BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Dissertation

CHARACTERIZATION OF IMMUNE RESPONSES INDUCED BY *N. MENINGITIDIS* PORB AND ITS USE AS A VACCINE ADJUVANT

by

ANDREW PHILIP PLATT

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My family, who were always behind me, even when they were more concerned about my next committee meeting than I was.

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CHARACTERIZATION OF IMMUNE RESPONSES INDUCED BY \textit{N. MENINGITIDIS} PORB AND ITS USE AS A VACCINE ADJUVANT

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ANDREW PHILIP PLATT

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Major Professor: Lee M. Wetzler, M.D., Professor of Medicine and Associate Professor of Microbiology

ABSTRACT

Vaccines play an essential role in public health. Adjuvants increase immunogenicity for many of these vaccines by stimulating the innate immune system: driving cytokine secretion to induce local and systemic pro-inflammatory states, upregulating costimulatory molecules on antigen presenting cells (APCs), and increasing antigen uptake and presentation to better engage T cell responses. \textit{Neisseria meningitidis} porin PorB is a Toll-Like Receptor 2 (TLR2) ligand with broad immune stimulating functions and can act as a vaccine adjuvant. Our lab is interested in characterizing how PorB activates the immune system and how these effects relate to its adjuvant activity. An understanding of adjuvant functions will allow for the rational, rather than empiric, design of future vaccines. Here we further investigate the effects of PorB on the innate immune
system as it may apply to the adjuvant activity of the porin. In a direct test of adjuvanticity we show that without the presence TLR2 the adjuvant activity of PorB, measured by antigen-specific IgG production, is diminished in immunized mice while loss of MyD88 entirely ablates PorB adjuvant activity. We demonstrate costimulatory molecule upregulation in response to PorB stimulation and its dependence on TLR2. We show that stimulation with PorB increases antigen uptake by APCs and drives APC migration to draining lymph nodes, which appears to be dependent on TLR2 and not on MyD88. Finally, we use systems vaccinology to uncover complex regulatory networks and dynamics. The inclusion of PorB as an adjuvant in a multi-injection vaccine formulation has two major effects on expression profiles in murine splenocytes. Vaccine preparations containing PorB as an adjuvant induce expression in inflammatory and immune signaling networks, in agreement with previous work, and accelerate the kinetics of the immune response, as demonstrated by induction of expression of cell cycle and proliferative genes and regulatory networks at earlier time points as compared to preparations not containing PorB. This systems biology approach reveals previously unappreciated aspects of reaction of the immune system to PorB. Together, these findings deepen our understanding of the immune response to PorB and offer potential insight into the mechanisms behind its adjuvanticity.
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<td>allophtocyanin</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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<tr>
<td>BMDC</td>
<td>bone marrow dendritic cell</td>
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<td>BMDM</td>
<td>bone marrow derived macrophage</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>BUSM</td>
<td>Boston University School of Medicine</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DOC</td>
<td>deoxycholate</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>GMI</td>
<td>geometric mean fluorescence intensity</td>
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<td>HEK</td>
<td>human embryonic kidney</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>IFNγ</td>
<td>interferon gamma</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<td>LN</td>
<td>lymph node</td>
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<td>LOS</td>
<td>lipooligosacharide from <em>N. meningitidis</em></td>
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<td>LPS</td>
<td>lipopolysaccharide from <em>E. coli</em></td>
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<td>M</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>MFI</td>
<td>mean fluorescence intensity</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>min</td>
<td>minute</td>
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<td>ml(s)</td>
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<td>μl</td>
<td>microliters</td>
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<td>mM</td>
<td>millimolar</td>
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<td>MPL</td>
<td>monophosphoryl lipid A</td>
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<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
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<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>OCT</td>
<td>optimal cutting temperature</td>
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<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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PBS  phosphate buffered saline
PE   phycoerythrin
PorB porin B from Neisseria meningitidis
PRR  pattern recognition receptor
PSF  point spread function
rGM-CSF recombinant granulocyte-monocyte colony-stimulating factor
SDS-PAGE sodium dodecyl sulfate – polyacrylamide gel electrophoresis
TCR  T cell receptor
TLR  toll like receptor
TMB  3,3',5,5'-tetramethylbenzidine
TNFα tissue necrosis factor alpha
Introduction

Vaccines have proven to be one of medicine's most effective tools in the fight against infectious diseases. From early variolation and Jenner's cowpox experiments, through the development of attenuated vaccine strains, to modern adjuvanted subunit vaccines, infection by dozens of once life-threatening pathogens can now be prevented. There remains, however, a pool of infectious diseases that have resisted all efforts to design effective vaccines. Malaria, HIV, tuberculosis, and a swath of emerging pathogens all present unique challenges to vaccine design. The largely empiric methods that have been used in the development of our current vaccines have been inadequate to this challenge. As immunologists we are left with a need to better understand how the vaccines and tools that we do have work, before we can attempt to rationally design new vaccines.

Any vaccine that can provide long-lasting immunity must work with both the innate and the adaptive arms of the immune system. These two branches must work in concert to recognize and respond to a vaccine, and produce a robust immune response. In addition to sensing the presence of invading pathogens and acting as a first line of defense, the innate immune system is responsible for shaping, to a significant degree, the nature of the adaptive immune response. It has become apparent that there are qualitatively different forms of adaptive immunity that have evolved to respond to the wide variety of infectious diseases humans have been exposed to. Determining how strongly
and down which of those paths the adaptive response will react is determined in large part as the innate immune system recognizes and responds to features of the pathogen or vaccine. Recent research in innate immunity has made significant progress in understanding the pathways that lie between recognition of, and reaction to, a pathogen and the final nature of the immune response.

Cells of the innate immune system recognize pathogens through an array of Pattern Recognition Receptors (PRRs). On the surface of innate immune cells the Toll-Like Receptors (TLRs), C-Type Lectins (CLEC) and other receptors are specific for various Pathogen Associated Molecular Patterns (PAMPs). Internal to the cell are additional TLRs, viral nucleic acid sensing helicases, and the NOD-Like Receptors (NLRs) that can signal through the inflammasome. The proliferation of these sensors in the literature has resulted in a nearly overwhelming number of possible targets for vaccine and adjuvant design. Mimicking the structures of native ligands, designer PAMPs have become the go-to choice for new vaccine adjuvants. A number of popular options are available to the researcher. Monophosphoryl Lipid A (MPL) is a detoxified variant of LPS derived from Salmonella, a TLR4 ligand, and is perhaps the leading candidate making its way through clinical trials. [1] CpG DNAs are short oligomers of nucleic acids with methylation designed to mimic bacterial chromosomes, and a TLR9 agonist. The saponin QS-21 and related members are plant-derived adjuvants whose mechanism of adjuvanticity is still being elucidated even as they have already entered clinical trials in a number of vaccine formulations. Making
sense of the wide variety of possible responses may require the use of systematic tools for rational vaccine design. Our lab studies the outer membrane Porin proteins of *Neisseria* species, which we have shown to be ligands of TLR2 [2].

An introduction to the study of adjuvants lies in the intersection between vaccines and innate immunity. I will begin with how the selection of vaccine components can influence aspects of the immune response, including the T helper profile [3]. From there, I will discuss the underlying innate targets of the vaccine that can effect these changes, focusing on dendritic cells [4], their subtypes [5], and the tools available to vaccine designers to select subsets of cells [6]. This will also include an analysis of emerging targets, including specific vehicles, formulations, and vaccine adjuvants. Finally, I will step back to take a more holistic approach, and report on the use of systems biology as a tool for vaccinologists in predicting and analyzing innate immune responses.

**Adjuvants and vaccines**

The “dirty little secret” [7] of vaccines has long been trace contamination with PAMPs: ligands for a wide range of extra- and intra-cellular Pattern Recognition Receptors (PRRs) [8]. The innate immune system has evolved multiple classes of PRRs, including the well-known TLRs [9] and NLRs [10]. Binding of these receptors by PAMPs activates cells of the innate immune system, which in turn can promote a stronger response by cells of the adaptive immune system [11-13]. Pathogen-derived molecules have therefore come under
intense scrutiny as potential adjuvants for vaccines, especially subunit vaccines where the antigen itself is not highly immunogenic [14, 15]. Extensive evidence exists of immune stimulation through TLR signaling which demonstrated increased chemokine and cytokine production, up-regulation of co-stimulatory molecules and cell proliferation [16, 17]. Common members of signaling networks, especially MyD88 [18-20], have also been observed to play a critical function in responses to PAMPs [21, 22]. Currently, adjuvant selection for vaccines is determined primarily through extensive and costly empirical clinical testing of multiple adjuvant systems [23]. From a rational vaccine design standpoint, it should be possible to pick and choose among the vast array of available PAMPs for specific vaccine adjuvants each tailored to a desired outcome [3, 8]. For this to be possible, however, a more detailed knowledge of the molecular mechanisms behind each ligand, and the skewing of the immune response generated by the presence of those ligands, is required. To this end, I set out to characterize, in more detail, the pathways activated by one particular TLR2 ligand, the outer membrane protein (OMP) Porin B (PorB) from *Neisseria meningitidis* [24].

Our laboratory has focused on investigating PorB and its interactions with the innate immune system. We have identified PorB as an agonist of TLR2/TLR1 heterodimers [2, 25] and reported its ability to act as an adjuvant when used in conjunction with a wide array of antigens [26, 27]. As a member of family of gram negative porins, PorB forms a trimeric β-barrel structure on the outer membrane
of the bacteria, and serves as a pore for ion exchange [24, 28]. In addition to identifying PorB as a TLR2/1 agonist, we have also made initial characterizations of the innate and adaptive response to the adjuvant in vitro; PorB increases surface expression of MHCII and CD86 on murine DCs and B cells and stimulates the release of Interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNF-α) [29-32]. We have also studied Mitogen Activated Protein Kinase (MAPK) activation in response to PorB in B cells, focusing on the Erk1/2 pathway [33, 34]. Demonstrating that these effects are TLR2 dependent is, therefore, of interest in confirming the molecular mechanisms underlying the adjuvanticity of PorB.

**Manipulating cytokine profiles**

A full review of the adaptive responses observed in Th1 [35, 36] versus Th2 [37] or Th17 [38] skewed immune responses, plus the impact of additional T helper subtypes such as the T follicular helper (Tfh), [39] is beyond the scope of this introduction. In brief, Th1 responses are driven by one family of cytokines including IFN-γ and IL-12, while Th2 profiles are observed in response to alternative factors, classically IL-4. Other cytokines, such as IL-10, can suppress the immune response by generating T regulatory (Treg) cells. [40] Early investigation into the roles pathogens play in this process observed Th1 responses to viral infections, while bacteria and helminths elicited more balanced or Th2 profiles. Further work has uncovered the transcription factors T-bet and GATA-3 as central to the differentiation of Th1 and Th2 CD4+ T cells respectively. With the discovery of the PRRs, efforts were made to classify the
nature of the T helper response to ligands of each receptor. While some trends have emerged, enough conflicting results have been reported that the field requires extensive review to encompass the full body of work [12]. However, recent work in the development of vaccines designed to generate cytokines that tend to drive such responses in one direction or another, and the innate immune mechanisms behind the secretion of these cytokines, can be addressed here.

As desirable as robust cytokine responses are in animal models as demonstrators of efficacy and immunogenicity, they must be carefully managed as novel products move towards the clinic. Very high cytokine responses, and their associated adverse effects, including pain, inflammation and injection site reactions, are the reason highly potent experimental adjuvants like Complete Freund's Adjuvant (CFA) and unmodified LPS are non-viable as clinical adjuvants. Overly aggressive stimulation of the innate immune system by adjuvants has also been linked to the development of autoimmunity; adjuvants have been used intentionally for this purpose in animal models [41, 42]. Conversely, sensitivity to innate cytokine responses may be a distinguishing feature of some live attenuated vaccine strains, as demonstrated by work on a Japanese encephalitis virus vaccine candidate [43] in which the increased immunogenicity of the attenuated strain was linked to its ability to elicit more robust cytokine responses than the pathogenic strain.

Most modern vaccine design efforts, and almost all of those towards viral pathogens, have been focused on generating primarily Th1 responses. [39, 44]
This is done with the goal of developing a robust cellular immunity [45] in addition to long-lasting humoral immunity while the avoiding allergic or tolerogenic [46] and auto-immune type responses [47] that are more closely affiliated with the Th2 and Th17 phenotypes respectively. Although almost all current vaccines offer protection through humoral immunity [48], design strategies for pathogens that have resisted efforts to date are focused on cellular immunity, based on the hypothesis that this may be protective in cases where humoral immunity has proven difficult to elicit or non-protective.

Skewing of the adaptive immune response by a vaccine to a desired T-helper profile begins with the innate immune system. The two most readily available tools for this purpose are the selection of adjuvant(s) and delivery vehicle, which together with the antigen of choice make up a subunit vaccine formulation. Once in vivo, differentiation of antigen specific CD4+ T cells into a Th1 or Th2 response is driven by local cytokine production and the expression of cell surface molecules by members of the innate immune system, especially in the context of peptide presentation on MHC [3]. While attenuated, inactivated or Virus Like Particle (VLP)-like vaccines (including DNA vaccines) may blur the lines between antigen, adjuvant, and vehicle, in the case of subunit vaccines these effects on the innate immune system can be ascribed to the adjuvant and/or vehicle even in the absence of antigen [49, 50]. Within the GSK Adjuvant Series (AS01-AS04), the combination of alum and MPL known as AS04 and used in the HPV vaccine elicits TNF-α and IL-6 plus additional chemokines [51].
The combinations of MPL and the saponin QS-21 in liposomes (AS01) or an oil emulsion (AS02) have been compared in head-to-head tests for their ability to elicit responses from various CD4+ T cell subsets [52-54]. AS02-adjuvanted vaccines have been observed to drive production of IL-2, TNF-α and IFN-γ, all associated with Th1 responses [55]. This work was done using antigens from Mycobacterium tuberculosis, and despite the secretion of Th1 cytokines, no antigen-specific CD8+ cytotoxic T cells were detected. In some studies AS01 has been observed to generate higher numbers of IL-2 and IFN-γ producing CD4+ T cells when compared to AS02 [52]. Both AS01 and AS02 were observed to generate larger cytokine responses than the antigen, a VZV glycoprotein, paired with alum or given without adjuvant in saline. Similarly, a report on the innate effects of the AS04 adjuvant formulation (MPL + alum) showed increased production of the cytokines TNF-α and IL-6, but not IFN-α, when compared to alum alone [51]. Reports of this nature on AS04 are particularly relevant given its recent approval for use in prophylactic vaccines for HPV. Moreover, these effects were observed to be specific to the innate immune system, affecting dendritic cells, but not T cells. Working on the TLR2 agonist Porin B (PorB) from Neisseria meningitidis [2], our lab has shown it to elicit a combination of Th1 and Th2 associated cytokines [29].

These papers do highlight one somewhat common deficiency in most reports on modern adjuvants: because of the relatively recent proliferation of new adjuvants, most comparisons in the literature are made to alum, with saline as a
negative control. This does have the advantage of providing a widely comparable reference point, and in highlighting the improvements of novel adjuvants over the standard of care. Novel vaccines, however, are not choosing between alum and a single novel adjuvant, but from the whole spectrum of new options. Alum may even inhibit key Th1 cytokine responses like IL-12 [56], leading to artificially high differential responses when it is used as a control for other adjuvants. Head-to-head comparisons between novel adjuvants, while rarer, thus provide more relevant information in researchers interested in candidate vaccine formulations.

The increased effectiveness of AS01 was also observed in human subjects vaccinated with three different GSK Adjuvant Systems, where the liposomal formulation outperformed two different oil-water emulsions using HIV derived antigens as determined by antibody titers and CD4+ T cell responses [53]. However, as reasonable as these observations are in assessing the performance of each system from a theoretical perspective, the usefulness of these observations as clinical correlates of protection has not been established. The occasionally contradictory nature of head-to-head results, especially when using different antigens and model systems, has made generalizable predictions difficult, if not impossible. As discussed later in this introduction, it may be that systems vaccinology offers the best hope for tools to handle this complex problem.

**Dendritic Cells**
Dendritic cells (DCs) play an essential role in communication between the innate and adaptive arms of the immune system. In the peripheral tissues, DCs sample the environment. Upon antigen capture and the presence of a danger signal, DCs undergo maturation and migration to the secondary lymphoid tissues, where they present antigenic peptides on MHC to T cell populations, directing the development of the cellular immune response. Presentation of antigen to T cells, expression of costimulatory factors, and secretion of various cytokines and chemokines are all essential functions in strengthening the adaptive immune response [57]. In this section, I will cover various new approaches both to targeting vaccines to dendritic cells and eliciting desired responses from those DCs.

A wide range of professional APCs reside in the body, each with a specificity and affinity for certain innate immunity pathways. Of particular interest are the various subtypes of dendritic cells. These have been observed to carry out particular functions, including migratory and resident populations, or with affinities for CD4+ vs. CD8+ T cells. Expression of PRRs, expression of costimulatory factors including CD40, CD80 and CD86, and secretion of cytokines, have all been observed to vary between DC subtypes. Upregulation of some or all of these factors by migratory or lymph node resident DCs has been taken to be a measure of activation of the innate immune system by vaccine formulations [51]. Clarification of the roles of each subtype and identification of markers that can be used to target these types is therefore a highly useful field of
work. Given the known differences between the behavior of e.g. plasmacytoid DCs (pDCs) in mice and humans, identifying comparable subtypes between the two species is also necessary to translate experimental formulations in clinically relevant candidates. Towards this end, the identification of a migratory DC population in humans capable of cross-presentation, identified by high CD141 expression and similar to the well-studied CD103+ population in mice, is of particular note [4].

"Dendritic cell vaccines" per se have also gathered a great deal of attention. These vaccines are composed of ex vivo antigen-pulsed and expanded autologous dendritic cells transferred back to the donor, with the intent to present those antigens to the adaptive immune system in a highly selective manner. The primary use of this technology to date has been in the development of cancer immunotherapies. [58] While the research presented here does not involve dendritic cell vaccines per se, many of the same response elicited in vitro when DCs are activated are also desirable as in vivo responses to vaccine injections [59-61].

Active targeting of antigens to dendritic cells is a rapidly advancing field. Antigen-IgG constructs have been used for over 25 years to deliver proteins of choice to dendritic cells by fusing antigens to antibodies specific for dendritic cell surface molecules [62]. More recently, targeting to specific DC subsets has been achieved with the use of antibodies or ligands specific for surface molecules with restricted expression [6, 63, 64]. As dendritic cell subsets have been observed to
process antigens in varied manners, targeting antigens toward particular subsets can be used to shape the immune response to a desired end [5]. While a large proportion of research is currently directed towards enhancing Th1 and cellular responses, efforts solely towards robust antibody responses have also been investigated.

