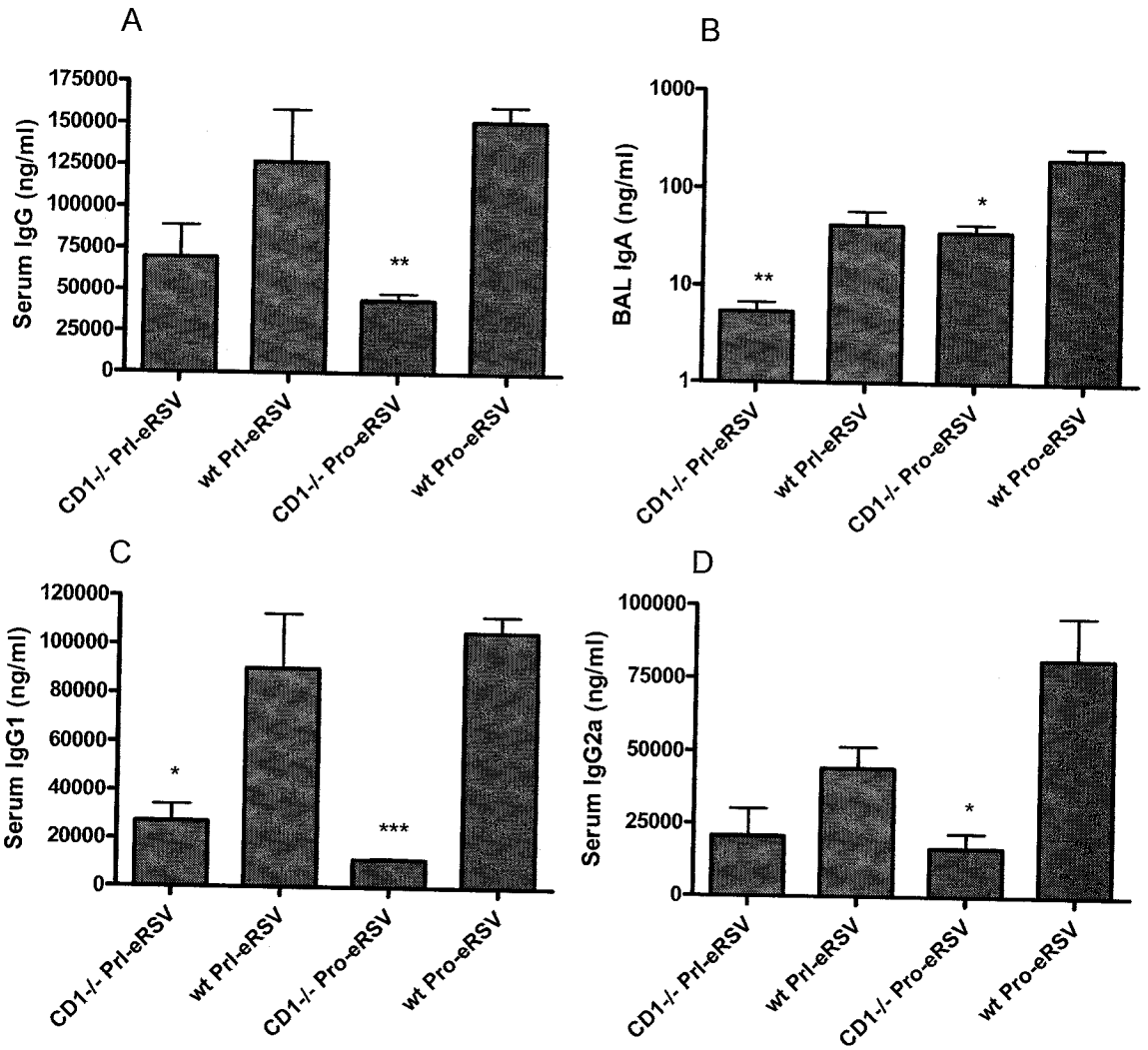


Figure 2

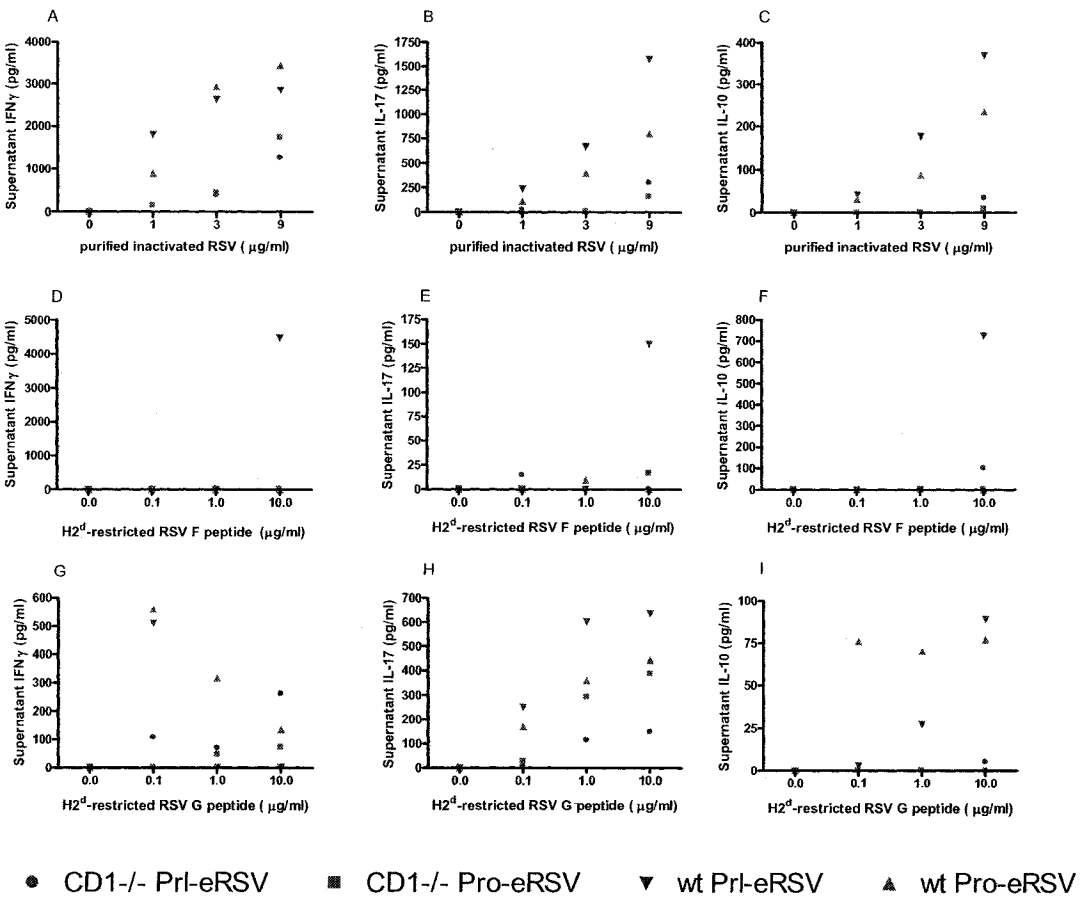

A
B
C
D
E
F
G
H
I
Supernatant IFNγ (pg/ml)
Supernatant IL-17 (pg/ml)
Supernatant IL-10 (pg/ml)
purified inactivated RSV (μg/ml)
H2d-restricted RSV F peptide (μg/ml)
