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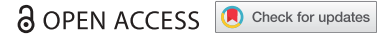
Neisserial PorB immune enhancing activity and use as a vaccine adjuvant

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ABSTRACT

Our laboratory has focused on Porin B (PorB), an outer membrane protein from *Neisseria meningitidis* and TLR2 ligand-based adjuvant, to characterize specific molecular and cellular pathways involved in improved immune responses induced by vaccine adjuvants. PorB's ability to form micellar nanoparticular multi-molecular organized structures and its interaction with Toll-like receptor 2/1 complexes likely accounts for its potent adjuvant activity. Downstream from this stimulation, we have observed enhanced antigen uptake in antigen presenting cells (APC), greater antigen deposition in secondary lymphoid organs, and promotion of germinal center reactions. In mice, antigen-specific IgGs were increased after PorB adjuvanted vaccination using the model antigen ovalbumin (OVA). Likewise, this formulation resulted in more IL-4 and IFN-γ positive T cells. Mice that received PorB adjuvanted vaccinations benefitted from lower bacterial burdens when challenged with recombinant *Listeria monocytogenes* expressing OVA. Mouse models lacking MyD88 signaling in various APC types helped identify macrophages as an essential cell type for the adjuvant activity of PorB. We believe the work presented here provides examples of the mechanistic studies required to understand how vaccine adjuvants are contributing to the establishment of protective immunity.

Vaccines are arguably one of the most important public health breakthroughs in modern medicine. Since the observation that live cowpox infection in dairy maids conferred immunity to smallpox, the field of vaccine development has made great strides in understanding the mechanisms by which immunological protection and memory are induced. With this knowledge, an evolution and diversification of the components used in vaccine formulations occurred. Immunologists have used both live and attenuated or killed pathogens as the antigen component of vaccines. These formulations are not without their respective risks, which range from a reversion of live pathogen to its virulent form or a failure to establish protective immunity by killed-pathogen. Subunit vaccines avoid these potential pitfalls by utilizing component portions of pathogens (either native or recombinant) and adjuvants to provide immune protection to the individual. Adjuvants are substances included in vaccine preparations to help promote an immune response to poorly immunogenic antigens and possibly skew the type of adaptive immune response that develops, potentially improving protection.[1] Aluminum salts are one of the earliest adjuvants added to vaccines and remain widely used today despite an incomplete understanding of their mechanism. In the past two decades, interest in understanding the mechanism of action of aluminum salts brought into focus an important question for vaccine-induced protection: How does the innate immune system influence the adaptive immune system to confer immunological memory and how do adjuvants effect this interaction[2,3]? The discovery of pathogen-associated molecular patterns (PAMPs) and

consequently, pattern recognition receptors such as Toll-like receptors (TLRs), provided the opportunity for well-defined cellular pathways to be exploited in order to gain further understanding about the cellular kinetics between innate and adaptive immunity.[4] Our laboratory has focused on the use of Porin B (PorB), an outer membrane protein from *Neisseria meningitidis*, as a TLR2 ligand-based adjuvant.[5,6] This commentary will focus on different mechanisms of action for adjuvants and the advantages of PorB which represents a single adjuvant that targets multiple mechanisms of action.

Role and mechanism of vaccine adjuvants

From a biological systems standpoint, adjuvants have relevance in every aspect of the immune response to a vaccine.[7] During the initial injection of the vaccine, adjuvants can act as proinflammatory effector molecules which promote the local secretion of proinflammatory cytokines and recruitment of antigen-presenting cells (APCs).[8] One such example is MF-59, an oil-in-water emulsion of squalene oil used in the current flu vaccinations.[9] MF-59 has been shown to increase innate cell recruitment to the injection site.[10] However, this characteristic is not unique to MF-59 as investigators have shown an increase in innate cell recruitment after administrations of other oil-in-emulsion compounds[11] or inorganic scaffolding.[12] The recruitment of innate cells to the injection site allows for antigen within the vaccine composition to be taken up via APCs and transported to secondary lymphoid organs such as lymph nodes, commencing the adaptive