Among the ligands used for targeting, those recognized by PRRs, and in particular the TLRs, have been of especial interest. Direct conjugation or indirect attachment of an antigen or vehicle to a TLR ligand offers the attractive combination of targeting to, and activation of, an APC upon binding. Direct conjugation of TLR ligands to antigens has been widely used as a means of enhancing immune responses to a greater extent that observed when mixing the antigen and adjuvant before injection. Conjugation of the TLR9 ligand CpG to Ova was observed to escape the requirement for a helper T cell population in generating a robust CTL response [65]. Experimentation with other TLR ligands as conjugated adjuvants has included the TLR7 agonist imidazoquinolines, observed to promote cross-presentation [66], and the TLR5 agonist flagellin [67]. Influenza HA fusion proteins based on flagellin have proceeded to Phase I clinical trials, where they showed reasonable safety profiles and immunogenicity [68]. Conjugation of the antigen with adjuvant additionally allows for a substantial reduction in the antigen dose required to elicit a robust response, a not insignificant factor in translational work focused on developing clinically viable vaccine formulations.
The C-type lectins are a family of carbohydrate-binding proteins with roles from cell-cell adhesion and motility to pathogen recognition and immune response. Lectins have emerged as a rich set of candidates for targeted delivery of antigens to specific DC subsets. The CD8+ DEC205+ family of DCs reside in the T cell zone of secondary lymphoid tissue and specialize in the presentation of antigen to CTLs [5]. DEC205 has recently been used for targeting an HIV vaccine designed to induce cellular immunity [69], and a DNA vaccine to two self antigens [70]. The lectins CLEC9A [71] and macrophage galactose-type C-type lectin (MGL) [72] have also been targeted and their responses characterized. Recently, effort has been made to conduct head-to-head comparisons to some of these lectins [69]. Comparable responses were observed when using fusion constructs of the HIV p24 protein and mAbs to Langerin, DEC205, and CLEC9A. As the lectins have their own natural ligands, investigation has also been made into the relative efficacy of using monoclonal antibodies or native ligands for targeting. Using DC-SIGN as the DC-expressed target, mAbs were found to be the most effective in driving the presentation of antigenic peptides on Class I MHC, and equivalent to the HIV ligand gp120 while superior to carbohydrates for presentation on Class II MHC [73].

If the science of cytokines and T helper cells is moving into the bedside, the study of dendritic cell responses to vaccines remains within the basic sciences. New dendritic cell populations are still being discovered and their role in the innate immune response fleshed out. Reagents are still being developed
that can target vaccines to specific DC subsets, either to surface molecules that
define certain subsets or based on the pattern of expression of PRRs. The next
step will be to understand the influence of incorporating such reagents on the
nature of the immune response to vaccines. Comparisons will need to be made
between antigen-IgG fusion constructs that target extremely specific cell
populations but may not have pro-inflammatory properties, mixed constructs like
those targeting the C-type lectins that can be both specific and agonistic, and
less specific adjuvants like TLR agonist fusion proteins with broad specificities for
innate immune cells but robust inflammatory properties.

**Antigen presentation and APC migration**

Generating the proper cytokine milieu and targeting an antigen to the
correct class of DC are necessary steps in the functioning of a vaccine, but they
are not sufficient for robust interaction between the innate and adaptive immune
systems. For antigen presentation to occur, at least two more conditions must be
met. First the antigen must be taken up by the APC, processed by proteolytic
machinery, and presented in the context of MHC plus necessary costimulatory
factors. Second, the APC itself must be in a location, generally the secondary
lymphoid tissue, where it can interact with a sufficient number of naïve T cells to
insure it is able to find a cognate member of the adaptive immune system
capable of recognizing the presented antigenic peptides. An analysis of these
factors is essential in determining the theoretical efficacy of new vaccine
formulations. Each of these factors will be discussed in turn below.
In order to stimulate a CTL response, APCs must present antigenic peptides in the context of Class I MHC. Dendritic cells are able to use multiple pathways for this purpose. One of the key factors to eliciting cellular to a vaccine is access to one or more of these pathways. During infection with live attenuated viruses, or transfection with DNA or RNA vaccines, expression by APCs of foreign proteins allows direct loading of peptide onto MHC through the endogenous processing pathway. In subunit and inactivated vaccines, APCs are able to capture, process, and present antigen on Class I MHC through a process known as cross-presentation [74]. The importance of various elements of the vaccine formulation in antigen processing and presentation has been well recognized. The original work in the field demonstrated not only the importance of PAMPs, in this case TLR ligands, but also the physical association of the PAMPs with the antigen, as regulation of the antigen processing machinery is done at the scale of individual endosomes [75, 76]. Recent analyses of vaccine formulations first focus on the internalization of soluble antigens in vitro or in vivo as measured by the acquisition of fluorescently labeled antigens [51]. Demonstrating this effect for subunit vaccines has involved work with antigen-adjuvant fusion constructs as discussed above, to some success.

Migration of antigen-loaded APCs to draining lymph nodes where they are able to efficiently stimulate cognate T lymphocytes is an essential process in the functioning of the innate immune system. New basic science tools are being developed to study these interactions, even as the efficacy of individual
adjuvants and vaccine formulations are being studied. The primary technique is to use fluorescently labeled antigens and to characterize the changes in the antigen-positive cell population in draining lymph nodes after vaccination. AS04, for instance, was shown to increase the number of antigen-positive DCs when compared to vaccination with alum alone, as well as the expression of some costimulatory molecules (CD40, but not CD86) [51]. Easily performed by flow cytometry, this technique also allows for characterization of multiple features of the antigen-positive cells, including expression of surface costimulatory molecules. However, it is unable to say anything about the association of APCs with lymphocytes, and is also restricted in representing a single, static point in time. The use of two-photon microscopy has allowed researchers to observe single-cell interactions between APCs and lymphocytes in vivo and in real time [77]. As the use of this technology has progressed, it has expanded from very theoretical investigation of the biomechanics and physiology of the innate response [78] into a tool to study the processes of responses of the innate immune system to pathogens [79] and subunit antigens [80]. In one study, the addition of a TLR4 agonist (LPS) to OVA-coated latex beads was observed to both increase migration of antigen-bearing cells to draining lymph nodes and extend interactions between those DCs and Ova-specific D011.10 T cells [81]. The ability of two-photon systems to study intact in vivo and ex vivo explants under conditions that maintain the organ structure of the lymph nodes is essential for these studies. If two-photon systems excel in teasing out specific cellular and
molecular interaction, *in vitro* imaging using IVIS systems [82] offers a systemic approach to analyzing the innate response to vaccines [83]. Working with a luciferase expressing viral vector, researchers were able to demonstrate that a TLR3 agonist was able to prevent the CNS spread of Venezuelan equine encephalitis virus.

**Role of IL-1β and MyD88**

The role of the cytokine interleukin 1 beta (IL-1β) and the inflammasome in innate immune responses to vaccines has been extensively studied. Whether alum activates the inflammasome *in vivo*, and whether this plays an essential role in its adjuvant activity, has been hotly debated [84, 85]. Interest in the inflammasome is by no means restricted to alum, however, and new adjuvant candidates are regularly screened for IL-1β release dependent on one or more inflammasome intermediaries [86]. As the downstream mediator of the IL-1 receptor, as well as an integral component in the signaling cascades of a number of PRRs and other signaling pathways, MyD88 plays a crucial role in the functioning of the innate immune system. This has been demonstrated for pathogens like malaria [22], adenovirus [87], and influenza [18], and for vaccine adjuvants [21, 88, 89] including our work demonstrating the role of MyD88 in responses to PorB [25]. As such, the effects of various vaccine formulations on MyD88-dependent processes have been studied intensely by our lab and others [9, 90].
As discussed above, there is a great deal of interest in designing or selecting vaccine formulations, specifically adjuvants and vehicles, which drive Th1 responses from the innate and adaptive immune systems. Recent work looking at the induction of a number of the key cytokines in this process has underscored the importance of MyD88 in driving both the induction of cytokines including IL-1β and Type I interferons, and the cellular response to them [88]. MyD88 has also been shown to be essential to the CD8+ CTL response to adenovirus-vector vaccines [87]. The authors conclude that this due to TLR ligands stimulating the release of IL-12. Work is currently ongoing to map out pathways beyond the dogma of MAPK and NF-κB activation by which MyD88 signaling can regulate innate immune activation and the generation of inflammatory responses [21].

Non-TLR ligand adjuvants are also capable of activating innate immunity through MyD88. The ISCOMATRIX adjuvant [91] has been shown to activate APCs and enhance cross-presentation of subunit vaccine antigens to CD8+ T cells in a MyD88 dependent manner [20]. The oil-in-water emulsion MF59 has also been shown to require MyD88 for its adjuvant effect, despite neither signaling through a TLR nor requiring the NLRP3 inflammasome that might generate IL-1β [89]. MyD88 has thus been shown to be essential for both humoral responses to vaccines in the generation of and signaling by cytokines and in cell-intrinsic processes such as antigen presentation. Additionally, as our lab and others have shown, many adjuvants are capable of directly stimulating
cells of the adaptive immune system, and MyD88-mediated pathways often play a role in such processes, as seen in the stimulation of B cells by VLPs [18] or PorB [25]. Conditional MyD88 knockouts in the relevant cell lines may be required to tease apart the compound roles played by this widely prevalent protein. It should be noted there are situations in which intact MyD88 signaling pathway is not essential for responses to vaccines. In particular TLR3 and TLR4 can both signal through the alternate TRIF/TRAF pathway. The MenC vaccine, for instance, has been reported to be effective in MyD88 KO mice [92]. In this case, alternative TLR4 signaling is likely responsible for activating the innate immune system.

Other formulation considerations

Designing a modern vaccine often requires the selection of one or more adjuvants. Historically the choice was simply whether to use alum or not; no other adjuvants were clinically licensed. More recently, the available choices have broadened to include additional vehicles, including oil emulsions such as Novartis’s MF-59, and TLR agonists such as the MPL in GlaxoSmithKline’s (GSK) AS04 combination formulation of MPL + alum [51]. Experimentally, of course, a far wider array of adjuvants and vehicles are available, with specificities for almost any PRR desired. Selection of a single candidate for regulatory approval, however, remains a largely empirical process, as seen in the efforts to select a single formulation for an effective malaria vaccine [23]. Current efforts to characterize the innate immune responses to adjuvants specific for each of the
various PRRs, and the nature of the adaptive response they generate, may however allow for more rational vaccine design in the near future. Difficulties arise, however, in the heterogeneous responses observed to single adjuvants, as seen in recent reports of Th17 responses to the TLR4 and TLR2 ligands [47, 93], usually used as examples of inducers of Th1 and Th2 responses. [3] Choices of model, either between strains of mice, or when transitioning from murine to non-human primate and/or human models, in addition to variability in route of vaccination [44], associated vehicles, and antigen, all complicate direct comparisons. PRRs specific to various adjuvants also see expression outside of the innate immune system, which can further complicate comparisons. Work in our lab has shown route-dependent effects of the TLR2 ligand PorB, as seen in its interaction with airway epithelial cells [94] when given as an intranasal vaccine to F. when given as an intranasal vaccine to F. tularensis [26]. Similarly, we have also observed it to have a direct effect on the adaptive immune system, where it triggers the tyrosine kinase and Erk pathways, leading to NF-κB activation in murine B cells [33]. Teasing out the innate-specific effects of vaccine components from their interactions with outside systems remains an ongoing challenge in the field.

**Vehicle design to trigger innate immunity**

In the past two decades, the design and selection of adjuvants has been at the forefront of subunit vaccine design. Given their ability to trigger Pattern Recognition Receptors, and the known effects PRRs have on the innate immune
system, this is of no surprise. Recently, however, an increasing focus has been placed on other aspects of the vaccine formulation. Specifically, the nature of the vehicle carrying the antigen and adjuvant has been the topic of a great deal of study, both in the development of new vehicles, and elucidating the contributions of said vehicles to the nature of the innate and adaptive immune response. In addition to the classical adsorption of antigen onto alum [14, 95-97], oil-water emulsions [44, 54, 96, 98, 99], liposomes [52, 100, 101], and more complex nanoparticles [49, 102] have all been studied.

There are numerous roles believed to be played by the vehicle in a vaccine formulation. The initial hypothesis for the effectiveness of alum, and one still discussed for new vehicles, is a depot effect whereby the association of the antigen with the vehicle keeps it held at the injection site, preventing washing out of the antigen and allowing more time for phagocytosis by APCs or interactions with the BCR of surveying B cells. Certain vehicles are theorized to affect antigen uptake and processing by APCs. This effect has been well studied in regards to TLR ligands and adjuvants [75, 76], and may play a role in some vehicles as well. Finally, certain vehicles may be associated with greater releases of cytokines or increased expression of costimulatory molecules on the surface of innate immune cells.

**Systems biology and assaying innate cells**

A discussed here, systems biology offers a suite of tools for analyzing the complex regulatory and signaling networks involved in the immune response to
vaccines. With knowledge of these networks, one can make queries of a model based on various assumptions about a vaccine candidate. The output of such a model can then be used as-is, as a hypothesis for the nature of the immune response that vaccine would provide. More thoroughly, if the nature of a protective response to the pathogen in question is known, it is possible to compare multiple vaccine candidates to determine which one would be hypothesized to have a response most closely mimicking that of known resistance to infection. From a more basic research approach, systems biology can allow researchers to examine the regulatory network itself, to understand which components are most essential in establishing robust immune responses [103]. From there it may be possible to proceed with more rational vaccine design targeted towards such essential or high-yield pathways.

One heavily used aspect of systems biology has been to mine the genomes of pathogens to find strong candidate antigens [104]. From such highlighted antigens vaccine design can then progress using a restricted set of proteins or peptides, greatly accelerating the process of vaccine development in a process often termed “reverse vaccinology” [105]. Common approaches include looking for evolutionarily conserved genes that will resist antigenic drift and selection, and identifying peptides or proteins with epitopes likely to be presented on Class I & II MHC, or that may generate protective antibody responses [106]. While I did not investigate antigen selection, the tools
developed for it [103] have applications in our work on those aspects of systems biology that relate to innate immunity, its pathways, and its functions.

There is an extraordinary wealth of information available relating the myriad interconnections between elements of the innate immune system. Signaling pathways, cell-cell communications, and regulatory networks have all been reported on for thousands of genes, proteins, and cells. Simultaneously, expression profiling using microarrays and deep sequencing can give us transcriptomes of tissues and cells of interest following stimulation with any vaccine formulation desired [96]. The challenger for the researcher, then, is finding and using the correct tools of the former to analyze the data of the latter. Given the large number of possible outcomes measurable for any adjuvant or formulation, head-to-head comparisons have been difficult in the past short of empirical clinical trials. Such trials, while the gold standard in selecting a final formulation with which to seek regulatory approval [23], are prohibitively expensive for all but the most promising vaccine candidates, and may slow the introduction of more experimental adjuvants. In contrast, systems biology approaches provide very broad analyses of vaccine formulations, allowing comparisons across as many outcomes as the researcher wishes to choose. A recent comparison of alum against MPL, for instance, was able to confirm narrow cytokine results with broad transcriptome evidence supporting an increased innate immune response to the TLR 4 ligand [3]. Similarly, a comparison of MF-
59, alum and CpG DNA used as adjuvants and sampling the injection site identified a core of 168 genes regulated by all three [96].

Systems biology approaches can also be used to study the efficacy of individual vaccines in various patient populations. A study of vaccine responses in children, including a comparison to atopic responses to allergens, demonstrated the importance of the Th1/IFN networks. In the immunogenic response to the diphtheria/acellular pertussis/tetanus and pneumococcal polysaccharide conjugate vaccines this network was balanced in the Th2 response, whereas it was blocked in the allergenic response [107]. Given the heterogeneity of the human patient population in contrast to inbred mouse models, describing responses across a range of backgrounds will be important in predicting the efficacy of vaccines that might be given to children, the elderly, or patients with compromised immune systems. Descriptive research like this works well to describe important regulatory networks and the key players among them. To be of greater use in the design of new vaccines and allow for greater in silico development, however, predictive models need to be accurately forecast the response to changes in vaccine formulation.

**Neisseria meningitidis porin PorB**

Molecules purified from pathogens and their derivatives can have many of the properties desired in adjuvants, driving cytokine release [108], adjuvant uptake [75], and APC activation [109]. This has led to the advancement of many such PAMPs as adjuvant candidates. Research in our lab, and work performed
for this dissertation, focuses on a protein purified from *Neisseria meningitidis*, a Neisserial major outer membrane protein, which is a porin and is termed Porin B (PorB). PorB is an outer member membrane protein and comprises 50-60% of the outer membrane protein content on the meningococcus [26, 27]. As a member of family of gram-negative porins, PorB forms a trimeric β-barrel structure on the outer membrane of the bacteria, and serves as a pore for ion exchange [24, 28]. When isolated and purified, PorB in suspension forms nano-scale structures known as proteosomes [24, 110]. Proteosomes are all-protein micelles, formed in the absence of any lipids, the presence of which would make the structure a liposome. The immunologic activity of the porins was first identified when using outer membrane vesicle preparations as vaccine candidates; it was recognized that PorB was capable of stimulating the immune system in the absence of other adjuvants [111, 112]. We have identified PorB as an agonist of TLR2/TLR1 heterodimers [2, 25] and reported its ability to act as an adjuvant when used in conjunction with a wide array of antigens, including proteins [111], polysaccharides [30], and liposaccharides [26]. In addition to identifying PorB as a TLR2/1 agonist, we have also made initial characterizations of the innate and adaptive response to the adjuvant *in vitro*: PorB increases surface expression of MHCII and CD86 on murine DCs and B cells and stimulates the release of Interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNF-α) [29-32]. We have also studied Mitogen Activated Protein Kinase (MAPK) activation in response to PorB in B cells, focusing on the Erk1/2 pathway [33, 34].
Despite the advances our lab and others have made in understanding the response of the immune system to PorB, many areas of its activity remain unexplored. The purpose of this thesis will be to address these other aspects of PorB immune stimulation and to answer questions that remain outstanding. Of note, while CD86 upregulation had been shown to be essential for PorB adjuvant activity and CD86 upregulation in response to PorB has been shown to be dependent on TLR2 and MyD88, as discussed above, the overall dependence of the adjuvant activity on TLR2 and MyD88 had not been conclusively demonstrated. This work also examines the effect of PorB on macrophage activation and activation marker expression, as has been examined for dendritic cells [90]. Characterization of the innate immune stimulating effects of PorB, to date, has focused on upregulation of cell surface proteins and secreted cytokines. While antigen uptake, processing and migration of APCs are known to be important for innate immune communication with adaptive immunity, the effect of PorB on these pathways had not yet been investigated, therefore, the effects of PorB on these pathways shall also been examined in this dissertation.

As discussed above, systems vaccinology has recently greatly expanded the range of tools available to study the response of the immune system to vaccine formulations. A survey of the transcriptome response to PorB was performed to enable an initial survey of the regulatory networks affected by PorB to potentially reveal previously unknown pathways and signals that could be related to PorB's adjuvant activity. Development of this resource would also allow
for future comparisons of PorB to other adjuvants. Analysis across the breadth of the multi-injection schedule was a new approach that had not previously been undertaken for any adjuvanted vaccine, and by doing so, would allow us to uncover previously unappreciated modes of regulation that would remain unseen if the responses to PorB at only a single time-point were investigated. Together, these goals allowed an approach that remained hypothesis-driven while still allowing for the discovery of unexpected results.

Conclusions

The adjuvant and vaccine development field has been making rapid progress in recent years thanks to a greater understanding of the roles played by innate immunity and their importance in the robust functioning of vaccines. There are multiple pathways through which innate immunity can influence the efficacy of a vaccine, and rational vaccine design must incorporate as much of this knowledge as possible. Once, it was sufficient simply to include an adjuvant or vehicle that would activate at least one PRR or otherwise activate the innate immune response. Now new research has shown that the selection of vaccine components plays an important role in cytokine profiles, targeting the vaccine to specific APCs, influencing antigen uptake, processing and presentation, and potentially other factors too numerous to classify by traditional molecular biology. With the sometimes bewildering array of options now available to vaccinologists, choosing the formulation that will interface with the innate immune system in
such a way as to elicit the desired protective response has become much more daunting.