H2d-restricted RSV F peptide (μg/ml)
H2d-restricted RSV G peptide (μg/ml)
CD1-/- Prl-eRSV
CD1-/- Pro-eRSV
wt Prl-eRSV
wt Pro-eRSV

Figure 3

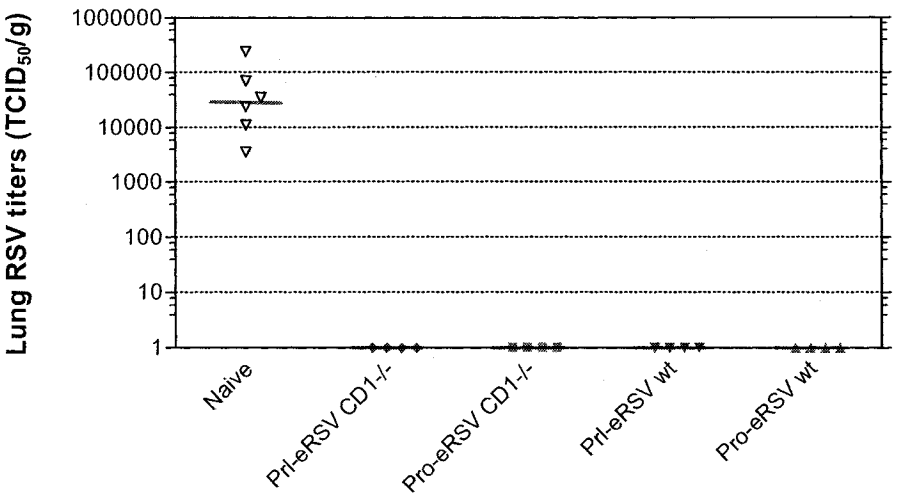
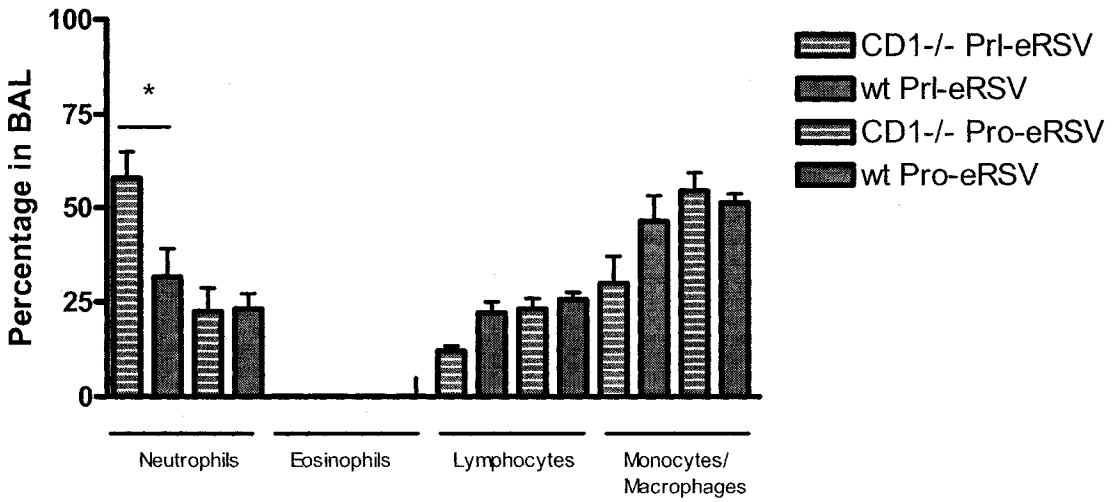