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immune response. Accordingly, the extent of antigen trafficking to lymph nodes can be modulated using specific adjuvants. One such example is synthetic oligodeoxynucleotides containing unmethylated CpG which are used as TLR-9 ligand-based adjuvants.[13] When biochemically linked to a lipophilic tail, CpG has been shown to increase the accumulation of antigen within draining lymph nodes.[14] These studies emphasize the importance of the innate immune system during an immune response to a vaccine and highlight the need to determine how stimulation of these cells with adjuvants leads to better adaptive responses.

One of the most important influences an adjuvant has on the adaptive pathway is its ability to enhance responses of B cells and T cells within the lymph node. This stimulation relies on specific signaling pathways which help activate APCs, B cells, and T cells. TLR ligands, particularly as adjuvants, have been very well studied for this purpose. Specifically, these molecules can increase the expression of co-stimulatory factors on the surface of each of these cell types. The TLR-ligand interaction on APCs initiates intracellular signaling which can result in increases in CD40, CD80, CD86, and MHCI/II expression.[15] CpG has been shown to increase the expression of CD40, CD80, and HLA-DR on naïve B cells when stimulated in vitro.[16] There is also evidence that CD4$^+$ T cells can be directly activated by CpG and increase their expression of OX40 as well as the proliferative cytokine IL-2.[17] Furthermore, TLR ligands have been appreciated for their role in promoting the survival of activated CD4$^+$ T cells.[18] Co-stimulatory factors alone, however, are insufficient to fully activate the adaptive immune response as cytokine expression and various modes of cytokine-dependent signaling are required. Cytokine expression, from both APCs and T/B cells, creates a unique microenvironment which allows a skewing of the adaptive immune response towards more humoral or cell-based effector functions.[19] Again, TLR-based adjuvants have been well studied in this area. Generally, extracellular TLR adjuvants (TLR1, TLR2, TLR5, and TLR6) produce inflammatory cytokines whereas intracellular TLR adjuvants (ligands for TLR3 and TLR7/8)

primarily produce interferon cytokines, in vitro and in vivo.[15] With subunit vaccine research entering its third decade, there is now, more than ever, a need for studies that go beyond empirical observations and truly explore the mechanisms by which different vaccine compositions impart immunological memory. We are convinced that these studies are imperative for future vaccine development efforts and must include multiple adjuvants within their study designs to truly decipher mechanistic pathways.

Neisserial porin as a vaccine adjuvant

Our laboratory has focused on a specific TLR2-based ligand adjuvant, PorB, from Neisseria meningitidis. The past two decades of studies using PorB have elucidated how its structure influences its adjuvant activity in vitro and in vivo. In 2005, our lab enhanced a purification procedure to isolate PorB without contamination from lipooligosaccharide or other potential contaminants. This procedure, utilizing ion-exchange chromatography, gel filtration chromatography, and affinity chromatography, purified native PorB without any contaminants determined by silver stain and Western blot.[20] The final protein extraction was then dialyzed to remove detergent and to allow the formation of proteosomes (protein micelles devoid of detergent, approximately 10 nm in diameter (Figure 1). From our studies, we have found that proteosome assembly is likely necessary for PorB's activity as an adjuvant. These structures, described as "micellar multi-molecular organized structures", nanoparticulate in nature, allow for PorB to have a similar structure as oil-in-water emulsion adjuvants and may be partially responsible for its potent adjuvant activity. In vitro studies indicated that PorB proteosomes signal through TLR1/TLR2 complexes and allow for IL-8 production from TLR2 expressing HEK293 cells.[6] Further studies examined the effects of PorB on APCs. We found that PorB can induce dendritic cell (DC) maturation, and enhances in vitro antigen presentation.[21] In addition, PorB stimulation of bone marrow-derived macrophages (BMDMs) in vitro was shown to increase the production of
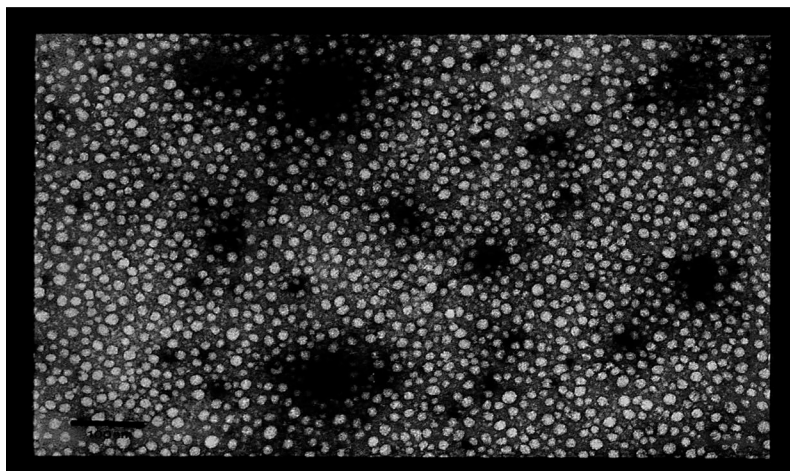