Manipulating cytokine profiles in vaccine responses is a means to an end. With the identification of the transcription factors that regulate T helper subtypes, it has become possible to predict, with some confidence, the skewing of the response to some vaccine adjuvants. While "standardized" cytokine profiles have been developed for the most commonly used experimental adjuvants, comparisons between adjuvants are still difficult due to the relative scarcity of such reports in the literature, often conducted under a wide variety of experimental conditions. Multi-component formulations and adjuvants that elicit mixed responses also present difficulties. More challenging still is that while theoretical models may predict that one type of a response or other will be more effective in vaccines, robust correlates of protection do not exist for many of the pathogens with vaccines in development. From the perspective of the vaccinologist, then, the field appears to be at a tipping point. A great deal of basic science knowledge has been accumulated; the challenge is now to successfully apply it to translational and clinical applications in a rational, rather than empirical, manner.

As the above discussion has pointed out, there are an ever-increasing number of factors that must be considered in the design of a modern vaccine. Accurately assessing the impact of individual elements of a vaccine formulation, much less their interactions, may already be moving past the abilities of
traditional molecular biology. Systems vaccinology offers the promise of tools to handle the multitude of pathways involved in the response to complex vaccines and their implications for a heterogeneous patient population. The current state of the field has shown promise in identifying key regulatory pathways in vaccine responses and proposing hypotheses for the differences observed in responses within or between patient groups. Progress remains to be made, however, in efforts to predict the efficacy of novel vaccines or changes to current vaccine formulations.

The eventual goal of vaccine research is the development of protective vaccines for epidemiologically relevant diseases. Many of the most intensely studied targets are widespread pathogens that have resisted previous efforts to create fully protective vaccines, including Tb, HIV and malaria. Indeed, some positive progress is being made on these fronts. However, there exist a far greater number of emerging infectious diseases waiting in the wings, not to mention efforts underway to develop therapeutic vaccines to cancers. If any progress is to be made against these highly variable diseases in a rational manner, it will be by applying the lessons learned in the design of current vaccines. Although the antigens we use may bear no resemblance to each other, the elements of the innate immune system that interact with our vaccines will stay the same. By better learning how to harness the incredible power of innate immunity to recognize, alert and inform, we will be ready to meet these challenges.
Methods

Mice

Six week old female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). MyD88 KO [113] and TLR2 KO mice [114] (on the C57BL/6 genetic background) were a gift from Dr. S. Akiria (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). All mice were maintained within the Laboratory Animal Science Center (LASC) at Boston University School of Medicine under specific pathogen free (SPF) conditions. The Boston University Institutional Animal Care and Use Committee approved all research on animal models.

Subcutaneous vaccinations and serum collection

C57BL/6, TLR2 KO and MyD88 KO mice were vaccinated subcutaneously as described previously [29]. Vaccines were delivered via 28G needle (BD Biosciences) underneath the skin at the nape of the neck, behind the ear. Vaccine formulations used 10 µg of lyophilized chicken egg Ovalbumin (Ova) with or without 10 µg PorB in 100 µL PBS per mouse. Control mice were given sham vaccines containing only 100 µl PBS. 3 doses were given on days 0, 14 and 28; blood was obtained from the tail vein on days -1, 13, and 27. On day 42, mice were humanely euthanized with CO₂ and terminal bleeds were obtained via cardiac puncture. Sera were purified by centrifugation and were frozen at -80 °C until use.
PorB and Ova Purification

PorB was purified from *N. meningitidis* strain H44/76 Δ-1/4 that lacks PorA and RMP (reduction-modifiable protein) [115] as described previously [24]. Bacteria were plated on gonococcal agar plates containing 1% Isovitallex (Becton Dickinson) and grown overnight at 37°C with 5% CO₂. Colonies were inoculated in 50ml of liquid GC medium [110] containing 1.5% proteose peptone (Becton Dickinson), 1% Isovitallex, 0.5% sodium chloride, 0.4% potassium phosphate dibasic and 0.1% potassium phosphate monobasic (all Sigma Aldrich). Liquid cultures were incubated at 37°C with shaking for seven hours, then split 1:4 into 4 new cultures (50 ml final volume) in new liquid GC medium and incubated for another 7 hours at 37°C with shaking. Each 50 ml culture was then added to 1.5 ml of liquid GC media and incubated with shaking at 37°C overnight. Bacterial pellets were obtained by ultracentrifugation (Sorvall RC-5C Plus Centrifuge, Sorvall Products, Newton, CT, USA) at 5000 rpm for 20 minutes.

Crude protein extracts were obtained by resuspension of the pellet in 1 M sodium acetate containing dithiothreitol (Sigma Aldrich) as an antiprotease. 5% Zwittergent 3-14 (Calbiochem, San Diego, CA, USA) and 0.5 M calcium chloride were added to the slurry in a water bath sonicator (Branson 3200 Ultrasonic Cleaner, Branson Ultrasonics Company, Danbury, CT, USA). Ethanol was added to a final 20% v/v concentration to precipitate DNA, LOS, and other debris. The suspension was separated by ultracentrifugation as above, and
the total protein precipitated by adding 100% ethanol to the supernatant to a final concentration of 80% v/v and overnight incubation at 4°C.

The pellet was resuspended in buffer containing 50 mM Tris, 10 mM EDTA, 5% Zwittergent and 0.02% sodium azide, adjusted to pH 8.0 (loading buffer). Ion-exchange purification was performed using DEAE-Sepharose and CM-Sepharose (Amersham, GE Healthcare, Buckinghamshire, UK) columns (2.5 x 10 cm, Econo column; Bio-Rad, Hercules, CA, USA) at a flow rate of 1.8 ml/min. [24]. Flow-through was collected until the 280 nm absorbance returned to baseline, and the protein precipitated as above in 80% v/v ethanol and resuspended in buffer containing 100 mM Tris, 10 mM EDTA, 0.2 M sodium chloride, 0.05% Zwittergent, and 0.02% sodium azide, brought to pH 8.0 (washing buffer). Next, gel filtration chromatography was performed with a Sephacryl S-300 (Amersham) column (2.6 x 180 cm) at a flow rate of 0.25 ml/min. The porin containing fractions were identified by Coomassie staining of SDS–PAGE gels. Porin containing fractions were pooled and precipitated as above, and resuspended in loading buffer, but at pH 7.6. Removal of endotoxin and lipoproteins was performed using a Matrex Celluline Sulfate (Millipore, Billerica, MA, USA) column (2.5 x 10 cm).) [24]. The column was washed with washing buffer at pH 7.5, and a linear gradient of 0.2–0.5 M NaCl was applied. Porin containing fractions were detected and precipitated. Final resuspension was done in 10% d-octyl-glucoside (DOG) (Anatrace, Affymetrix, Santa Clara, CA, USA) with 10 mM Hepes, pH 7.2. Solubilized preparations were dialyzed
against 3 changes of PBS with 0.02% azide (>5 × 10^{10} times the original volume of the sample over 72 h) to remove the detergent [24]. During dialysis PorB forms lipid-free micelles known as proteosomes [110]; this preparation will be referred to as “PorB” from this point forward. Protein concentrations were measured using a BCA protein assay reagent kit (ThermoFisher Scientific, Waltham, MA, USA) as per the manufacturer’s protocol.

PorB activity was confirmed by stimulating HEK cells that had been stably transfected with a vector expressing TLR2, TLR4 or an empty vector [116] as has been described previously [117]. 5 × 10^5 cells were plated in each well of a 24 well plate (Corning) and stimulated with 10 μg/ml PorB for 24 hours. Activity was measured as the ability to induce IL-6 in TLR2 transfected cells, but not those containing the empty vector or TLR4. IL-6 was measured from the supernatant using an ELISA kit (BD OptEIA, Becton Dickson) following the manufacturer’s protocol. Pam3CSK4 (Invivogen) was used as a TLR2 positive control, N. meningitidis lipooligosaccharide (LOS) was used as a TLR4 positive control, and TNFα (Sigma Aldrich) was used as a universal positive control.

Ovalbumin was derived from chicken egg whites by freeze-drying followed by lyophilization and resuspension of the total protein in sterile PBS. Resuspended Ova was sterilized by passage through a 0.22 μm filter. Ova concentration was measured by the BCA assay as described above. Endotoxin contamination of Ova and PorB was monitored by silver staining of SDS-PAGE gels. Contamination was detected as ladder-pattern banding in PorB lanes.
equivalent to endotoxin positive controls. *E. coli* LPS (Sigma-Aldrich) and *N. meningitidis* LOS were used as positive controls.

**Coomassie Staining**

Coomassie solution was made by mixing 45 ml dH$_2$O with 45 ml methanol, 10 ml acetic acid, and 0.25 g Coomassie Brilliant Blue (Sigma Aldrich). Sonication was used to solubilize the Coomassie dye, and the solution was passed through Whatman 3MM filter paper (Whatman, Middlesex, UK) to remove any particulates. SDS-PAGE gels were added to Coomassie solution and incubated at room temperature for at least 4 hours. The gel was then washed with changes of dH$_2$O and de-stained with a solution of 25% propanol and 10% acetic acid in dH$_2$O until visible bands were clear. Gels were preserved by equilibrating in 4% glycerol and 10% ethanol, then dried on a frame (Owl Separation System, Portsmouth, NH) between two sheets of gel drying film (Promega, Madison, WI, USA).

**Silver Staining**

SDS-PAGE gels were placed in a well-cleaned glass petri dish containing 50 ml of a 40% methanol, 10% acetic acid solution and allowed to equilibrate with gentle rocking at room temperature for 30 minutes. Gels were then washed twice for 15 minutes with a solution of 10% ethanol and 5% acetic acid. Silver stain oxidizer concentrate (Bio-Rad) was dilute 1:10 with ddH$_2$O to a total volume of 20 ml, and incubated with the gel for 5 min. Gels were then washed 2 x 5 min
with ddH$_2$O. Silver stain reagent (Bio-Rad) was diluted 1:10 with ddH$_2$O to a total volume of 20 ml, and incubated with the gel for 20 min. Gels were then washed with ddH$_2$O for 1 min. Developer solution was made immediately prior to use by mixing 5 g of silver stain developer (Bio-Rad) with 150 ml ddH$_2$O. Wash water was removed from the gels, and 25 ml developer solution was added. Once the developer solution turned cloudy, it was removed, and fresh developer added. This process was repeated until bands were clearly visible on the gels, and the reaction stopped by adding 25 ml of 5% acetic acid. Then gel was then equilibrated in dH$_2$O and then dried as described above for Coomassie gels.

**Antibody and chemokines assays**

Mouse sera were assayed for antigen-specific immunoglobulins by enzyme-linked immunosorbent assay (ELISA) as previously described [29]. Immulon 2HB 96-well plates (ThermoFisher) were coated with 100 μl Ova (5 μg/mL) in coating buffer (0.2 M sodium carbonate in PBS, pH 9.4) and incubated overnight at 4°C. Lanes for the standard curve were coated with 100 μl goat anti-mouse F(ab')$_2$ (Sigma Aldrich) at 10 μg/ml under the same conditions. Plates were washed 3x with washing buffer (PBS + 0.05% Tween20 (ThermoFisher). 200 μl blocking buffer (PBS + 5% BSA (Sigma Aldrich) + 0.05% Tween20) was added and plates were incubated for 1 h at 37°C, then washed as above. Sera were sequentially diluted in washing buffer starting at 1:50 and added to the previously coated wells, and incubated overnight at 4°C. Standard curves were
generated using control mouse IgG (Sigma Aldrich) beginning at 800 ng/ml and
diluted using serial dilutions down to 24 pg/ml. Plates were washed as above,
then 100 µl of 1:10,000 dilution alkaline phosphotase-conjugated anti-mouse IgG
(Sigma Aldrich), or 1:5,000 dilution anti-subtype IgG (Sigma Aldrich) were added.
After 3 washes with washing buffer as above, the color was developed with 100
µl one-step p-nitrophenyl phosphate (Pierce, Rockford, IL) for 15 min and the
optical density (OD) at 405 nm was measured on an ELx800 reader (Bio-Tek
Instruments, Inc., Winooski, VT). A standard curve was made using the
colorimetric values obtained for total IgG control samples by plotting the average
of each control IgG concentration, finding the linear portion of the curve on a log-
log transformed graph, and calculating the regression line over that range. The
equation obtained for the regression line was then used to calculate the
concentration of IgG in each sample. IgG subtypes were reported as the optical
density (OD) of a 1:50 dilution of serum in PBS.

Generation of bone marrow derived macrophages (BMDM)

BMDMs were generated from the femurs and tibias of C57BL/6, TLR2 KO
and MyD88 KO mice [118],[118]. Following the removal of muscle tissue, marrow
was flushed from the bones with RPMI 1640 (Gibco, Life Technologies,
Carlsbad, CA, USA). Single cell suspensions were generated by disruption using
a 25G needle and passage through a 70 µm nylon mesh (ThermoFisher
Scientific). Erythrocytes were removed with lysis buffer (0.15 M ammonium
chloride, 0.05 M potassium bicarbonate, 0.5 mM EDTA), washed in PBS, and the
remaining cells pelleted, then plated in RPMI 1640 supplemented with 10% FBS
(Cellgro), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), 100 μg/ml
streptomycin (Sigma-Aldrich) and 20% 0.22 μm-filtered L929 (a M-CSF secreting
cell line) conditioned media. Cells were plated in 10 cm bacterial plastic
(ThermoFisher) plates. Media was changed every 3 days. Washing of the plates
with PBS was used remove loosely attached and unattached cells, purified
macrophage progenitor cells remained by adherence to the plastic. Before
experiments cells were removed from the plates by washing with 0.05% trypsin
and 0.53 mM EDTA (Cellgro) then seeded at the appropriate density.

**Removal of endotoxin protein contamination from LPS and LOS**

Lipoprotein contamination was removed from LPS and LOS samples
using double phenol extraction, as has been previously described [119]. *E. coli*
LPS (Sigma Aldrich) or *N. meningitidis* LOS (gift of Mike Apicella) was
resuspended in endotoxin-free water containing 0.2% triethylmine (TEA) (Sigma
Aldrich). Deoxycholate (DOC) (Acros Organics, ThermoFisher Scientific) was
added to a final concentration of 0.5%, followed by the addition of an equal
volume of water-saturated phenol. Samples were vortexed intermittently for 5
min, and the phases were allowed to separate at room temperature for 5 min.
Samples were placed on ice for 5 min, followed by centrifugation at 4°C for 2 min
at 10,000 x g. The top aqueous layer was transferred to a new tube and the
phenol phase was subjected to re-extraction with 500 µl of 0.2% TEA/0.5% DOC. The aqueous phases were pooled and re-extracted as above with 1 ml of water-saturated phenol. The pooled aqueous phases from the second extraction were adjusted to 75% ethanol and 30 mM sodium acetate (Sigma Aldrich) and allowed to precipitate at -20°C for 1 h. The precipitates were centrifuged at 4°C to 10 min at 10,000 x g, washed with 1 ml of cold 100% ethanol, and air dried. The precipitates were resuspended in 500 µl of 0.2% TEA.

BMDM Stimulation and IL-1β measurement

C57BL/6 BMDMs were seeded in 24-well plates (Corning) at 5 x 10^5 cells/well. 24 hours later, selected wells were stimulated in triplicate with one of the following TLR ligands: E. coli LPS double extracted in phenol chloroform to remove lipoprotein contamination, (100 ng/ml) (Sigma-Aldrich), Pam3CSK4 (100 ng/ml) (Invivogen, San Diego, CA, USA) or N. meningitidis porin PorB (10 µg/ml). 5 hours after stimulation, selected wells were stimulated with 5 mM ATP for 30 minutes. After 30 minutes, all supernatants were harvested and analyzed with the mouse IL-1β/IL-1F2 ELISA kit (R&D Systems)) using a modified protocol. 

Immulon 2HB plates (ThermoFisher) were coated overnight with 100 µl of 4 µg/ml of rat anti-mouse IL-1β (R&D Systems). Plates were washed 3x with PBS and wells were blocked with PBS for 1 hour at room temperature. Plates were washed and 200 µl of known concentrations of recombinant IL-1β (R&D Systems) or 1:2 dilutions of supernatant in PBS were added to the coated wells.
for 2 hours at 37°C. Plates were washed and 100 μl of 2.5 μg/ml biotinylated goat anti-mouse IL-1β (R&D Systems) was added to the wells. Plates were washed 3x with PBS and 100 μl streptavidin-HRP (R&D Systems) was added to the wells,, then incubated for 1 h at 37°C. After washing the plates 3x in PBS, 100 μl of a 1:1 mixture of H₂O₂ and TMB (R&D Systems) was added as a substrate for 20 minutes at room temperature. Absorbencies were read at 450 nm as above. IL-1β concentrations in the standard curve were used to calculate a regression line from the linear portion of the graph. The linear regression line was used to calculate the concentration of IL-1β in each supernatant sample.

**Generation of bone marrow dendritic cells (BMDC)**

BMDCs were generated from the femurs and C57BL/6, TLR2 KO and MyD88 KO mice as has been previously described [90, 118, 120]. Cells were isolated as above for BMDMs, but plated into 24 well plates (Gibco) in media containing 10% FBS (Cellgro), 20 ng/ml rGM-CSF (Sigma Aldrich), 50 μm 2-ME (Gibco), and 20μg/ml gentamycin (Sigma Aldrich). Cells were seeded at 5 x 10⁵ cells / ml. 700 μl of media was removed every 2 days, and replaced with fresh media containing rGM-CSF. On day 4, cells were split 2:1 and re-seeded in 24 well plates. Experiments were begun on day 6 without further re-seeding of the cells, to avoid additional handling and incidental activation of the DCs.

**Cell Stimulation and Antigen Uptake**
C57BL/6, TLR2 KO, and MyD88 KO BMDMs were seeded in 24-well plates (Corning) at $2 \times 10^5$ cells/well. 24 hours later, ovalbumin purchased with an Alexa-594 label attached (Molecular Probes, Life Technologies, Carlsbad, CA, USA) was added to the wells at 5 $\mu$g/ml. At this time, the cells were also stimulated with one of the following TLR ligands: E. coli LPS double extracted in phenol chloroform to remove lipoprotein contamination, (100 ng/ml) (Sigma-Aldrich), Pam3CSK4 (100 ng/ml) (Invivogen, San Diego, CA, USA) or N. meningitidis porin PorB (10 $\mu$g/ml). Cells were allowed to rest for 2, 4, 6, or 24 hours. Cells were detached with 200 $\mu$l of 4 mg/ml lidocaine HCl (Sigma) and 10 mM EDTA for 10 min at room temperature, followed by gentle scraping. Cells were washed twice in PBS and examined by flow cytometry. For fluorescent microscopy, cells were grown as above, but plated onto Lab-TekII chamber slides (Nalge Nunc, Naperville, IL, USA) at $1 \times 10^5$ cells/well. Cells were stimulated for 6 hours with 10 $\mu$g/ml Alexa-594 labeled Ova (Ova-A594) and the indicated TLR agonist or media controls. After stimulation, slides were washed 3 times with PBS and dehydrated with 2 washes of 95% ethanol and 2 washes of 100% ethanol. Two final washes were performed in xylenes, anti-fade mounting media (SlowFade with DAPI, Invitrogen) was added, and a coverslip (Corning, Corning, NY, USA) was placed over the slide. Slide corners were fixed with nail polish. Slides were stored at 4°C and covered until ready for use.