Lung RSV titers (TCID50/g)
1000000
100000
10000
1000
100
10
1
Naive
Prl-eRSV CD1-/-
Pro-eRSV CD1-/-
Prl-eRSV wt
Pro-eRSV wt

Figure 4


Percentage in BAL
100
75
50
25
0
*
Neutrophils
Eosinophils
Lymphocytes
Monocytes/
Macrophages
CD1-/- PrI-eRSV
wt PrI-eRSV
CD1-/- Pro-eRSV
wt Pro-eRSV

6.General Discussion and Conclusion

The need to develop a safe and effective vaccine against respiratory syncytial virus (RSV) is as pressing as ever. Past experience has taught us that there are significant risks associated with RSV immunization, most notably the development of exacerbated natural disease following immunization with the formalin-inactivated RSV (FI-RSV) vaccine [1,2]. The reproduction of this pathology in animal models has provided us with a model for this immunopathologic event, suggesting that an aberrant Th2-biased response contributes to the enhanced pulmonary inflammation [3]. Many questions remain regarding the mechanism of natural RSV infection itself including the inefficiency of the natural immunity to protect against re-infection and the immunopathology caused by an overzealous immune response to infection in some children. Perhaps the single most important question regarding immunization against RSV is the following: can a vaccine do 'better' than a natural viral infection that does not give rise to complete or durable immunity? The subject of my thesis was to demonstrate that such a vaccine may indeed be feasible through the judicious use of novel adjuvants that exploit our current knowledge of mucosal and innate immunity.

In our model, we used either the proteosome or Protollin adjuvants to formulate vaccines using an enriched subunit RSV antigen (eRSV) and immunized mice via the intranasal route. An obvious benefit of intranasal vaccines against respiratory diseases is the induction of antigen-specific immunity at the site of initial viral exposure/invasion. When tackling a virus such as RSV, which can repeatedly re-infection the upper

respiratory tract over the lifetime of an individual, a strong mucosal response may prevent colonisation of the upper airways and limit the risk of subsequent spread to the lower respiratory tract. Unfortunately, while mice tend to elicit life-long mucosal immunity against respiratory pathogens, immune effectors in the respiratory tract are thought to be short-lived in humans. However, a number of intranasal vaccines against other respiratory viruses have progressed through the various stages of clinical development and show great potential. For example, a subunit influenza vaccine formulated with proteosomes successfully protected against influenza A in double blind-randomized trial [4]. Nevertheless, intranasal immunization is a relatively new field and more investigation is required to better characterize this route of immunization in humans.

Proteosomes [5] or Protollin, formulated with LPS [6], possess a number of key features that make them ideal for intranasal immunization. First, they are particulate in nature. Vaccine antigens associated with particles are less vulnerable to degradation in the respiratory mucosa [7]. In addition, specialized antigen sampling cells in the nasopharynx associated lymphoid tissue mucosa have been demonstrated by several groups [7-9]. Although the mucosa of the respiratory tract has not been as fully studied as the gut mucosa, a number of findings suggest that M cells [10], or even DCs can sample antigens directly from the nasopharyngeal passage [7]. A significant attribute of proteosome-based adjuvants is the presence of PAMPs on either the Neisserial porins or the incorporated Shigella LPS (in Protollin-based vaccines). The NALT and overlying follicular-associated epithelium (FAE) as well as epithelial cells of the nasopharyngeal