Figure 1. Electron micrograph of PorB proteosomes.
Micellar multi-molecular organized clusters of PorB termed "proteosomes". The scale bar is in the lower left corner and measures 100nm. Each proteosome is approximately 10nm in diameter. This electron micrograph was generated by Dr Lee Wetzler

IL-6, IL-1β, and TNF-α. Interestingly, and similar to the DC studies, PorB treatment also increased surface expression of CD40, CD54, CD69, and CD86 costimulatory factors in BMDMs.[22]

PorB has also been shown to increase total antigen-specific IgGs including IgG1, IgG2b, and IgG3 subtypes when used as an adjuvant.[23] These data suggest that PorB promotes both a Th1and Th2 antibody response, which is an ideal situation for producing a diverse array of protective antibodies. To decipher which innate cell type(s) were required for the PorB-induced increase in antibody response, our lab used transgenic mice to conditionally remove TLR-signaling in multiple professional antigen-presenting cell types. Specifically, we used Cre-Lox recombination to prevent MyD88 expression, the essential adaptor molecule for all extracellular TLRs, in APCs: macrophages (Lysm^cre), dendritic cells (CD11c^cre) or B cells (CD19^cre). PorB, CpG, and aluminum salts (alum) were all used to delineate cellular kinetics and mechanisms. Loss of MyD88 signaling resulted in a significant decrease of antigen-specific IgGs from all cre-floxed mice treated with TLR-based adjuvants with the greatest decrease observed in animals without macrophage MyD88 signaling.[23] Intriguingly, the germinal centers of these mice were also significantly decreased in both size and number when compared to wild-type control animals with intact MyD88 signaling. These studies emphasized the role of MyD88 signaling within macrophages for antibody production and germinal center formation, regardless of the TLR-based adjuvant used. A recent publication from our lab focused on the ability of PorB to increase antigen trafficking to the lymph node and cross-presentation of antigen by dendritic cells.[24] This observation led to our exploration of PorB's effect on CD4+ and CD8+ T cell populations. Most recently, our group has shown that PorB adjuvanted vaccination of mice using the model antigen ovalbumin (OVA) results in an increase of IL-4 and IFNγ positive CD4+ and CD8+ T cells. Most strikingly, when challenged with a recombinant intracellular pathogen expressing OVA, *Listeria monocytogenes* (rLmOVA), mice that received PorB adjuvanted OVA vaccination benefitted from lower bacterial burdens and an increase in survival when compared to OVA vaccination alone.[25] Together, these data highlight the unique characteristics of PorB which interacts with TLR1/2 complexes and signals in a MyD88-dependent fashion. As a vaccine adjuvant, PorB increases the expression of co-stimulatory factors and cytokines by APCs, increases antibody production, improves antigen trafficking to the lymph node, and promotes a broad T cell response including effective cross-presentation allowing for both CD4 and CD8 T cell responses.[23–25] A visual representation of PorB's effects in relation to vaccine adjuvanticity is displayed in Figure 2.