Dendritic cell antigen uptake experiments were performed as above, with the exceptions that DCs were not re-seeded at a lower density prior to
stimulation. Additionally cells were dislodged with vigorous pipetting prior staining with anti-CD11c PE (Becton Dickinson) for flow cytometry.

Fluorescent Microscopy

All fluorescent microscopy images were obtained on a Nikon deconvolution wide-field Epifluorescence system (Nikon, Tokyo, Japan) with a mercury-halide light source. Filters sets used were UV excitation/blue emission for DAPI, yellow-green excitation/red emission for Alexa-594. Images were captured using 40x and 60x oil immersion objectives. All images were captured using NIS Elements (Nikon) and analyzed using ImageJ (NIH). For absolute intensity comparisons, 14 µs exposures were used, based on the optimal exposure for the brightest samples. For high resolution and deconvolution imaging auto-exposure settings were used. Z-stacks for deconvolution were obtained by bracketing the in-focus region of the monolayer and applying 300 nm spacing to the slices. At least 10 slices were obtained for each sample. Deblurring was performed using the Parallel Iterative Deconvolution plug-in for ImageJ on a 16-core workstation with 32 GB of RAM. Theoretical Point Spread Functions (PSF) were generated using the Diffraction PSF 3D plugin, using the appropriate settings for each z-stack. Deconvolution was done using the 3D spatially invariant method and the pre-set parameters of the plugin.

Hock Vaccinations
A hock vaccination model to examine recruitment to draining lymph nodes [121] was used under the advisory of the BU IACUC. Briefly, mice were gently restrained, and a 31 G needle used to inject 10 μl of the designated vaccine into the lateral aspect of the ankle, avoiding all major blood vessels. Half of the mice were vaccinated with PBS in one ankle and 5 μg Ova-A594 in the second. The other half of the mice were vaccinated with 5 μg Ova-A594 in one ankle and 10 μg PorB + 5 μg Ova-A594 in the second. This allowed the contralateral ankle of each mouse to be used as an internal control, while also controlling for systemic versus localized effects of including PorB as an adjuvant.

**Lymph Node Preparation**

24 hours after hock vaccinations, mice were CO₂ euthanized, and the draining popliteal lymph nodes (LNs) removed by dissection. LNs were torn with tweezers and digested using 30 μl Collagenase D (ThermoFisher) in 1 ml PBS with Ca++ and Mg++ for 30 minutes at 37° C with gentle agitation to allow for easier separation of dendritic cells. A single cell suspension was generated by grinding the remaining tissue using a 70 μm nylon mesh (ThermoFisher). Cells were washed in PBS, counted, and stained as above for analysis using flow cytometry.

**Sectioning for Fluorescent Microscopy**
For fluorescent microscopy, mice were given hock vaccinations as described above. 24 hours post injection mice were euthanized, and the draining popliteal LNs removed by dissection. Nodes were embedded in Optimal Cutting Temperature (OCT) medium (Richard Allan Scientific, Kalamazoo, MI, USA) in molds (ThermoFisher) and flash frozen in an ethanol and dry ice mixture. Prepared nodes were stored at -80°C until ready for use. Sectioning was performed on a cryostat (Microm HM 550, Microm International GmbH, Walldorf, Germany). Blocks containing nodes were removed from molds and placed on mounts with a drop of OCT medium, which was allowed to freeze while the blocks equilibrated to the -20°C internal temperature of the cryostat. 20 μm slices were made until the node was visible at the surface of the block. 8 μm sections were obtained, and placed on lysine treated slides (Colorfrost Plus, ThermoFisher). Sections were air dried at room temperature for 1 hour, then fixed in acetone at -20°C for 8 minutes. Sections were dehydrated in successive ethanol baths of 2 x 5 minutes in 95% ethanol, followed by 2 x 5 minutes in 100% ethanol. Dehydrated sections were washed with 2 x 5 minute baths in xylenes, then mounted and counterstained in medium containing DAPI (SlowFade with DAPI, Invitrogen). Light microscopy was performed on a widefield epifluorescent microscope as described above.

Flow Cytometry
Flow cytometry [122] was conducted on a BD LSRII flow cytometer (Beckton Dickinson Biosciences, San Jose, CA). In brief, cells were harvested with gentle scraping and transferred to FACS tubes (Becton Dickinson Biosciences, San Jose, CA). Cells were washed with cold FACS buffer (0.2% BSA + 0.01% sodium azide in PBS) then stained for 30 minutes on ice with 0.5 μg of the indicated fluorescently labeled antibody in 150 μl of cold buffer. Analysis was performed in FlowJo (Tree Star, Ashland, OR, USA). Antibodies used: FITC or PE rat IgG2a, FITC anti-IAb, PE anti-CD11b, FITC or PE anti-CD11c, FITC anti-CD14, FITC anti-CD54, FITC or PE anti-CD40, FITC anti-CD89, FITC or PE anti-CD86, FITC anti-TLR2, and FITC anti-TLR4 (Beckton Dickinson Biosciences, San Jose, CA and Caltag, Burlingame, CA). Forward and side scatter gates were used to identify live cells.

Vaccinations for microarray experiments

Mice to be used in microarray studies were vaccinated on a 3 x 2 week schedule as described above. For the preliminary microarray experiment, C57BL/6 mice were separated into groups of three for each vaccine formulation. The formulations were 10 μg Ova, 10 μg PorB, 10 μg Ova + 10 μg PorB, or mock vaccinated with PBS only. All vaccines were delivered in 100 μl of PBS subcutaneously behind the neck. 24 hours after the third vaccination, mice were sacrificed and their spleens removed. Spleens were immersed in 1 ml RNAlater (Qiagen, Venlo, Netherlands) to preserve RNA content. RNA was extracted using
RNEasy kits (Qiagen) according to the manufacturer's instructions. Approximately one third of the spleen (~20 mg) was diced with sterilized scissors and then disrupted using a tissue homogenizer (PRO Scientific, Oxford, CT, US) for the initial processing steps. RNA collected from each sample was purified and stored at -80°C until all samples were collected. Purified RNA was checked for quality and concentration on a Nanodrop system (Thermo Fisher Scientific) to insure that all samples met the Microarray Core's minimum requirements for concentration and purity. Purified RNA was delivered to the Boston University School of Medicine (BUSM) Microarray Core for reverse transcription and analysis on Affymetrix Mouse Gene 1.0 microarray chips (Affymetrix, Santa Clara, CA, US). On delivery of the RNA to the Core, each sample was assayed using an Agilent Bioanalyzer to obtain a more detailed analysis of its composition. All of the samples were determined to meet the standards of the Core. Data analysis was performed in cooperation with an in-house bioinformaticist.

For the second microarray experiment, mice were divided into sample groups by number of injections and vaccine formulation received, resulting in a total of 10 different conditions. C57BL/6 mice were given 1, 2 or 3 injections of a vaccine formulation spaced 2 weeks apart. Formulations were 10 µg Ova, 10 µg PorB or 10 µg Ova + 10 µg PorB. Control mice were given three injections of PBS only. All vaccines were delivered in 100 µl of PBS subcutaneously behind the neck. 24 hours after the indicated number of vaccinations, mice were
sacrificed and spleens collected. To avoid any effects of RNA treatment, spleens were immediately processed for RNA purification using RNEasy kits as described above. Terminal exsanguination via cardiac puncture was also performed. Serum was purified using BD Microtainer serum separator tubes (Becton Dickinson). RNA was delivered to the BUSM Microarray core for analysis as described above.

**Microarray Analysis**

Statistical analysis of the first microarray experiment was conducted in collaboration with the BUSM Microarray core. Statistical analysis of the second microarray experiment was performed in collaboration with Adam Gower of the Clinical and Translational Science Institute (CTSI) of BUSM. Raw Affymetrix CEL files were normalized to produce gene-level expression values using the implementation of the Robust Multiarray Average (RMA) [123] in the "affy" package [124] included within in the Bioconductor software suite (version 2.10.0) [125] and an Entrez Gene-specific probeset mapping from BrainArray (version 14.0.0) [126, 127]. Empirical Bayesian (moderated) $t$ tests and linear modeling were performed using the limma package [128], and analyses of variance (ANOVAs) were performed using the sva package [129]. Correction for multiple hypothesis testing was accomplished using the Benjamini-Hochberg false discovery rate (FDR) [130]. Unbiased clustering analysis was performed to group samples by similarity of expression profiles. All statistical analyses were
same human Entrez Gene identifier were collapsed into a single value by averaging. After trimming genes with no human homologs and collapsing repeated genes 16,000 human gene IDs were left in each ranked file. GSEA software from the Broad was used for all analyses. Ranked lists were probed against the collected gene sets from Biocarta, Reactome and KEGG. This generated an initial list of ~2,800 gene sets. Parameters were set in GSEA according to the suggested guidelines to exclude any gene set with fewer than 15 or more than 500 genes represented in the gene lists, leaving ~2,000 sets. A
random seed was generated for each analysis, and all experiments were done with 1,000 repetitions.
Results

Chapter 1: TLR2 and MyD88 dependency of PorB immune activation and adjuvant activity

Our lab and others have previously demonstrated that PorB stimulates the innate immune system and acts as an adjuvant. We have also shown that PorB activates TLR2 [2] and induces pro-inflammatory signaling pathways in a TLR2 and MyD88 mediated manner [90]. We were interested, therefore, in determining the extent to which inflammatory processes in response to PorB, and the adjuvant activity of PorB, were dependent on intact TLR2 and MyD88 signaling. I investigated the ability of PorB to increase cell surface protein expression in macrophages using TLR2 KO cells. To test for overall adjuvant activity, I vaccinated TLR2 and MyD88 knockout mice with Ova with or without PorB to determine the extent of adjuvanticity conveyed by PorB in the absence of each gene.

Cell surface markers are upregulated on BMDMs following stimulation with PorB

Expression of cell surface proteins on APCs is both a measure of activation and, in cooperation with T cells, a mechanism of communication between the innate and adaptive arms of the immune system [109, 135]. We examined proteins associated with T cell stimulation, (CD40, CD86)) [136-138] and for APC motility (CD54) [135, 139], as well as other markers of BMDM activation (CD14, and CD69) [109],[109]. We have previously shown that PorB
induces upregulation of CD86 and MHCII on APCs, but not CD80 [30]. BMDMs from C57BL/6 or TLR2 KO mice were stimulated with PorB, or Pam₃CSK₄ or LOS as positive controls, for 24 hours, then stained for surface proteins and analyzed by flow cytometry. All five cell surface proteins were upregulated by PorB in cells from WT mice. CD14, CD40 and CD86 were not upregulated in cells from TLR2 KO mice following PorB stimulation, relative to unstimulated cells from TLR2 KO mice (Fig. 1). Unexpectedly, CD54 and CD69 were partially upregulated in cells from TLR2 KO mice following stimulation with PorB. All five markers were expressed at baseline in TLR2 KO cells stimulated with Pam₃CSK₄. All five molecules were upregulated in BMDMs from WT mice following stimulation with the TLR4 ligand LOS. LOS induced upregulation remained constant in cells from TLR2 KOKO mice.

Upregulation of CD14, CD40, CD54, CD69 and CD86 indicates that stimulation with PorB places macrophages in an activated state from which they would be well positioned recruit antigen-specific T cells and engage the adaptive immune system. Loss of CD14, CD40 and CD86 up-regulation in response to PorB in TLR2 KO cells demonstrates that TLR2 is essential in these signaling pathways, and may play a role in the partial loss of adjuvant activity by PorB observed in TLR2 KO mice below. That CD54 and CD69 were still partially upregulated by PorB in TLR2 KO cells, even when Pam₃CSK₄ induced upregulation was entirely ablated, implies that PorB may act partially through a TLR2 independent pathway. This finding may be related to the observation below.
that PorB still retains some measure of adjuvant activity even in TLR2 KO mice \textit{in vivo}. 
Figure 1: Upregulation of co-stimulatory cell-surface proteins by PorB is partially TLR2 dependent. BMDMs from WT or TLR2 KO mice were stimulated in culture with PorB, Pam3CSK4 or LOS, then stained for markers of activation and examined by flow cytometry. In WT cells, PorB increased surface expression of CD14, CD40, CD54, CD69 and CD86 as compared to unstimulated cells. Knockout cells had decreased expression of CD14, CD40 and CD86, but still had higher expression of CD54 and CD69 when compared to basal levels. In the latter two cases, the TLR2 ligand Pam3CSK4 had no effect on TLR2 KO cells. Histograms are from one representative sample of at least 3. Data represents one of two experiments.
Stimulation of cells by TLR ligands may induce increase expression in the cell of the cognate receptor to the ligand [9, 140]. To investigate the possibility that PorB creates such a positive feedback loop, we determined if there was increased surface expression of TLR2 and/or TLR4 on BMDMs following stimulation with PorB or LOS. Cells were stimulated with 10 μg/ml PorB or 100 ng/ml LOS as above in vitro for 24 hours and then assayed by flow cytometry. Stimulation with either PorB or LOS led to increased surface expression of TLR2 as compared to unstimulated controls; neither ligand increased surface expression of TLR4 (Fig. 2). This demonstrates that stimulation of innate immune cells with PorB has the potential to include a positive feedback loop specific for TLR2. Establishment of a positive feedback loop would aid in robust activation of the innate immune system, a desired feature in a vaccine adjuvant.
Figure 2: PorB increases surface expression of TLR2. C57BI/6 mouse BMDMs were stimulated with PorB or LOS. Stimulation lead to increased surface expression of TLR2 following stimulation with either TLR ligand when compared to media controls. Neither ligand, however, induced surface expression of TLR4. Histograms are from one representative sample of at least 3. Data represents one of two experiments.
PorB alone is not sufficient to activate the inflammasome

Activation of the inflammasome is a potential mechanism of action for adjuvants that is of significant interest [85, 141]. It is also a pathway known to be MyD88 dependent [142]. Stimulation with PorB was investigated to determine if PorB alone was sufficient to activate the inflammasome in vitro. Release of cleaved IL-1β requires activation of both signals for the inflammasome: increased pro-IL-1β synthesis and cleavage of pro-IL-1β by activated Caspase 1 [141]. C57BL/6 BMDMs were stimulated with PorB, LPS or Pam3CSK4. After 5 hours, exogenous ATP was added to half of the wells to trigger activation of Caspase 1 [142]. In the absence of ATP, none of the TLR ligands stimulated the release of IL-1β as measured by ELISA. However, increased mature IL-1β following ATP co-stimulation was observed for all of the TLR ligands tested including PorB (p<0.05) (Fig. 3). Failure to observe mature IL-1β release after stimulation with PorB alone indicates that activation of the inflammasome likely does not play a significant role in the adjuvant activity of PorB.
Figure 3: PorB stimulates IL-1β release only in the presence of ATP. WT BMDMs were stimulated with PorB, LPS, TNFα, or Pam3CSK4 at the indicated concentrations for 5 hours. After 5 hours, ATP was added to half the cells for 30 minutes. Supernatants were collected and analyzed by IL-1β ELISA. IL-1β release was not observed for PorB, LPS or Pam3CSK4 in the absence of ATP. In the presence of ATP, significantly increased IL-1β release was observed for all three TLR ligands (p<0.05). Data is representative of two independent experiments.
MyD88 and TLR2 are necessary for the maximal adaptive immune response to PorB

Previous work by our lab has shown that PorB is a TLR2 agonist [2], and that it functions as an adjuvant in vaccines [26]. We were interested in demonstrating that this adjuvant effect was dependent on intact TLR2 and MyD88. Previous research had shown that many immune stimulating functions of PorB were dependent on TLR2 and MyD88, but we had yet to conclusively demonstrate that signaling through these proteins is essential for overall adjuvanticity. To determine whether TLR2, or its downstream adapter protein MyD88, are required for the adjuvant activity of PorB, mice lacking either gene were vaccinated and antigen-specific antibody production was measured. TLR2 KO or MyD88 KO mice, in addition to WT C57BL/6 mice, were vaccinated with 10 µg Ova or a combination of 10 µg Ova + 10 µg PorB. Vaccines were administered subcutaneously (s.c.) on days 0, 14, and 28. The level of the humoral immune response generated towards the antigen (Ova) was then determined. Blood was collected from the mice on day 42, and the serum assayed by ELISA for Ova-specific IgG. Consistent with previous work, WT mice had significantly higher concentrations of Ova-specific IgG when vaccinated with Ova + PorB as compared to Ova alone (p<0.001) as determined by a two-tailed Mann-Whitney test, used due to the non-parametric distribution of IgG titers. TLR2 KO mice vaccinated with Ova + PorB had significantly lower concentrations of Ova-specific IgG than WT mice given the same vaccine, but higher levels of
IgG than TLR2 KO mice vaccinated with Ova alone (p<0.05). (Fig. 4a). For all mice, anti-Ova IgG in pre-immune sera was below the limit of detection (data not shown). Despite the slight retention in adjuvanticity of PorB in the TLR2 KO mice, the substantial decrease in IgG from the WT mouse model demonstrates the importance of TLR2 signaling to the adjuvant effects of PorB. It is also not clear if the small increase in antibody levels observed after vaccination with PorB as an adjuvant in TLR2 KO mice would correspond to any protective effect were these experiments repeated in a challenge model. In contrast, MyD88 KO mice vaccinated with Ova showed no increase in the level of Ova-specific IgG when PorB was added to the vaccine formulation. This demonstrates that intact MyD88 signaling is essential for the adjuvant activity of PorB, confirming the initial hypothesis. Taken together with the results of the TLR2 KO vaccinations, these data suggest that PorB may poses some TLR2 independent, MyD88 dependent, adjuvant activity that has not previously been reported.