passage are particularly responsive to PAMPs, which they recognize via their TLRs [10]. The downstream signaling cascades triggered by TRL-PAMP interactions can not only upregulate the development of adaptive immune responses, but also favour the polarization of T-helper cells towards a Th1 phenotype. Thus proteosome-based adjuvants are not only particularly well-suited for NALT cell recognition, they also contribute directly to abrogating the Th2-response historically associated with enhanced RSV pathology. In addition to TLR ligands, proteosome-based adjuvants also carry co-purified lipids from Neisseria. Endogenous and exogenous lipids are presented by CD1d to CD1d-dependant NKT cells, an innate-like pathway that has only recently been recognized for its potential role in augmenting the immunogenicity of co-administered antigens [11,12]. Although there are few indications in the literature suggesting that other lipids may activate this pathway to achieve optimal immunogenicity [13], we sought to investigate the possible role of this potent pathway in the immunogenicity elicited by the proteosome-based RSV vaccines.

In this work, we have successfully demonstrated that intranasal subunit proteosome-based vaccines can protect BALB/c [14] and C57Bl/6 [15] mice against RSV challenge without enhanced risk for Th2-driven adverse events. In our work with Protollin-eRSV in BALB/c mice, we showed that this vaccination strategy could elicit not only antigen-specific CD4+Th1 cells but also antigen-specific CD8+ T cells that produced abundant IFNγ. Interestingly however, these specific immunological attributes were not required for protection in this model, probably as a result of the very high neutralizing antibody titers generated. However, in preliminary studies using C57Bl6 mice, the Proteosome-

eRSV vaccine did not protect mice against challenge at the doses tested, suggesting that in a more stringent mouse model, LPS-mediated upregulation of TLR activity was indeed required for protection. We were able to confirm this hypothesis by studying our vaccine formulations in TLR2, TLR4 and MyD88-/- mice (manuscript submitted). In these studies, the contribution of TLR4 and the MyD88 adaptor protein to the protection elicited by Protollin-eRSV was significant. Surprisingly, TLR2 made no contribution. Despite having a direct impact on protection, the polarization of T-helper 1 cells and immunoglobulin isotype switching, MyD88 had no impact on the overall level of antigen-specific antibodies. These results are consistent with the recent finding that other innate mechanisms may be involved in the generation of adaptive immune responses [16]. In our protection model, our results also suggested that intact MyD88 signalling was required for the downregulation of eosinophils and the recruitment of neutrophils to the lungs. These findings may indicate that MyD88 is involved in a number of other pathological conditions implicating the recruitment of granulocytes to the lungs, such as infectious diseases, allergy and asthma. Another interesting aspect of our MyD88 work is that despite being required for protection and the regulation of pulmonary granulocyte, MyD88 did not appear to be required for the early events leading to pro-inflammatory cytokine production and co-stimulatory molecule upregulation. This observation may be the result of the dual signaling capacity of the TLR4 receptor, which can bypass the TIRAP/MyD88 pair and utilize the TRAM/TRIF adaptor proteins instead to achieve late NF-κB upregulation and TNF-α production. It is interesting to speculate that this phenomenon is related to the hypothesis of differential recruitment kinetics of the adaptor proteins to the plasma membrane by PIP2 or N-myristoylation [17,18]. This

phenomenon would establish a direct link between phospholipid metabolism and TLR signaling, implying that a number of factors that influence cell lipids could also impact TLR signaling downstream pathways and immune response outcomes.

Our work in CD1-/- mice, deficient for the CD1d-NKT cell pathway, raises the novel concept that the lipids present in proteosome-based vaccines and possibly many other lipid-containing adjuvants, may play a significant role in the immunogenicity of vaccines. These results suggest an alternative innate mechanism of TLRs to support and augment immunogenicity [16]. Importantly, our results also suggest that the TLR and CD1-NKT pathways may act synergistically to achieve superior T-helper cell responses. Those augmented responses were primarily of Th1 phenotype, suggesting that the collaboration of TLR and CD1-NKT cells further enhances the Th1-bias provided by TLR ligation. These findings pave the way for further research into the relationship between vaccine adjuvants, lipid metabolism and TLR activation.

The work presented herein demonstrates the feasibility of a safe and effective proteosome-based RSV vaccine administered via the intranasal route. Our investigation of the mechanisms employed by proteosome-based adjuvants suggests that Protollin-eRSV vaccine efficacy is mediated via the LPS-TLR4-MyD88-dependent pathway. The finding that MyD88 is involved in pulmonary granulocytic recruitment may have broad implications in other pathological lung conditions. Finally, our work also suggests that the immune rsponse to vaccines can be strongly influenced by the CD1d-NKT cell

pathway and that this pathway may be a powerful new tool for lipid-containing vaccine antigens or adjuvants.

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