Conclusion

Optimization of vaccine compositions for the protection of humans against disease is an ongoing effort with the long-term goal of global disease eradication. Adjuvants play a critical role in this optimization, particularly for weakly immunogenic antigens like those used in subunit vaccines. Accordingly, there is a need to reveal the potentially diverse mechanisms by which novel adjuvants influence both the innate and adaptive arms of the immune response. Understanding how adjuvants stimulate a specific immune response will serve to both inform vaccine design efforts and answer some of the most fundamental questions in immunology. Here, we have described PorB, a molecule with unique characteristics as an adjuvant that improves both humoral and cell-based immunity. Beyond this, our studies of PorB have yielded insights regarding intracellular signaling, cellular dynamics, and cross-presentation of antigen during an adjuvant associated immune response. By continuing these types of studies, and communicating findings in a consistent manner,
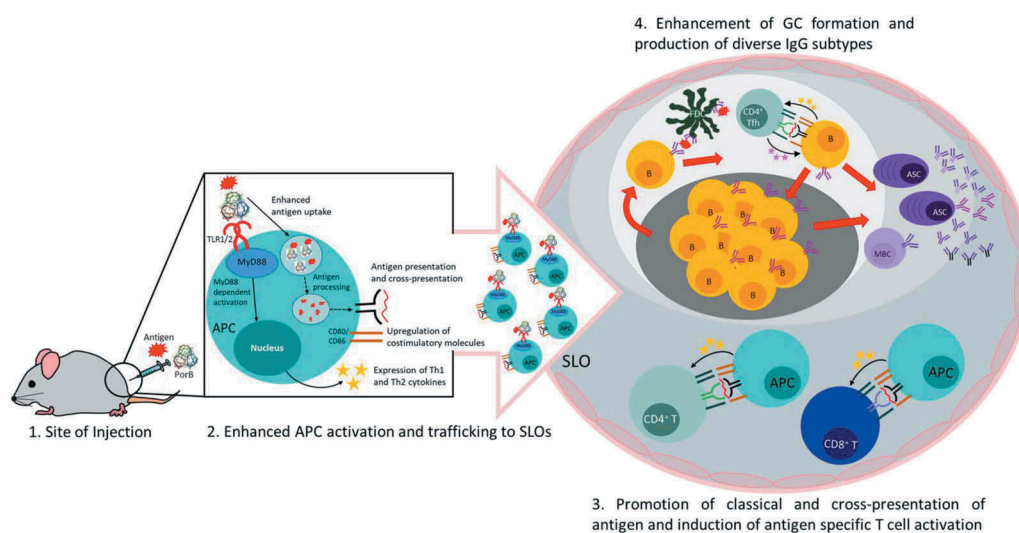

Figure 2. Effects of PorB as an adjuvant during vaccination.
(1) PorB recognition by TLR1/2 on antigen presenting cells (APCs) at the site of injection leads to TLR2 and MyD88-dependent signaling for increased costimulatory molecule expression and secretion of Th1 and Th2 cytokines as shown by Platt et al[22] and Mosaheb et al.[23] (2) Enhanced APC activation promotes trafficking of APC-antigen to secondary lymphoid organs (SLOs) and (3) antigen uptake and processing for classical and cross-presentation inducing activation of antigen-specific CD4+ (as shown in green) and CD8+ T cells (as shown in blue).[24] (4) PorB enhances germinal center (GC) formation and production of both Th1 and Th2 IgGs.[23,24]Follicular dendritic cell (FDC); CD4+ T follicular helper cells (Tfh); Memory B cell (MBC); Antibody secreting cell (ASC)

the vaccine field will continue to advance, allowing for improved vaccines. The prospect of reducing disease burden is exciting and of utmost importance especially when considering diseases for which there is no efficient vaccine such as HIV, TB, and malaria.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

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