We further investigated the dependence of the adjuvant activity of PorB on TLR2 to determine if it would be similar for the individual IgG subtypes. Subtyping IgG can additionally be used to characterize the immune response to PorB as a marker of potential skewing of the response towards a Th1 or Th2 phenotype [143]. The relative amount of OVA specific IgG1, IgG2b, IgG2c and IgG3 was determined in sera obtained from vaccinated mice two weeks after the third immunization (Day 42). The OD at a single, fixed dilution of 1:50 was determined as quantitation of IgG subtypes could not be performed in the
absence of suitable purified IgG subtype standards. WT mice vaccinated with Ova + PorB demonstrated an immune response dominated by IgG1 and IgG2b (Fig. 4b). These subtypes, in turn, were significantly suppressed in the responses of TLR2 KO mice given the same vaccine. IgG1 and IgG2b are both associated with a Th2 type response [144]. As both subtypes decreased in expression in TLR2 KO mice vaccinated with PorB as compared to WT mice administered the same vaccine formulation, it can be concluded that both the adjuvant activity of PorB and the T helper phenotype of the response it induces are dependent on TLR2-mediated processes. Interestingly, immunization of TLR2 KO mice resulted in a statistically significant (p<0.05) increase in IgG2c production as compared to WT mice, possibly indicating that the non-TLR2 mediated adjuvant activity of PorB may skew more towards a Th1 type response, as IgG2c induction is related to production of Th1 type cytokines (i.e. IL-12) [145]. However, the biological relevance of the phenomena is currently unclear.
Formulation and Background

**A**

Anti-Ova IgG (ug/mL)

- WT OVA
- WT OVA + Pore
- TLR2 KO OVA
- MyD88 OVA
- MyD88 OVA + Pore

**B**

- IgG 1
- IgG 2b
- IgG 2c
- IgG 3
Figure 4: Increases in antigen-specific IgG by PorB is dependent on TLR2 and MyD88. (a) Concentration of IgG antibody to Ova in C57Bl/6 mice as measured by ELISA in serum on from day 42. WT C57Bl/6, TLR2 KO and MyD88 KO mice were vaccinated on days 0, 14 and 28 with Ova, Ova + PorB or sham (PBS). Vaccines that included PorB showed significantly less elevation in IgG over vaccines that did not include PorB in TLR2 KO mice, and no detectable effect in MyD88 KO mice. None of the mice had detectable antibodies to Ova prior to vaccination (data not shown). (b) OD of specific subtypes of anti-Ova IgG at a 1:50 dilution of serum as measured by ELISA. In WT mice, IgG1 and IgG2b were the dominant subtypes indicative of a Th2-type response. These in turn decreased in the TLR2 KO mice, consistent with the total IgG data. n=4, data represents one of two experiments.
Chapter 1: Conclusions

In summary, I set out for this first Aim to characterize aspects of the immune stimulating activity of PorB and the reliance of these effects on intact TLR2 and MyD88 signaling. Evidence of increased cell surface expression of proteins in response to stimulus with PorB was expanded to include macrophages, as has been previously been demonstrated for dendritic cells [90] and B cells [33]. In contrast, it was discovered that stimulus with PorB alone is not sufficient to induce release of mature IL-1β. Surprisingly, while previous work in our lab has demonstrated that TLR2 is required for signaling in response to stimulus with PorB, it was observed that expression of CD54 and CD69 was still increased in response to stimulus with PorB in macrophages derived from TLR2 KO mice. This response to stimulation with PorB in the absence of its canonical receptor demonstrates that more of the molecular biology of PorB's interactions with the immune system have yet to be elucidated.

One of the goals of this research is to better understand the activity of PorB as a vaccine adjuvant. As discussed above, the responses of purified immune cell types to stimulation with PorB has been studied for dependence on intact TLR2 and MyD88. To expand these results to the use of PorB as an adjuvant, I examined the adaptive immune response in mice lacking TLR2 or MyD88 following vaccination with PorB as an adjuvant, and found that the loss of TLR2 significantly decreased the adjuvant effect of PorB, while the loss of MyD88 entirely ablated it. The presence of any adjuvant effect of PorB in the
absence of TLR2 was unexpected, and reinforces the conclusion reached from the observation of increased surface protein expression in cells derived from TLR2 KO mice, that significant questions about the immune stimulating effects of PorB remain to be answered.
Chapter 2: PorB induced antigen uptake and APC migration

Antigen uptake, processing and presentation have long been recognized as a key series of events in the communication between the innate and adaptive immune systems. Various TLR ligands have previously been shown to increase activity through these pathways, and this has been hypothesized to play a role in their ability to act as vaccine adjuvants. Given these reports, we were interested in determining if PorB was also capable of interacting with antigen handling. If so, indicate another pathway that could plausibly explain some of the adjuvant activity of PorB. To this end, I investigated a number of the early events in antigen presentation by APCs, including antigen uptake and migration of APCs from the periphery to the draining lymph nodes.

PorB increases antigen uptake in vitro at earlier time points than alternative TLR ligands

The effect of PorB on antigen uptake and APC trafficking was examined, as this could be directly related to the PorB adjuvant activity we have previously described. Increased antigen uptake and processing has been attributed to other TLR ligands [75, 76]. If the same effects could be observed for the nano-scale particles formed by PorB [24] then this could play a role in enhancing presentation by the innate immune system and subsequent activation of adaptive immunity during vaccination. A fluorescently tagged version of the same antigen used in vaccination experiments was chosen to allow for ease of comparison between in vivo and in vitro experiments. Alexa-594 has the advantage of being
stable to long-term light exposure as well as easily differentiated from the common fluorescent tags (FITC, PE, APC) used in FACS.

BMDMs from WT C57BL/6 mice were seeded in tissue culture wells and stimulated with Alexa-594 labeled Ovalbumin alone or in the presence of PorB on control TLR ligands. Pam3CSK4 was used as a TLR2-TLR1TLR1 heterodimer control, and E. coli LPS as a TLR4 control. In cells stimulated with Ova-A594 alone essentially 100% of BMDMs demonstrated fluorescence above background; change in geometric mean intensity (GMI) was therefore used to interpret the effects of TLR ligand co-stimulation. At 2, 4 and 6 hours after stimulation, cells stimulated with Ova-A594 plus PorB demonstrated significantly (p<0.05) higher Ova uptake than cells stimulated with Ova alone or Ova in combination with LPS or PAM3CSK4 (Fig. 5). At 2, 4 and 6 hours, LPS and PAM3CSK4 had no significant effect on Ova uptake. At 24 hours after stimulation, WT cells treated with any of the three TLR ligands had equivalent GMI under the same conditions. The Mann-Whitney test was used for all statistical analyses due to the non-normal distribution of data.
A

![Bar chart A](chart.png)

B

![Bar chart B](chart.png)
Figure 5: Stimulation of murine BMDMs with PorB increases early uptake of antigen in vitro. A) Geometric Mean Intensity (GMI) of the Alexa-594 signal from BMDMs stimulated with Ova-A594 and the indicated TLR ligand, for 2, 4, 6 or 24 hours. At 2, 4, and 6 hours, cells stimulated with PorB took up more Ova than cells stimulated with Ova alone or either LPS or Pam3CSK4. At 24 hours, cells stimulated with any of the TLR ligands had taken up equivalent levels of Ova-A594, and were all higher than cells given Ova alone. B) PorB driven antigen uptake in a TLR2 dependent and MyD88 independent manner. BMDMs derived from TLR2 KO mice show less uptake of Ova after 6 hours in the presence of PorB than WT-derived BMDMs under the same conditions. Conversely, BMDMs derived from MyD88 KO mice and stimulated with PorB for 6 hours demonstrate equivalent Ova-A594 uptake to WT BMDMs also stimulated with PorB. * p<0.05 n=4 data represents one of three experiments.
I next hypothesized that the increase in antigen uptake in response to PorB stimulation was dependent on TLR2 and MyD88. Previous work with these knockout lines has shown that intact TLR2 and MyD88 signaling are required for other PorB-driven inflammatory processes, including upregulation of cell surface proteins and secretion of cytokines [25, 90]. The above experiments were therefore repeated using macrophages derived from TLR2 KO or MyD88 KO mice. Loss of PorB driven increases in antigen uptake in these cells would indicate that the knocked out protein played a role in this process. As it has been shown above that both of these proteins were required for the full adjuvanticity of PorB, loss of increased antigen uptake in either knockout line could imply that increased antigen uptake may play a role in the adjuvant effects of PorB.

In BMDMs derived from TLR2 knockout mice PorB did not increase antigen uptake (Fig. 6). At 2, 4 and 6 hours post stimulation, there was no significant difference in antigen uptake between TLR2 KO BMDMs stimulated with Ova-A594 alone and cells co-stimulated with PorB, Pam₃CSK₄, or LPS. After 24 hours of stimulation there was no significant difference in antigen uptake between BMDMs stimulated with Ova-A594 alone and cells co-stimulated with PorB or Pam₃CSK₄. Across all time points, ANOVA comparison of cells stimulated with Ova alone and in conjunction with PorB showed no significant difference (p>0.05) in Ova-A594 uptake. Cells stimulated for 24 hours with the TLR4 ligand LPS, however, showed increased Ova-A594 uptake as compared to cells stimulated with Ova alone. As both PorB and Pam₃CSK₄ are TLR2 ligands,
this confirms a role for TLR2 in the observation that PorB increases antigen uptake. Moreover TLR2 appears to be essential for both the rapid antigen uptake driven by PorB and the slower antigen uptake observed in co-stimulation with Pam₃CSK₄. This effect is receptor specific, as loss of TLR2 had no effect on the TLR4 ligand LPS.
Figure 6: Antigen uptake in TLR2 KO BMDMs. Macrophages derived from TLR2 KO mice were stimulated with Ova-Alexa594 with or without the presence of TLR ligands PorB, Pam₃CSK₄, and LPS. After 2, 4 or 6 hours post stimulation, none of the TLR ligands had a significant effect on antigen uptake in the BMDMs as measured by flow cytometry. After 24 hours of stimulation, only macrophages co-stimulated with the TLR4 ligand LPS showed an increase in antigen uptake above macrophages stimulated with Ova-A594 alone. ANOVA analysis of all time points reveals no significant effect (p>0.05) of co-stimulation with PorB or Pam₃CSK₄. *: p<0.05 for Ova + LPS, n=4 data represents one of two experiments.
MyD88 is a key downstream mediator for the TLR2 signaling pathway, and the loss of MyD88 has ablated all signaling, inflammatory and adjuvant functions of PorB in which it has been studied to date [25, 90, 146]. As such, I hypothesized that the loss of MyD88 would entirely ablate the increased antigen uptake by BMDMs observed in the presence of PorB. When this hypothesis was tested, however, the loss of MyD88 showed no effect on increased antigen uptake when BMDMs were stimulated with PorB, Pam₃CSK₄ or LPS (Fig. 7). In parallel with results in cells derived from WT mice, at 2, 4 and 6 hours, cells stimulated with PorB showed increased Ova-A594 uptake as compared to cells stimulated with Ova alone. After 24 hours, cells stimulated with PorB, Pam₃CSK₄ or LPS all showed increased Ova-A594 uptake as compared to cells stimulated with Ova alone. ANOVA analysis comparing cells stimulated with Ova alone and cells stimulated with PorB confirms a significant increase (p<0.05) in Ova-A594 uptake across all time points.

Contrary to the initial hypothesis, MyD88 does not appear to play a role in mediating the increase in antigen uptake by BMDMs observed in the presence of PorB. Taken together with the results using cells derived TLR2 KO mice (Fig. 5b), I propose a TLR2-dependent, MyD88-independent effect of PorB. This pattern of regulation has not previously been reported for any activity of PorB. On hypothesis that could explain these effects would rest on the particulate nature of PorB proteosomes, whereby interaction of multiple PorB epitopes on the surface
of the cell, combined with particle recognition effects [147] is sufficient to induce antigen uptake.
Figure 7: Antigen uptake in MyD88 KO BMDMs. Macrophages derived from MyD88 KO mice were stimulated with Ova-Alexa594 with or without the presence of TLR ligands PorB, Pam3CSK4, or LPS. ANOVA analysis of all time points confirms that cells co-stimulated with PorB take up significantly (p<0.05) more Ova-A594 than cells stimulated with Ova alone. By 24 hours, stimulation with all TLR ligands is sufficient to increase Ova-A594 uptake over stimulation with Ova alone. *: p<0.05, n=4, data represents one of two experiments.
PorB drives a significantly increased fraction of BMDCs to take up antigen

During vaccination, dendritic cells play a key role in antigen trafficking and presentation to T lymphocytes [51, 57]. Given the finding that PorB increases antigen uptake in macrophages, I hypothesized that our observations of antigen uptake in macrophages would be replicated in dendritic cells as well. Dendritic cells were stimulated in vitro under the same conditions as macrophages. As the differentiation process did not induce 100\% CD11c positive cells, all samples were co-stained with hamster anti-mouse CD11c PE to gate on DCs. In contrast with the results we observed experimenting with macrophages, not all of the DCs took up the antigen, as determined by flow cytometry. For this reason, changes in the uptake of antigen were measured as an increase the fraction of Ova-A594 positive, CD11c positive cells (Fig. 8). The effects of stimulation with TLR ligands were therefore defined as samples for which a higher fraction of DCs were antigen positive as compared to cells given Ova-A594 alone for the same period of time.
Figure 8: PorB increases the fractions of DCs positive for Ova-A594, but not the quantity of Ova taken up into the cell. Dendritic cells were stimulated with Alexa-595 labeled Ova in the presence or absence of PorB. After 6 hours, cells were harvested, stained with αCD11c and examined by flow cytometry. A) Control cells mock stimulated with PBS, showing 68% of cells are CD11c positive. B) DCs (gated on CD11c+ cells) stimulated with 5 μg/ml Ova-A594. C) DCs stimulated with 5 μg/ml Ova-A594 + 10 μg/ml PorB. 62% of cells are positive for antigen, versus 51% of cells not stimulated with PorB. Figures are representative flow cytometry plots showing gating analysis. Data are summarized in Fig. 9.
At 4 and 6 hours in WT C57BL/6 cells, the addition of PorB to Ova in the media increased the fraction of Ova-A594 positive DCs above the background uptake of cells given Ova alone (Fig. 9). Neither LPS nor Pam3CSK4 had this effect. After 24 hours of stimulation, both PorB and LPS increased the fraction of DCs that took up Ova from the media. The parity between TLR ligands at 24 hours is similar to that observed for BMDMs, although it should be noted that at no time point did Pam3CSK4 increase the fraction of antigen-positive BMDCs. This may be due to a previously observed effect specific to murine BMDCs in which stimulation with the soluble TLR2 ligand Pam3CSK4 did not increase antigen processing and presentation, while stimulation with a particulate TLR2 ligand derived from Mycobacterium bovis did [148]. The addition of PorB also took longer to take effect in BMDCs, with no effect observed until 4 hours of treatment, in contrast with BMDMs where an effect was observable within 2 hours of treatment. As responses to TLR stimulation in DCs were delayed in BMDCs as compared to BMDMs, as seen in the response to PorB, 24 hours may also not have been enough time to observe the effect of Pam3CSK4 on antigen uptake in BMDCs.
Figure 9: PorB increases antigen uptake in dendritic cells. BMDCs were stimulated with Ova-A594 and TLR ligands; antigen uptake was measured by flow cytometry. Antigen uptake was measured as the percentage of CD11c positive cells that had a signal in the Alexa-594 channel above background. At 4 and 6 hours after stimulation a significantly higher percentage of BMDCs took up Ova-A594 when in the presence of PorB as compared to co-stimulation with LPS or Pam$_3$CSK$_4$, or when given Ova alone. After 24 hours of stimulation BMDCs stimulated with PorB or LPS had similar fractions of Ova-A594 positive cells, both higher than cells stimulated with Pam$_3$CSK$_4$ or Ova alone. *: p<0.05, n=3, data represents one of three independent experiments.
PorB drives increased antigen uptake in DCs in a TLR2 dependent, MyD88 independent manner

With the confirmation that stimulus with PorB increased antigen uptake in DCs similar to how it affected macrophages, I next repeated the DC antigen uptake experiments using cells derived from TLR2 KO and MyD88 KO mice. As with the antigen uptake experiments on macrophages, using the knockout cell lines would confirm that the observed effect of increased antigen uptake was dependent on the known TLR2 binding activity of PorB. The results of the experiments using DCs derived from knockout mice were in line with those observed in macrophage cells. Cells derived from TLR2 KO mice did not increase their uptake of antigen in response to stimulus with PorB in comparison to cells stimulated with Ova alone at any time point, and at 24 a higher fraction of cells took up antigen after stimulus with LPS (Fig. 10). This confirms that increased antigen uptake in response to stimulation with PorB is a TLR2 dependent process. Dendritic cells derived from MyD88 KO mice and stimulated with PorB did take up more antigen than cells given Ova alone at 4, 6, and 24 as, comparable to the results obtained from DCs derived from WT mice (Fig. 10). At 24 hours, dendritic cells derived from MyD88 KO mice and stimulated with LPS took up more antigen than cells given Ova alone. These results confirm the TLR2 dependence, and the MyD88 independence, of the increase in antigen uptake observed in macrophages is recapitulated in DCs.
Figure 10: PorB drives antigen uptake in DCs in a TLR2 dependent and MyD88 independent manner. Dendritic cells derived from TLR2 KO or MyD88 KO mice were stimulated in vitro with Ova-Alexa594 with or without PorB, Pam3CSK4 or LPS for 2, 4, 6 or 24 hours. A) Cells derived from TLR2 KO mice show no increase in antigen uptake in response to stimulation with PorB at any time point. At 24 hours stimulation with LPS is sufficient to increase antigen uptake as compared to cells given Ova alone. B) Cells derived from MyD88 KO mice show increased antigen uptake in response to stimulation with PorB at 4, 6, and 24 hours, and increased antigen uptake in response to stimulation with LPS at 24 hours. *: p<0.05, n=6, data represents one of two experiments.
Increased antigen uptake in response to PorB is visible under fluorescent microscopy

I next determined if the effects of PorB on antigen uptake observed by flow cytometry could be confirmed by fluorescent microscopy. The six hour time point was chosen, as this was when the largest effects of PorB on antigen uptake were observed in the flow cytometry studies. Widefield image comparisons demonstrate that qualitatively more antigen was taken up by cells co-stimulated with PorB as compared to cells given Ova alone, or along with LPS (Fig. 11). These results agree well with those obtained by flow cytometry. The effect of PorB on antigen uptake may appear greater in imaging studies than it does using flow cytometric analysis due to the greater dynamic range of the flow cytometer.
Figure 11: 40x fluorescent micrographs of WT BMDMs stimulated for 6 hours with Ova-A594 with or without TLR ligands. A) Alexa594-labeled Ovalbumin (10 μg/mL) B) Ova + LPS (100ng/mL) C) Ova + PorB (10 μg/mL). All images captured with a 14ms exposure; cells stimulated with PorB fluoresce brighter. Cells counterstained with DAPI. (Insets): Maximum intensity projections of the A594 channel made using deconvolution of z-stacks of regions of interest of A-C. There may be a change in the intra-cellular distribution of fluorescent antigen, with diffuse staining throughout the cytoplasm cells stimulated with Ova alone or in combination with LPS, and a more punctate distribution in the presence of PorB. Images representative of fields from three separate experiments.
In determining the subcellular distribution of Ova under each stimulation condition, it can be observed that in cells given Ova alone or in conjunction with LPS, the Ova appears diffusely distributed throughout the cytoplasm. In contrast, in cells co-stimulated with PorB, the Ova appears collected into punctate structures. It is possible that these represent endosomal structures, presumably phagolysosomes.

**PorB drives increased cellular recruitment to secondary lymphoid tissue when used as a vaccine adjuvant**

We were interested in determining if the adjuvant activity of PorB could be explained in part by increased recruitment of APCs to secondary lymphoid organs. Delivery and presentation of antigen in lymph nodes plays an essential role in the development of adaptive immunity and thus represents a possible pathway through which PorB may mediate a portion of its adjuvanticity. I chose a hock vaccination model to allow for each mouse to serve as its own internal control. In this model APCs from the vaccinated hock migrate to the draining popliteal lymph on the same leg that received the vaccine but do not cross the midline to the contralateral lymph node. This was confirmed by the absence of any antigen-positive cells in mice given labeled antigen in one leg only (data not shown).

Popliteal lymph nodes draining hocks vaccinated with Ova + PorB contained significantly (p<0.02) more cells 24 hours after vaccination than those
draining hocks vaccinated with Ova alone (Fig. 12). Draining lymph nodes from hocks vaccinated with Ova alone, in turn, contained more cells than lymph nodes draining hocks mock vaccinated with PBS. Vaccination of the contralateral leg with Ova + PorB versus PBS had no effect on the number of cells in lymph node draining a site vaccinated with Ova alone. Based on the absence of contralateral increases in lymph node cell count it is demonstrated that the effect of PorB on increased migration is local, rather than systemic.
Figure 12: Cell counts from the draining popliteal lymph nodes of mice given hock vaccinations of Ova with or without PorB. Draining popliteal lymph nodes were collected 24 hpi. Draining lymph nodes of hocks that had received PorB in addition to Ova had significantly higher numbers of cells than those receiving Ova alone or PBS. There was no increase in cellularity after receiving PorB in the contralateral hock. *p<0.02, n=8 data represents one of two experiments.
Vaccination with PorB as an adjuvant increase the fraction of DCs in the draining lymph node that contain antigen

I next hypothesized that the inclusion of PorB into the vaccine formulation would have the same effect on APCs in vivo as it did in vitro. I chose to examine dendritic cells for this study as they play a critical role in antigen presentation within the lymph node. In lymph nodes draining hocks vaccinated with Ova + PorB, a higher percentage of dendritic cells were found to be antigen positive, as determined by the presence of an Ova-A594 signal above background, when compared to lymph nodes draining hocks that had been vaccinated with Ova alone (Fig. 13). Mock vaccination with PBS resulted in no antigen-bearing dendritic cells even when the contralateral leg was vaccinated with Ova, confirming an absence of midline crossover and the validity of the internal controls (data not shown).
Figure 13: Vaccination with PorB as an adjuvant increase the fraction of antigen-positive dendritic cells in the draining lymph node. C57BL/6J mice were given vaccine injections in the left and right rear hock: 10 μg Ova-A594 to the left hock, 10 μg Ova-A594 + 10 μg PorB to the right hock. 24 hours post injection, mice were sacrificed and single cells suspensions from the draining lymph nodes were stained for CD11b and CD11c and examined using flow cytometry. CD11b/CD11c positive DCs were gated, and the proportion of A594+ cells quantified. Lymph nodes draining sites that had received adjuvanted vaccines had a significantly higher percentage of DCs carrying antigen. *: p<0.005 n=4, data represents one of two experiments.
A greater number and wider distribution of antigen bearing cells are visible in lymph nodes after vaccination with PorB as an adjuvant. For a more detailed investigation of APC and antigen trafficking to draining lymph nodes following vaccination I made fresh frozen sections of popliteal lymph nodes following hock vaccination and examined them under widefield fluorescent microscopy. 8 μm sections were made and acetone fixation was used to preserve tissue structures. Sections from a lymph node draining a hock vaccinated with Ova alone revealed sparse antigen positive cells around the periphery of the lymph node (Fig. 14). In contrast, lymph nodes draining hocks vaccinated with Ova + PorB had more antigen positive cells, and those cells penetrated into the interior of the lymph node. Control lymph nodes draining hocks mock vaccinated with PBS showed no antigen present (not shown).
Figure 14: Fluorescent micrographs of 8 μm fresh frozen sections of popliteal lymph nodes of mice, 24 hours post vaccination in the hock. Ova-A594 fluoresces red, sections were counterstained with DAPI in blue. A) Section of a node draining an ankle vaccinated with 10 μg Ova-A594. A few peripheral cells are antigen positive. B) Section a node draining an ankle vaccinated with 10 μg Ova-A594 plus 10 μg PorB as an adjuvant. Antigen positive cells are present in greater numbers and have penetrated into the interior of the lymph node. Micrographs are representative images of 8 nodes examined for each vaccination condition.
Ch. 2 Conclusions

In summary, I demonstrate here that stimulation with PorB acts to increase antigen uptake and migration by cells of the innate immune system. While these processes are known to be important during the immune response, and have been studied with regards to other TLR ligands, the effects of PorB on these functions of the innate immune system has previously not been known. I examined the effect of stimulation with PorB on antigen uptake in macrophages and dendritic cells, and found that antigen was taken up more rapidly when the cells were stimulated with PorB, as compared to stimulation with other TLR ligands, or in response to antigen alone. Based on previous work in the lab showing the dependence of PorB immune stimulating functions on TLR2 and MyD88, I repeated the antigen uptake experiments in cells that lacked either gene, and found that TLR2 was essential for the increase in antigen uptake observed in response to PorB stimulation, while MyD88 was not. This was not expected, as MyD88 has been shown to be essential in all previous effects of PorB our lab has studied. This suggests that PorB interacts with the antigen uptake pathway through a MyD88 independent pathway; such a pathway could make for a future study of interest. The demonstration that both antigen uptake and APC migration can be modulated simultaneously by PorB suggests that these are viable targets for further refinement in vaccine design and should be considered in the development of next-generation vaccine adjuvants.
Chapter 3: Transcriptome analysis of mouse splenocytes when PorB is used as a vaccine adjuvant in a multi-injection vaccine schedule

Microarray experiments have the ability to survey the complete expression profile of a tissue or cell sample [96]. Combined with advances in systems biology analysis techniques, it has become possible to characterize the entire response of a system to stimulus to a depth previously unobtainable. I was interested in using these techniques to study the response of the immune system to vaccination when using PorB as an adjuvant. The purpose was to confirm previous observations of immune pathways activated by PorB and to potentially reveal inflammatory networks not previously known to be influenced by the inclusion of porin. In total, two microarray studies were performed: an initial experiment that examined the effect of PorB only after the third of three vaccinations, and a follow-up experiment that investigated the effect of PorB after each of the three vaccinations.

Microarray analysis of the effects of PorB inclusion in a vaccine formulation on expression of genes in splenocytes

In designing the first microarray experiment, I was interested in determining the effects of PorB on the immune system, both alone and in combination with Ova. The same three vaccination schedule was used that we have previously demonstrated highlights the adjuvant functionality of PorB [26]. Sample collection 24 hours after the third vaccination was chosen as it was expected to maximize the potential for innate immune signaling. While PBMCs
have been a common choice source for RNA in human microarray studies [149], splenocytes were chosen to maximize both the quantity of RNA that could be recovered and the range of possible cell types RNA would be recovered from.

Once the expression values for each gene had been obtained, I was interested in determining if there were any significant differences between the mice receiving each vaccine formulation. ANOVA analysis of all of the genes from the microarray revealed 289 genes significantly regulated at \( p<0.001 \) across all experimental conditions. This \( p \)-value cut-off was chosen in consultation with the biostatistician. Although the number of regulated genes was relatively small compared to previous studies in the field of vaccinomics [149, 150], it was decided to proceed with further analysis. A clustering analysis was used to determine the primary divisions between the vaccine formulations, as well as how their patterns of expression in each sample related to each other. Unbiased clustering analysis of the 289 regulated genes did group mice from each vaccine formulation together. However, the primary division observed was between those mice that had received Ova (i.e. the Ova alone and Ova + PorB formulations) and those mice that did not receive Ova (the PBS and PorB alone formulations). This result, implying that the most significant factor in the splenic response was the presence of Ova, rather than PorB, was unexpected. To pursue the original goal of determining the effects of PorB inclusion, a second analysis was performed specifically contrasting mice that did or did not receive PorB as a component in the vaccine formulation. 56 significantly regulated genes at
p<0.001 (Fig. 15) were identified for which expression differed between mice that did receive PorB and those that did not. This is a very small number of genes, especially given the magnitude of the difference in antigen-specific antibody titers known to result from the inclusion of PorB as a vaccine adjuvant. Given such a surprising result, I hypothesized that the major regulatory activity of PorB on splenocytes may have occurred at a time point other than after the third vaccination, and that a new experiment would be required to determine when these regulatory effects occurred.
Figure 15: Gene regulation after the third of three vaccine injections. Mice were vaccinated with 10 µg Ova, 10 µg PorB, or 10 µg Ova + 10 µg PorB. Control mice were mock vaccinated with PBS. Significantly regulated genes were defined as those for which expression differed between the two groups receiving PorB and the two groups that did not. Genes were clustered by pattern of expression, and fold change represented as relative to the average of PBS controls. n=3, experiment performed once.
Microarray analysis after each of three vaccine injections

Based on the results of the first microarray experiment, I hypothesized that the critical regulatory steps that occur when PorB is used as a vaccine adjuvant may be occurring some time before the third vaccine injection. To determine if this was the case, a second microarray experiment was designed to investigate the splenic response after each of three vaccinations. The vaccination schedule and the choice of the spleen as the source of RNA were kept the same for the reasons outlined above.

Statistical analysis of the microarray data began with a principle component analysis (PCA). PCA is used to identify major subpopulations within the samples within which gene expression is significantly different from all other samples. The two principle components PC1 and PC2 accounted from 25% and 14% of all inter-sample variability respectively in gene expression. Clustering of samples from one or more conditions would have indicated major systematic changes in gene expression between individual samples. As all of the samples were obtained from the same source tissue (spleen) no such clustering was expected. PCA revealed no clustering of samples or significant outliers due to technical effects (Fig. 16).
Principal Component Analysis (PCA) across all genes in all samples

Figure 16: Principle component analysis of all microarray samples.

PCA was applied to all 30 samples of the second microarray data set. Clustering was not observed in either of the two principle components.
Our initial analysis of the microarray data was concerned with the kinetics of the response within each vaccine formulation. I knew from the earlier microarray experiment that by the third injection there were relatively few genes that varied between mice that did and did not receive PorB as an adjuvant in their vaccinations. To determine if PorB had an effect earlier in the schedule of the vaccination than the third injection, two models of gene regulation over time were constructed. The first was a linear or "monotonic" model. This model selected genes that varied in a continuous direction over the course of the vaccination schedule. Specifically, Day 0 was given an ordinal value of 0, Day 14 an ordinal value of 1, and Day 28 an ordinal value of 2. Genes were then scored according to how their expression across all three time points within a single vaccination conditions correlated with the model. That is, either a significantly regulated gene's expression might start at one point after the first injection, increase after the second, and increase still further after the third. Alternately, it could decrease after the second and still further after the third. The former scenario was reported as a positive t value with a high absolute magnitude, while the latter was reported as a negative t value with a similarly large absolute magnitude. This model represented what could be considered a classic model of vaccine response, with each successive vaccination generating a larger and larger response.

In mice vaccinated with Ova alone, 927 genes were determined to be significantly regulated (p>0.005) in a monotonic fashion. (Fig. 17) The higher p-value cut-off of p<0.005 was chosen in consultation with Adam Gower, due to the
more stringent filtering done before statistical analysis and the increased power of the Bayesian model used. Many of the upregulated genes, those for which expression increased over the course of the vaccination schedule, were those involved in cell cycle control and regulation. This category included a number of proto-oncogenes including Brca1 and Brca2, as well as cyclin and Cdk genes. Elements of the ATPase associated with its function in lysosomes were also observed to be upregulated, which may be related to increased processing of antigen for presentation. Genes downregulated in mice vaccinated with Ova alone included a wide range of T cell associated genes, including CD3, CD4, CD8 and the transcription factors Foxo1 and GATA3.

In mice vaccinated with Ova + PorB, only 77 genes were determined to be regulated (p<0.005) in a monotonic fashion (Fig. 17). Only six of these genes overlapped with those monotonically regulated in mice vaccinated with Ova alone. No significant patterns within the regulated genes were observed.
Figure 17: Regulated genes under a monotonic model. Gene expression from mice vaccinated with Ova alone or Ova + PorB were fit to a monotonic model of regulation following each of three injections (p<0.005).
The second model constructed was a V-shaped or "revertant" model. In developing this model, it was hypothesized that the major regulatory events initiated by PorB as an adjuvant occurred by the second vaccination. After the third vaccination, then, it would be expected that many of those genes would only be expressed at a level equivalent to the first vaccination. Such a pattern of expression would explain why in the first microarray experiment relatively few genes were seen that showed differential regulation after a third injection with the inclusion of PorB in the vaccine formulation: with the key regulatory steps completed, expression of those genes could have returned to a level commensurate with a vaccination that did not include the adjuvant. To model this predicted behavior gene expression levels at Days 0 and 28 were grouped together, and compared to day 14. Genes were designated as regulated in a revertant manner if their expression at Day 14 was significantly different that at Day 0 and Day 28 within a single vaccine formulation. A positive t value represented expression that increased at Day 14 and then declined, while a negative T value indicated expression decreased at Day 14 and then recovered.

In mice vaccinated with Ova alone, 69 genes were determined to be significantly regulated (p<0.005) in a revertant fashion. No significant patterns to the genes were observed. In mice vaccinated with Ova + PorB 384 genes were determined to be regulated (p<0.005) in a revertant fashion (Fig. 18). Upregulated genes, i.e. those for which expression peaked at day 14, included lysosome-affiliated elements of the ATPase proton pump, as seen in
monotonically regulated genes for mice vaccinated with Ova alone. Expression of multiple components of immunoglobulins, including heavy, light and constant regions, also peaked at Day 14. Downregulated genes included Calmodulin and components of the extracellular matrix. There were no genes regulated in a revertant manner in both mice vaccinated with Ova alone and mice vaccinated with Ova + PorB (Figure 19).
Figure 18: Genes regulated in a revertant manner in mice vaccinated with Ova + PorB. Mice were vaccinated with 10 μg of Ova + 10 μg of PorB and given 1, 2 or 3 injections 2 weeks apart. A) Regulated genes were defined as those which fit a model in which expression on Day 14 was significantly higher (red) or lower (blue) on Day 14 than on Days 0 and 28. Columns are individual mice. B) Gene Set Enrichment Analysis output for 4 genes sets determined to be positively enriched under the revertant model in mice vaccinated with Ova + PorB. Gene sets are representative of the inflammatory, immune signaling, and lysosomal pathways generally determined to be enriched in a revertant manner when PorB is included as a vaccine adjuvant.
Figure 19: Regulated genes under a revertant model. Gene expression from mice vaccinated with Ova alone or Ova + PorB were fit to a revertant model of regulation following each of three injections (p<0.005). There were no genes overlapping between the two formulations.
Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was chosen to interpret the large number of diverse genes returned as significant over the multiple comparisons. Additionally, the initial statistical analysis returned a large number of genes expected to be in related ontology categories at slightly under statistical significance. Using enrichment analysis of the entire genome made it possible to aggregate these genes to uncover statistically significant conclusions. GSEA analysis was performed for the each time course models for the Ova alone and Ova + PorB vaccine formulations, as well as pairwise comparisons between the two formulations at each time point. For each of the time course models, enrichment was determined as positive or negative: positively enriched or upregulated sets were those for which the genes in the set clustered with large positive t statistics, while the inverse held for negatively enriched sets. This was interpreted as general up or down regulation respectively of the pathway represented by those genes within the model. For the pairwise comparisons, t statistics represented the over or under expression of the gene in mice vaccinated with Ova + PorB relative to mice vaccinated with Ova alone. “Positive” enrichment thus indicated that the genes in the set were expressed higher at that time point in mice vaccinated with Ova + PorB relative to Ova alone.

Using the monotonic model there were 264 positively upregulated genes sets in mice vaccinated with Ova alone as defined by an FDRq<0.25. Many of these gene sets, including every member of the top twenty most enriched sets,
were related to the cell cycle and proliferative processes. In total at least 87 gene sets dealt with replication and associated processes, while only 3 were involved in inflammation or responses to pathogens. Additionally there were 392 negatively regulated genes sets in mice vaccinated with Ova alone. Negatively regulated sets included T cell processes, TLR signaling cascades, chemokines and innate immunity, and the MAPK and NF-κB signaling pathways. In contrast, there were no enriched gene sets in either direction under the monotonic model in mice vaccinated with Ova + PorB.

Using the revertant model there were 244 positively regulated (peaking at Day 14) genes sets in mice vaccinated with Ova + PorB at an FDRq<0.25. These sets covered TLR signaling, endocytosis and lysosomal digestion, cell proliferation, and signaling through multiple cytokines. Inflammatory signaling cascades predominated, including nine of the top twenty pathways (Fig. 18b). In addition to generalized inflammatory pathways, GSEA analysis also revealed similarities to signaling pathways for other receptors that have been targeted by experimental adjuvants. These included upregulation of genes seen in TLR3, TLR9, and RIG-I mediated inflammatory responses. At least 56 of these gene sets were related to immunity and inflammatory processes, with another 13 specific for proliferation. Additionally there were 132 genes sets negatively regulated under the revertant model in mice vaccinated with Ova + PorB. Downregulated gene sets included those for ion channels, cell adhesion and the ECM. Heatmap analysis of the Toll Receptor Cascade gene set positively
regulated under the revertant model in mice vaccinated with Ova + PorB shows a majority of the genes comprising the pathway peaking at Day 14, after the second injection (Fig. 20). This can also be seen in the GSEA output, demonstrating that the majority of the genes within the set have high positive t values under the revertant model.
A

Enrichment plot:

REACTOME_TOLL_RECEPTOR_CASCADES

B

O+P Day 0  O+P Day  O+P Day
28
Figure 20: Detailed analysis of a single gene set positively enriched in the revertant model of mice vaccinated with Ova + PorB. A) GSEA analysis of Reactome Toll Receptor Cascade gene set using a ranked analysis of the revertant model for mice vaccinated with Ova + PorB. The set is enriched for genes positively regulated after vaccination with Ova + PorB. This includes genes individually significantly regulated (p<0.005) as well as a number of genes just below the threshold for individual significance (0.005<p<0.01). B) Heat map of all of the genes in the Reactome Toll Receptor Cascade gene set. Gene expression was normalized across all mice vaccinated with Ova + PorB and fold changes calculated for each mouse. Genes were sorted by moderated t value using the revertant model.
I next determined the extent of the overlap between the Ova monotonic model and the Ova + PorB revertant model. Extensive similarities between the two would imply that the inclusion of PorB as an adjuvant resulted in an acceleration of the same regulatory events that would occur in mice vaccinated with Ova alone without activating any additional pathways. Only four genes were found to be significantly (p<0.005) regulated in both a monotonic fashion in mice vaccinated with Ova alone and in a revertant fashion in mice vaccinated with Ova + PorB. This is well within the probability of random chance, and suggests that there is no significant overlap between individual genes. However, as was shown in the initial gene set analysis, there exist a large number of gene sets enriched for upregulated genes under each of those conditions, even if few individual genes reached statistical significance. We therefore determined if there was significant overlap in the enriched gene sets overlap between the Ova monotonic and the Ova + PorB revertant models. 23 gene sets were positively enriched in both models. These gene sets included multiple members relating to the cell cycle, replication, and associated processes like DNA repair. This correlates with the initial analysis of gene sets from each model, wherein the revertant model of the mice vaccinated with Ova + PorB had all the cell cycle and proliferation gene sets of the mice vaccinated with Ova alone, but also were significantly enriched in inflammatory processes not observed in the monotonic model of mice vaccinated with Ova alone.
A detailed comparison was made between one gene set in the top 20 most highly enriched sets of both the Ova alone monotonic model and the Ova + PorB revertant model (Fig. 21). This was done to better understand the differences in the kinetics of the responses to each vaccine using a more easily manageable set of data. For this analysis the G2-M Checkpoint gene set from Reactome was used. From the GSEA data it is evident that the genes in the set are highly and uniformly regulated in a monotonic manner across the vaccination schedule in mice vaccinated with Ova alone. In contrast in mice vaccinated with Ova + PorB while the genes cluster towards positive correlation and fitting of the revertant model, it is not to the same extent as observed in their Ova monotonic counterparts. To examine this further a heatmap was made of the expression of all of the genes in the G2-M Checkpoint set and their expression in each of the mice across all of the time points for both the vaccine formulations. The average expression level for each gene across all of the samples was determined, and the fold expression of that gene in each of the samples relative to the average was plotted. In this analysis it is evident that in the mice vaccinated with Ova alone significant upregulation only occurs following the third vaccination. When the data for the mice vaccinated with Ova + PorB was examined, it was observed that the genes within the set were generally upregulated following the second vaccination, as predicted by the revertant model. However, one mouse demonstrated upregulation after only one vaccination, while another still showed increased expression after the third vaccination. While vaccination with PorB thus
does appear to increase the rapidity with which these genes were upregulated, it may also be introducing an element of variability to the response. It is this variability that may explain the lower t statistics for individual genes in mice vaccinated with Ova + PorB even while the gene set as a whole remained significantly enriched.
Figure 21: Comparison of gene expression between mice vaccinated with Ova alone and Ova + PorB. One gene set was selected that was positively enriched in both mice vaccinated with Ova alone under the monotonic model and mice vaccinated with Ova + PorB under the revertant model. A) GSEA output for the Reactome G2-M Checkpoint gene sets using ranked gene lists from both models. Genes cluster as highly significant under the Ova monotonic model, while they are less significant under the Ova + PorB revertant model. B) Heatmap generated from all the genes within the Reactome G2-M Checkpoint gene set for all of the mice vaccinated with each of the vaccine formulations sorted by moderated t value. It can be observed that clear partitioning is evident in the mice vaccinated with Ova alone. Mice vaccinated with Ova + PorB tend to show highest expression of these genes after the second vaccination although outliers are present.
The above results indicate a significant difference in the kinetics of the response to vaccines adjuvanted with PorB as compared to vaccines comprised of only the antigen. Two competing hypotheses could account for this difference. The first is that PorB does actually accelerate the observed response, resulting in larger and/or more rapid changes in gene expression in the mice that receive it. In contrast, PorB could be truncating the responses observed when mice were vaccinated with Ova alone, resulting in an earlier downturn in expression (Fig. 22a). To determine which of these hypotheses were correct, the rate of change of expression observed for each vaccine formulation was calculated.

To calculate the rate of change of gene expression following vaccination with each formulation those genes from mice vaccinated with Ova alone that fit the monotonic regulation mode were selected, as well as those genes from mice vaccinated with Ova + PorB that fit the revertant model. For genes from the Ova + PorB revertant model, the fold change in expression for each gene between the first and second vaccinations was calculated. As this change occurred over the two-week time period between vaccinations, the calculated value was the rate of change of expression per “interval” of time. The absolute value of fold induction was used to generate a one-tailed histogram. For genes from the Ova monotonic model, the fold change in expression between the first and third vaccinations was calculated. As this change in expression occurred over four weeks of time, rather than two, the change in fold expression were then halved to obtain the rate of
change over the same interval as the genes from the Ova + PorB model (Fig. 22b).
Figure 22: Rate of induction of genes after vaccination with Ova vs Ova + PorB. A) Model describing the two hypotheses tested. Ova + PorB Revertant represents gene expression in mice vaccinated with Ova + PorB over the course of three vaccinations for those genes expressed in a revertant manner. Ova Monotonic High and Ova Monotonic Low represent two possible patterns of gene expression in mice vaccinated with Ova alone. In Ova Monotonic Low, mice vaccinated with Ova alone achieve the same fold gene induction after three vaccinations that mice vaccinated with Ova + PorB achieve.
Monotonic Low, mice vaccinated with Ova alone achieve the same fold gene induction after three vaccinations that mice vaccinated with Ova + PorB achieve after two vaccinations. In Ova Monotonic High, mice vaccinated with Ova alone have comparable gene expression levels to mice vaccinated with Ova + PorB after two vaccinations, but continue to increase gene expression after the third vaccination. Differentiation between the two models is possible by determining the slope of each line, i.e. the rate of change of gene expression. B) The rate of fold induction for each gene regulated in a monotonic manner in mice vaccinated with Ova alone and each gene regulated in a revertant manner in mice vaccinated with Ova + PorB was calculated. Genes were plotted in a histogram with relative frequency on the y-axis to account for an unequal number of genes in each set. Mice vaccinated with Ova + PorB showed significantly higher rates of induction in gene expression as compared to mice vaccinated with Ova alone. *: p<0.0001.
Mice vaccinated with Ova + PorB had a higher median rate of change in gene expression than mice vaccinated with Ova alone, as measured by a non-parametric Mann-Whitney test (p<0.0001). This can be observed as the histogram of revertant Ova + PorB genes peaks to the right of the monotonic Ova alone genes (Fig 22b). The higher rate of induction when PorB is included in vaccine formulation confirms the hypothesis that PorB accelerates the kinetics of the vaccine response.

**Pairwise comparisons between Ova and Ova + PorB formulations**

In addition to studying the effects of PorB on the kinetics of the vaccine response, I was also interested in investigating specific pathways activated when PorB is present in a vaccine injection. To directly compare the effects of the inclusion of PorB as an adjuvant in the vaccine formulation at each time point pairwise comparisons were performed. At each time point, the expression of each gene was contrasted between mice that received Ova alone and mice that had received Ova plus PorB. A the suggestion of biostatistician Adam Gower this analysis was performed for just the genes in the top quartile by variance across all samples to increase statistical power.

24 hours after the first vaccine injection, 57 genes were expressed at significantly higher (p<0.01) levels in mice that had been Ova + PorB when compared to mice vaccinated with Ova alone. GSEA analysis using the moderated t statistics from this comparison revealed an additional 192 gene sets (FDR<0.25) that were significantly enriched in genes upregulated in the mice
that had received Ova + PorB (Table 1). These gene sets were predominately involved in cell cycle and proliferation. To determine the cause of the relatively large number of positively enriched gene sets given the small number of significantly regulated genes, the GSEA output was more closely examined. In many case the plurality of the genes within the set trended towards higher expression in the mice that had received the adjuvanted vaccine but individually did not reach the threshold for statistical significance. However, once taken in aggregate it appeared clear that these pathways were being enriched due to the addition of the adjuvant.
Table 1: Summary of regulated genes and gene sets using pairwise comparisons. Pairwise comparisons were made between mice vaccinated with Ova alone and Ova + PorB after 1, 2 and 3 injections with each vaccine formulation. Significantly regulated genes were defined as those for which the difference in expression between mice vaccinated with the two formulations had a p<0.01. Ranked lists using the moderated t values from the pairwise comparisons at each time point were used in Gene Set Enrichment Analysis.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 15</th>
<th>Day 29</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p&lt;0.01</td>
<td>p&lt;0.005</td>
<td>FDRq&lt;0.25</td>
</tr>
<tr>
<td>Ova</td>
<td>108</td>
<td>59</td>
<td>345</td>
</tr>
<tr>
<td>O+P</td>
<td>57</td>
<td>30</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>15</td>
<td>198</td>
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<td></td>
<td>53</td>
<td>28</td>
<td>107</td>
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After a single vaccination 108 genes were expressed at significantly lower levels in mice that had been vaccinated with Ova + PorB when compared to mice vaccinated with Ova alone. Using the same GSEA analysis as above, an additional 349 gene sets were determined to show enrichment in comparatively downregulated genes (Table 1). These gene sets included representatives of inflammatory processes, innate and adaptive immunity, and intra- and extracellular signaling.

By Day 14 and the second injection, the number of genes upregulated in mice that had received Ova + PorB relative to mice that had received Ova alone increased to 415, and the number positively enriched gene sets to 375 (Table 1). Specific genes more highly expressed in mice vaccinated with Ova + PorB include multiple components of the immunoglobulins and T cell receptors. Cell cycle related genes such as Brca1 and Brca2, as well as Cyclin E1, E2 and F and Cyclin dependent kinase (Cdk) 1 were also more highly expressed. Enriched gene sets include the same cell cycle and proliferation pathways observed after the first vaccination, but now also included gene sets involved in lysosomal degradation of proteins. A number of gene sets involved in T cell signaling through the T cell receptor (TCR) as well as CD28 and CTLA4 were also enriched in upregulated genes. This agrees well with previous research that has shown that PorB is able to convert T-independent adaptive immune responses to T-dependent responses. 257 genes were found to be expressed at lower levels after inclusion of PorB into the vaccine formulation, along with 187 gene sets.
These gene sets included elements of ion channel pathways, cell-cell adhesion, and cell-cell signaling. Interestingly IFN-α was observed to be downregulated with the inclusion of PorB in to the vaccine formulation.

Results after the 3rd and final injection mirrored those of the earlier microarray study. Only 53 genes were upregulated in mice vaccinated with Ova + PorB, as compared with mice vaccinated with Ova alone (Table 1). A total of 107 gene sets, however, could be identified as being enriched for positively regulated genes in the mice receiving Ova + PorB. These gene sets included innate and adaptive pathways, as well as MyD88 regulated events and gene sets identified as reactions to pathogens. At Day 28 only 29 genes were expressed at lower levels when mice received PorB in addition to Ova in their vaccine formulation. Differential gene regulation and enrichment in gene sets at each time point are summarized in Figure 23.
Figure 23: Comparison of relative expression between mice vaccinated with Ova and Ova + PorB after each of three vaccinations. Variations in individual gene expression (solid bars, left axis) due to the inclusion of PorB were highest after the second vaccination. GSEA (hashed bars, right axis) demonstrates a larger effect on the relative expression of gene sets due to the inclusion of PorB in the vaccine formulation after the first and second injections, decreasing by the third injection. As each gene may be included in multiple gene sets, and a gene set may be considered significantly regulated even if many of the genes comprising it are not individually significantly regulated, it is possible to have more genes sets regulated than individual genes.
The results after the final vaccination do not suggest that the third vaccination had no effect on the mouse at all. Mice vaccinated with either Ova alone or Ova + PorB each had over 500 genes differentially regulated to mock vaccinated mice after the third vaccination. Rather, then, by this point the two vaccinations appear to have developed similar patterns of expression (Fig. 23), while still maintaining significant effects relative to mock vaccinated mice.

We knew from the above analysis that expression of a number of gene sets peaked at Day 14 for mice given Ova + PorB, while mice given Ova alone expressed many gene sets highest at Day 28. I hypothesized this shift in expression within each vaccine formulation would be apparent when comparing formulation with each other. To make this comparison I looked for gene sets that were expressed higher at Day 14 in mice vaccinated with Ova + PorB when compared to Ova, and higher in mice vaccinated with Ova alone at Day 28. Remarkably, 148 gene sets matched these criteria, out of 375 expressed higher in Ova + PorB mice at Day 14 and 198 expressed higher in Ova alone at Day 28 (Fig. 24). These overlapping gene sets were overwhelmingly composed of those representing cell cycle and proliferation pathways.
A

O Alone Day 28
198 Gene Sets

O+P Day 14
148 Gene Sets
Cell Cycle and Proliferation

375 Gene Sets

B

43 Gene Sets
Proteosome

O+P Day 14
375 Gene Sets

O+P Day 0
192 Gene Sets

74 Gene Sets
Cell Cycle and Proliferation

O Alone Day 28
198 Gene Sets
Figure 24: Overlap between gene sets elevated after administration of a given vaccine formulation after each vaccination. Comparisons were made between mice vaccinated with Ova + PorB versus Ova alone at each time point. A) Overlap between gene sets positively enriched in mice vaccinated Ova + PorB after second the second vaccination and gene sets positively enriched in mice vaccinated with Ova alone after the third vaccination. B) Overlap between the above gene sets and gene sets enriched after the first vaccination with Ova + PorB.
Given the significant changes in relative expression between the time points I determined which gene sets maintained their expression across multiple time points. As has been shown, mice vaccinated with Ova + PorB had some similarities after one and two injections as well as with mice vaccinated with Ova alone after three injections. Performing an analysis of overlapping gene sets as described above revealed 74 gene sets in common between all three groups of mice (Fig. 24). These gene sets were predominately related to the cell cycle and proliferation. An additional 43 gene sets were found to be in common between mice given one and two vaccinations of Ova + PorB but not shared by mice given three injections of Ova alone. These gene sets included a number pertaining to formation and trafficking of lysosomes and proteasomal digestion. These findings agree well with earlier work demonstrating that PorB increases antigen uptake in APCs, likely for eventual antigen processing and presentation. While many genes showed similar expression after the first two injections of Ova + PorB, this changed drastically after the third injection. There were no gene sets enriched for upregulated genes after the third vaccination that were also upregulated after both the first and second injections of Ova + PorB.

Comparison of the mice after the third vaccination with Ova + PorB with earlier time points reveals that a substantial shift in gene expression occurs at the third vaccination. I determined how broad this effect was across the entire genome, rather than just those genes for which expression differed from mice vaccinated with Ova alone. To do this a new model was generated that grouped
expression on Days 0 and 14 together and looked for changes in expression as compared to Day 28. Only 67 genes were upregulated at Day 28 \((p<0.005)\) and 86 were downregulated, suggesting that this model does not describe the major trends in vaccination progression. However, 296 gene sets were found to be enriched for genes expressed lower after the third injection in mice vaccinated with Ova + PorB than after the first two vaccinations with the same formulation. Moreover, these gene sets were richly represented by innate and adaptive immune processes. These included the signaling cascades of both the T cell receptor and the B cell receptor, as well as TLR signaling. Additional pathways including IL-1, IL-7, CD28, and a number of downstream MAPK pathways were also represented. There were 103 gene sets enriched for genes expressed at higher levels after the third injection. These gene sets were a mix of ion channel activity and cell-cell interactions. The inclusion of a number of nerve and olfactory pathways in these gene sets suggest they may be noise generated by the large number of mouse olfactory genes present in the microarray which were collapsed when mapping mouse genes onto human homologs.

**Ch. 3 Conclusions**

My studies of the microarray analysis on the inclusion of PorB as a vaccine adjuvant reveal that the major regulatory events driven by PorB occur after the second of three injections, and include upregulation of genes and pathways responsible for cell division, replication, inflammation, and immune signaling. In contrast, mice vaccinated with Ova alone show a pattern of gene
regulation whereby gene expression increases steadily over the course of the vaccination schedule, with regulation occurring predominantly in genes and pathways related to cell division and replication only. As a result of these two factors, by the third injection, there are relatively few genes with differential expression between mice vaccinated with Ova alone as compared to mice vaccinated with Ova plus PorB. These results indicate the existence of a new paradigm for gene regulation over the course of multi-injection vaccination schedules when an adjuvant is used, and may need to be taken into account during the design of novel adjuvants and vaccine formulations.
Discussion

The use of immune adjuvants, other than alum, in human vaccines is beginning to be explored [14, 23, 101]. These adjuvants are essential for designing vaccines that avoid the potential dangers of attenuated or inactivated pathogens, while still eliciting strong protective immune responses. Therefore, there is great interest in developing and characterizing new adjuvant candidates with novel function and mechanisms of action [151-153]. Research into vaccine adjuvants has generally focused on identifying candidate adjuvants and measuring their efficacy in model systems [17]. While broad trends have emerged in the classes of compounds that are effective adjuvants [96], including the TLR ligands [16, 17], less work has been done to determine the specific molecular mechanisms by which individual adjuvants can be differentiated from one another. To address this issue, one of the aims of our lab’s research is to elucidate the mechanisms by which one specific TLR ligand, PorB, acts on the innate immune system and as a vaccine adjuvant. The unique set of pathways affected by each adjuvant is not fully understood, especially for newer adjuvants such as PorB [2, 27]. I set out to examine a number of possible pathways through which PorB may be stimulating the innate immune system; regulation of these pathways would represent possible mechanisms that could explain the adjuvanticity of PorB. Additionally, I hypothesized that the activation of these pathways occurs via signaling through the known TLR2 agonist activity of PorB.
In previous work, we have shown that the *N. meningitidis* OMP PorB acts as an adjuvant in vaccines, increasing the strength of adaptive immune responses to a range of antigens including polysaccharides, proteins, and *F. tularensis* LPS [26, 30]. We have also demonstrated that PorB is a ligand of TLR2/1 heterodimers [2] and requires Myo88 for efficient cell signaling [24, 90]. Analysis of the TLR2 dependent effects of PorB is therefore essential in linking its adjuvant activity to known pathways of innate activation. Such a demonstration would establish a clear causal chain, validating the proposed mechanism of adjuvanticity.

**Chapter 1: Innate immune activation and adjuvant activity of PorB**

The ultimate goal for any vaccine adjuvant is to elicit a robust antigen-specific response by the adaptive immune system [154]. As the canonical targets of many adjuvants, including PorB, are elements of the innate immune system, this requires efficient communication between the innate and adaptive arms. To this end, we were interested in the expression of surface proteins on APCs in response to PorB. Increased cell-cell communication has been identified as a mechanism of adjuvanticity for other adjuvants [51], and plays an essential role in the function of live attenuated vaccines that naturally include PAMPs [81, 137]. Previous research by our lab had identified MHCII and CD86 as upregulated on dendritic cells following stimulus by PorB [90]. As additional targets, proteins significant for T cell stimulation (CD40) [136], for APC motility (CD54) [139], and markers of BMDM activation (CD14, and CD69) [109] were chosen. Increased
surface expression in all of these proteins was observed following stimulation with PorB. If APCs stimulated by PorB have the same response in vivo, they would be well prepared to activate CD4 and CD8 T cells. This would corroborate previous work by our lab showing significant increases in the CD4 T cell dependent process of class switch recombination driven by the inclusion of PorB in vaccine formulations [26], and confirmed by the observation of significantly increased antigen-specific IgG in C57Bl/6 mice vaccinated with Ova + PorB as compared to mice vaccinated with Ova alone. Surprisingly, CD54 and CD69 expression in TLR2 KO BMDMs stimulated with PorB were still slightly elevated when compared to unstimulated cells. Additionally, while TLR2 KO mice vaccinated with Ova + PorB had anti-Ova IgG levels significantly below those of WT mice given the same vaccine, they still had higher antibody levels than TLR2 KO mice vaccinated with Ova alone. While we do not as yet have a confirmed mechanism for this weaker adjuvant activity, it does appear to be MyD88-dependent, as MyD88 KO mice vaccinated with Ova + PorB and anti-Ova IgG levels comparable to those vaccinated with Ova alone. A TLR2-independent mechanism of adjuvanticity for PorB could be important given the variability in TLR polymorphisms in humans and the role they can play in vaccine response [155, 156]. Even a sub-optimal response could afford some measure of protection in an individual who might otherwise be unprotected if they carried a TLR2 variant that was unresponsive to PorB.
The role of the inflammasome and IL-1β in vaccine adjuvant activity is a controversial topic, especially in regards to the mechanism of action of alum [85, 141]. In particular, while TLRs are known to increase transcription and translation of pro-IL-1β, activation of an NLR or AIM2 is still required to cleave pro-caspase-1 and begin the inflammasome cascade leading to the production of active IL-1β and IL-18 [10]. As *N. meningitidis* PorB is a pore-forming protein, and potassium efflux driven by exogenous ATP is sufficient to activate NLRP3 [157], we were interested to see if PorB alone would be sufficient to provide both signals to the inflammasome. This would provide a novel mechanism for a TLR-based adjuvant to stimulate the immune system and could possibly explain some of the TLR2-independent, MyD88-dependent effects we observed in other experiments. However, findings demonstrated that while PorB was indeed capable of promoting IL-1β release when given in combination with exogenous ATP, PorB alone, like Pam3CSK4 and LPS, failed to elicit a strong IL-1β response. The lack of IL-1β generated by treatment with PorB alone suggests an alternative explanation is needed for the weak adjuvant activity of PorB in TLR2 KO mice. The absence of inflammasome activation by PorB alone does not rule out the inflammasome playing a role in the adjuvant activity of PorB, but does imply that some other factor would be required to generate the second signal *in vivo*. Inclusion of such a factor into a vaccine formulation containing PorB could be an interesting avenue pursue.

Chapter 2: Antigen uptake and APC migration
In studying antigen uptake and trafficking, I expanded the scope of the innate immune network known to be affected by PorB. The role of TLRs and their ligands in increasing antigen uptake and processing has been well studied in the innate immune response to LPS and other PAMPs [75, 76]. The state of the field can now be extended to include PorB in the list of adjuvants known to affect antigen uptake. Our lab has previously demonstrated the importance of cytokine responses and TLR2 presence in the constellation of effects driven by PorB [25]. We have also shown that PorB upregulates activation markers and costimulatory molecules on APCs [90]. This research expands the scope of innate immune pathways known to be affected by PorB to include the antigen uptake and migration fields. The findings presented here support the hypothesis that increased antigen uptake in macrophages and dendritic cells is observed in response to stimulation with PorB. These results suggest that increased antigen uptake and APC migration could play a role in the adjuvant activity of PorB.

I first examined the ability of PorB to increase antigen uptake in macrophages and dendritic cells. It should be noted that a different measure of outcomes was required for macrophages and dendritic cells. In the BMDM experimental model essentially all of the macrophages took up at least some of the labeled Ova, even in the absence of a TLR ligand. This required the use of the geometric mean intensity as a marker; results were interpreted as an increase in the amount of Ova taken up by each cell. The conclusion, therefore, is that PorB increased the amount of antigen taken up by each cell at the earlier
time points. To explain the increased antigen uptake, it may be noted that PorB forms nanoscale proteosome aggregates in solution [24]. Physical size of particles has been implicated in modifying antigen uptake by APCs [147, 158, 159]. It is possible, therefore, that the physical size of the PorB proteosome accelerated the process of phagocytosis once the particle had attached to the surface of the cell via TLR2, with the labeled Ova antigen brought along through non-covalent adsorption to the proteosome. In this scenario the results observed in the presence of PorB would be similar to those of studies on intact pathogens, only using a much smaller delivery vehicle. In contrast, in dendritic cells the proportion of cells that took up the antigen increased in the presence of PorB. These results are consistent with the observation that the inclusion of PorB as an adjuvant increases the fraction of dendritic cells positive for antigen in draining lymph nodes. Together these results suggest that PorB may act in some way to convert dendritic cells from a quiescent state to one in which they actively take up antigen more rapidly than stimulation with a soluble TLR ligand.

In both cell types by 24 hours LPS (and Pam₃CSK₄ in macrophages) was equivalent to PorB in antigen uptake while still remaining higher than control cells given Ova alone. This suggests that either PorB acts to increase antigen uptake in an alternate manner to LPS or Pam₃CSK₄, or that it acts through the same pathways but in a more rapid manner. To differentiate between these hypotheses, we can look to the known effects of TLR ligands on antigen uptake and fit them where possible to the observed effects of stimulation with PorB. At
earlier time points the nature of antigen uptake in response to stimulation by PorB appears quantitatively different from that observed in response to stimulation with LPS. It was observed from the fluorescent microscopy studies that at 6 hours the distribution of Ova within macrophages appeared to differ between cells stimulated with LPS and PorB. This would not fit with a hypothesis that stimulation with PorB simply accelerates the same response seen in cells stimulated with LPS. Instead, at early time points, a TLR2 dependent, MyD88 independent process specific for PorB appears to drive increased antigen uptake in response to PorB. As mentioned above, the particulate nature of PorB proteosomes may be responsible for this observed effect. As a great deal of research has gone into the development of vaccine vehicles that incorporate PAMPs attached to nanoparticle structures [160-162], the demonstration that PorB may be utilizing particulate mechanisms to increase antigen uptake is significant because it represents a combination particulate vector and TLR ligand in a single molecule. At later time points, however, PorB, LPS and Pam3CSK4 may act through the same pathways to increase antigen uptake. Soluble ovalbumin is known to be internalized through mannose-receptor mediated endocytosis, and the mannose receptor is known to interact with TLR receptor dependent processes [163-165]. By the 24 hour time point, this effect could be responsible for the increased Ova taken up by cells stimulated with LPS and Pam3CSK4. As a TLR ligand, it is conceivable that PorB too increases mannose-receptor mediated endocytosis by the 24 hour time point. The above
observations of antigen uptake across all time points could therefore be explained if at early time points PorB acts to increase antigen uptake through TLR2 dependent particulate endocytosis while at later time points the synergistic activity of TLR signaling and mannose-receptor mediated endocytosis predominates for all TLR ligands. In this model PorB is uniquely suited to increase antigen uptake under multiple conditions as compared to alternate adjuvants.

Flow cytometry and microscopy studies jointly suggest a role for PorB in increasing migration of antigen-bearing APCs to draining lymph nodes from sites of vaccination. Given that PorB increases the percentage of antigen positive dendritic cells in the flow cytometry data while the prevalence of DCs out of all cells remains the same and the increase in the total size of the lymph node, it can be concluded that PorB must act to increase DC migration from the periphery. This is in contrast to the competing hypothesis that PorB acts solely to increase antigen uptake by resident DCs of the lymph node. As presentation of antigen by APCs to T cells in secondary lymphoid tissue is critical in robust adaptive immune responses, it is possible that increased migration of APCs may play a role in the effectiveness of PorB as a vaccine adjuvant. Further characterization of antigen-positive DCs in draining lymph nodes will allow us to better differentiate the relative effects of PorB immune stimulating activity on draining and resident DCs. Further study of frozen tissue sections may also aid in characterization of the antigen-positive cells within the lymph nodes and their
relation to other cell types. It would be of interest to determine if the inclusion of PorB into the vaccine formulation increased interactions between APCs and antigen-specific T cells.

Chapter 3: Transcriptome analysis and systems vaccinology

In my final aim, I analyzed gene expression in mice vaccinated with PorB and a test antigen (OVA) to better characterize the nature of the overall responses induced by PorB when used as a vaccine adjuvant. While a great deal has been revealed using vaccinomics to understand the responses to single injections of vaccines [103], much less is understood about the dynamics of multi-injections vaccination schedules. This aim therefore addressed two goals: opening a new region of the field by examining the course of a multi-injection vaccination schedule, and expanding the scope of known adjuvant regulatory profiles to include PorB.

Analysis of microarray data obtained only after three vaccination injections revealed minimal differences between mice vaccinated with Ova versus Ova + PorB. However, it is known that mice given the PorB adjuvanted vaccine produce significantly more antigen-specific antibodies by two weeks after a third vaccination, as seen in Aim 1. To explain this discrepancy it was hypothesized that key regulatory events driven by the adjuvant must have occurred earlier in the vaccination schedule. Our second round of microarray experiments thus focused on understanding the kinetics of the entire vaccination schedule to
determine both the effects of PorB as an adjuvant and when those effects were most apparent.

To examine the changes in kinetics observed when PorB is used as a vaccine adjuvant we constructed multiple kinetic models, and fit the expression profiles of mice given each vaccine to each model. The monotonic model described genes that either increased or decreased continuously over the course of the vaccination schedule. The revertant model, in contrast described genes that first increased in expression, then returned to their baseline levels. Gene regulation in mice vaccinated with Ova alone correlated well to the monotonic model ascribed to classic multi-injection dogma, but it is evident that such a model does not accurately describe the response to the adjuvanted vaccine formulation. Instead, it is found that a revertant model best describes the progress of the response to a PorB adjuvanted vaccine. More detailed analysis comparing the reactions individual mice within each group also suggests that the inclusion of PorB leads to a more heterogeneous response. That is, while gene upregulation trends earlier when an adjuvant is included, gene expression in some of the mice may peak after the first injection while other mice maintain elevated expression of relevant genes through the third injection. This revertant pattern of gene regulation represents a novel finding in the field of systems vaccinology, although it is not clear whether it applies only to regulation when PorB is used as an adjuvant, or if it could be representative of adjuvanted vaccines in general.
In addition to changes in the timing of gene regulation, recruitment of a significantly larger number of genes relating to innate immunity, TLR signaling, and inflammation with the inclusion of PorB as an adjuvant are also seen. As PorB is known to be a TLR2 agonist, this is not unexpected. However, this work does provide confirmatory evidence that these regulatory events do occur in the spleen, a secondary lymphoid organ, and are not restricted to localized activation of innate immune cells at the site of injection, where they have previously been studied [96]. Additional observations of T cell recruitment and activation also fall into line with previous research on the use of PorB as a vaccine adjuvant that demonstrate its ability to convert T independent adaptive immune responses to T dependent ones [30]. This pattern of innate immune and inflammatory gene induction by PorB is consistent with studies on other vaccines adjuvants [166, 167].

The question of whether the accelerated kinetics of gene expression profiles seen with PorB as an adjuvant can be generalized to include all adjuvants, or even all TLR ligand adjuvants, remains open. It was observed in our analysis of the GSEA results that the use of PorB as an adjuvant leads to the upregulation of genes comprising sets defined from those related to multiple other PRRs, including TLR3, TLR9 and RIG-I. Upregulated gene enrichment in these sets could imply that the responses observed after vaccination with PorB may also be seen in when the ligands for these other receptors are used as adjuvants. If so, this could imply that the kinetics observed in response to
vaccination with PorB as an adjuvant are generally applicable to vaccine adjuvants as a whole. The significant changes in responses observed after each injection imply that studying a single injection of a vaccine normally given in a multi-injection schedule does not accurately capture the entirety of the immune response as it develops over the course of the schedule. A single injection may be sufficient for many of the highly immunogenic vaccines that have been studied to date, and in these cases no longitudinal study would be required. However, both vaccines currently in use and in development are designed with the intent of requiring multiple injections in order to develop sufficient coverage and protection in the general population. Based on the current results, it would be expected that many of these vaccines, especially those employing adjuvants to complement less-immunogenic antigens, will not progress through gene regulation in a monotonic manner. Even if this has not been definitively established as a general characteristic of adjuvanted vaccines, the results presented here suggest that the potential for non-monotonic regulation needs to be taken into account.

Conclusions

The underlying theme of my work has been the characterization of the immune stimulating and adjuvant effects of PorB. I investigated multiple pathways through with PorB might stimulate the immune system for evidence of activity. I confirmed that treatment with PorB does increase surface protein expression on macrophages, while ruling out that PorB is sufficient to induce the release of IL-1β without additional stimulation. I demonstrated that stimulation
with PorB does increase antigen uptake by macrophages and dendritic cells, and also increases migration of antigen positive dendritic cells to draining lymph nodes. In areas where PorB was shown to have activity, I investigated whether this activity was TLR2 or MyD88 dependent. While in most cases both genes were required for the observed effect, a few surprising counter-examples were revealed as well. In particular, stimulation with PorB increased expression of CD54 and CD69 in the absence of TLR2, and increased antigen uptake in the absence of MyD88. These results, taken together with the observation that PorB still has a minimal adjuvant effect in mice lacking TLR2, suggest that immune signaling in response PorB is more complex than previously suspected.

While studies of the effects of PorB on cells in culture are essential for understanding the mechanics behind the activity of PorB, the eventual goal of this research is for its use as a vaccine adjuvant. To this end, it is essential to study the effects of PorB within a complete immune system, which requires animal models. In the research I have presented here, I directly tested whether PorB requires TLR2 or MyD88 to function as a vaccine adjuvant, and found that TLR2 is essential for maximal adjuvanticity of PorB, and that MyD88 is required for PorB to have any adjuvant effect at all. This agrees well with our lab's previous research into the requirements of PorB in stimulating individual pathways of innate immunity, and could support a hypothesis that they play a role in the adjuvant activity of PorB. In order to expand the scope of known immune stimulating activities of PorB, and to better understand the kinetics of the
immune response when PorB is used as an adjuvant, I examined the entire transcriptome of splenocytes during vaccination with PorB as an adjuvant using microarray techniques. This investigation revealed that, when PorB is used as an adjuvant, regulation in immune and inflammatory networks was observed that was not present in mice vaccinated with antigen alone. These in vivo results corroborate earlier findings in our lab into the innate immune stimulating effects of PorB in vitro. Additionally, when PorB was included as an adjuvant, gene regulatory events were accelerated, with expression in many gene sets peaking after the second of three vaccinations, while for mice that did not receive the adjuvanted vaccine expression in most genes was still increasing by the third vaccination. These results report new activity for PorB, and could suggest that some of its adjuvant effect is due to accelerated immune regulation.

My work expands our knowledge of the effects of PorB as an agonist of the innate immune system and as a vaccine adjuvant. I have characterized known immune responses in new cell types, and new responses not previously studied for PorB, both in wild type and knockout cells. These studies are important in that they both better characterize the responses induced by PorB, and suggest new hypotheses for mechanisms that could explain its adjuvant activity. In studying the adjuvant activity of PorB, I have demonstrated the necessity of TLR2 and MyD88 for PorB to have its full adjuvant effect, and in doing so, begun the process of determining which of the known immune stimulating effects of PorB are required for its adjuvanticity. This list of known
effects, in turn, has been expanded through the microarray analysis of gene regulation induced when PorB is used as a vaccine adjuvant. In observing accelerated gene regulation when PorB is used as a vaccine adjuvant, I have described an effect that is not only new for PorB, but has never been described in this manner for any vaccine adjuvant.

**Future Directions**

The broad direction of our lab’s research is to understand the functions of vaccine adjuvants in general, and PorB in particular. While the data presented here does extend our knowledge of the effects of PorB, it also opens up new avenues of research. As described above, evidence has been obtained that PorB possesses a TLR2-independent mechanism of adjuvanticity, based on the finding of increased antigen-specific antibodies when PorB is included in a vaccine given to TLR2 KO mice. While it is known that this adjuvanticity is ablated in MyD88 KO mice, the specific mechanisms at play are not yet known, although calcium signaling due to pore-forming activity by PorB has been suggested. A TLR2 dependent, but MyD88 independent, mechanism for PorB driven increased antigen uptake has also been observed. Further investigation into the interaction of PorB with antigen uptake may focus on demonstrating increased antigen processing and presentation on the surface of APCs in the presence of PorB, potentially related to the particulate nature of PorB proteosomes. Further investigation into the observation of potential changes to the intracellular distribution of Ova-A594 in the presence of PorB could be carried out through
labeling of endosomal compartments or analysis of peptide cleavage and presentation on Class I or Class II MHC. Given the increase in antigen-bearing APCs recruited to draining lymph nodes when PorB is included as a vaccine adjuvant, it would be interesting to determine if these APCs also associate more closely with antigen-specific T cells. Experiments using labeled T cells and either fresh frozen sections or two-photon microscopy [78, 81] have been used in the field to investigate similar questions.

The transcriptome analysis of the inclusion of PorB as a vaccine adjuvant could expand in multiple directions. Having established a profile for PorB as an adjuvant over the course of three injections, it would be interesting to expand the scope of the project to include additional adjuvants of relevance to modern vaccinology. Repeating existing experiments with additional adjuvants would enable highlighting the differences between each, and determining if the accelerated kinetics we observed in response to PorB are consistent across some or all adjuvants. A more comprehensive comparison between adjuvants could also allow for the rational design of future vaccines and the selection of specific adjuvants tailored to meet specific vaccine requirements for immune stimulation, innate and adaptive responses, or other criteria. Taking a more focused approach to the data thus far collected, the studies on PorB presented so far have uncovered regulation of a number of targets of interest. In particular multiple miRNAs have been observed that exhibited either revertant regulation when PorB was included as an adjuvant or were consistently expressed at higher
levels when PorB was included in the vaccine formulation. Experimentation to confirm the regulation of these miRNAs, and to determine their role, if any, in the immune response to PorB, would be a highly novel finding in the field of adjuvant immunology.
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Curriculum Vitae

Andrew Platt  
aplatt@bu.edu  
(503) 432-5988  
Born: 1985

Education

Graduate
Boston University School of Medicine  
Dept. of Microbiology  
Wetzler Lab  
Enrolled in the combined MD/PhD program

Undergraduate
Williams College, Williamstown, MA  
B.A. in Chemistry with Honors, Cum Laude  
Major: Chemistry  
Cumulative GPA: 3.72/4.0; Major GPA: 3.77/4.0  
Exeter College, Oxford University, England  

High School
Oregon Episcopal School, Portland, OR  
GPA 3.65, graduated Cum Laude

Research Experience
Boston University School of Medicine  
Graduate Student, Department of Microbiology  
Advisor: Prof. Lee Wetzler

• Investigated the effects of the Neisseria meningitidis outer membrane protein PorB in its use as a vaccine adjuvant
• Further refined the importance of receptor and signaling molecules TLR2 and MyD88 on the adjuvant activity of PorB
• Demonstrated that costimulation with PorB increases antigen uptake and migration by APCs
• Carried out a systems biology analysis on the effects of PorB when used as an adjuvant in a multi-injection vaccination schedule using microarray technologies

Williams College, Department of Chemistry  
Senior Thesis Research Student  
Title: Micelle Formation in Valine Containing Block-Copolymers  
Advisor: Prof. Sarah Goh

• Designed amphiphilic PEG-peptide block copolymers for micellar drug delivery vehicles
• Synthesized copolymers through solution phase peptide chemistry
• Quantified reaction conversions through the use of a small molecule probe for more reliable end group determination by NMR
• Improved aggregation studies by investigating alternative fluorophores for increased compatibility with our system
• Initiated experiments to probe the effects of peptide structure on micelle formation

Oregon Health & Sciences University
Research Assistant Summer 2006
Supervisor: Dr. John Morrison
• Examined neurotrophin levels in rat retinas in response to elevated intra-ocular pressure as a model system for glaucoma
• Applied protein measurement techniques including immunohistochemistry and Western blotting to quantitatively examine this relationship

Freightliner Corporation, R&D/E Department, Materials Division
Intern Summer 2005
Supervisor: Alan Prentice
• Analyzed corrosion resistance and structural strength of materials and structures used in large truck manufacturing
• Verified testing protocols and designed tests and fixtures to measure specific variables

Williams College, Department of Chemistry
Research Assistant Winter 2004
Supervisor: Prof. Chip Lovett
• Designed primers for site-directed mutagenesis of proteins of unknown function
• Gained experience working with radio-labeled substrates

Portland State University, Department of Biology
Research Assistant Summer 2003, 2004
Supervisor: Dr. Anna-Louise Reysenbach
• Catalogued the microbiological diversity of hyper-thermophilic bacteria and archaea in a sample from a Costa-Rican hot spring
• Constructed a 16s ribosomal DNA library
• Isolated and sequenced unique clones and compared to known species
• Identified of novel species and determined of relative population levels
Teaching Experience
Boston University School of Medicine  
Tutor 2010-2013
• Tutored medical students in the Biochemistry and Diagnosis and Therapy course
• Designed tutoring plans to address specific student weakness in coordination with the tutoring office
• Assisted students with preparation for Step 1 of the USMLE Board Exams

Teaching Assistant
Williams College, Department of Chemistry  
Fall 2006
• Assisted in the laboratory instruction of 20 students for Chemistry 153: Concepts of Chemistry – Advanced Section
• Graded weekly pre-lab assignments and notebooks
• Instructed students in laboratory techniques including qualitative analysis and titration

Awards
Eagle Scout  
National Merit Scholar Finalist

Publications
Platt AP, MacLeod H, Massari P, Liu X, Wetzler LM. "Innate Immune Responses to Neisseria Porin PorB and Subsequent Vaccine Adjuvant Activity are TLR2 and MyD88 Dependent" in preparation.  
Platt AP & Wetzler LM “Innate Immunity and Vaccines” Accepted, Current Topics in Medicinal Chemistry