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The PorB porin from commensal *Neisseria lactamica* induces Th1 and Th2 immune responses to ovalbumin in mice and is a potential immune adjuvant

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Abstract

Porins from pathogenic Neisseriae are among several bacterial products with immune adjuvant activity. *N. meningitidis (Nme)* PorB, has been shown to induce immune cells activation in a TLR2-dependent manner and acts as a vaccine immune adjuvant. The PorB porin from *Neisseria lactamica (Nlac)*, a common nasopharyngeal commensal shares significant structural and functional similarities with *Nme* PorB. In this work we ask whether the immune adjuvant ability of porins from pathogenic Neisserial strains is a characteristic shared with porins from non-pathogenic Neisserial species or whether it is unique for bacterial products derived from microorganisms capable of inducing inflammation and disease. We evaluate the potential immune adjuvant effect of *Nlac* PorB in mice using ovalbumin (OVA) as a prototype antigen. Immunization with *Nlac* PorB/OVA induced high OVA-specific IgG and IgM titers compared to OVA alone, similar to other adjuvants such as *Nme* PorB and alum. High titers of IgG1 and IgG2b were detected as well as production of IL-4, IL-10, IL-12 and INF-γ in response to *Nlac* PorB, consistent with induction of both a Th1-type and a Th2-type immune response. OVA-specific proliferation was also determined in splenocytes from *Nlac* PorB/OVA immunized mice. In addition, B cell activation *in vitro* and cytokine production in response to *Nlac* PorB was found to be mediated by TLR2, in a similar manner to *Nme* PorB.

Keywords

Adjuvant; immune stimulation; commensal bacteria; porin

1. Introduction

Adjuvants are used for improving the specific immune response to vaccine antigens and for induction of immunological memory [1,2]. The addition of adjuvants to vaccine formulations makes it possible to reduce the amount of antigen and the number of immunizations needed, while improving the magnitude and the duration of the specific immune response. However, the majority of adjuvants are not accepted for use in humans [2]. Although several potential adjuvants are in experimental stages, the only licensed adjuvant in use for humans in the United States is aluminum and calcium salts [3]. We propose that the PorB porin from commensal *N. Lactamica* acts as an immune adjuvant and has the potential of being developed as a potent

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immune modulator derived from a non-pathogenic bacterium, which would positively affect ease of manufacturing. Examples of adjuvants are oil emulsions [4–6], squalene (MF59) [7], immune stimulating complexes (ISCOMs [8,9]) with Quil-A and both gram-negative bacteria and bacterial products [4,10–12]. These include DNA with immunostimulatory CpG motifs (one of the most powerful adjuvants [13]), *Bordetella pertussis* toxin [2,14], *Corynebacterium granulosum* derived P40 component [15], MPL [16], *Mycobacterium* and its components (Freund adjuvant)[4,17], Cholera toxin [18] and porins from various organisms such as *Fusobacterium nucleatum* [19], *Shigella* [20–22], *Salmonella* [22,23] and *Neisseria meningitidis* and *gonorrhoeae* [24–29].

Porins from pathogenic Neisseria species have adjuvant activity in humans and animals [34, 35]; this is due to activation of antigen presenting cells (APCs), and our group has shown that signaling via Toll-Like receptors (TLRs) [31,32,68] is required for this activity. Increased expression of CD86/CD80, MHC II and CD40 on the APC surface has been shown in response to porins from pathogenic Neisserial species (and other bacteria) [23,26,30–32], as well as B cell proliferation and increased antibody production [33]. Activated APCs release specific cytokines (i.e. IL-12, IL-4, IL-6) that proceed to guide the differentiation of T cells [36].

Toll-Like receptors (TLRs) have recently been described as specific surface receptors expressed by APCs for recognition of pathogen associated molecular patterns (PAMPs) [37–41]. However, TLRs detect specific molecules which are not exclusive to pathogenic organisms. For example, TLR4 recognizes LPS [37], which is present on all Gram-negative bacteria, including non-pathogenic bacteria, and TLR5 [42] recognizes flagellin [43], also expressed by most gut commensal [44]. Various TLR ligands have been shown to modulate the immune response [45–47] and act as vaccine adjuvants regardless of their pathogenic or non-pathogenic origin (i.e LPS, CpG DNA). Examples of non-pathogenic bacteria with adjuvant activity include *Bacillus firmus* [48], probiotic *Bacillus subtilis* spores [49] and some non-pathogenic intestinal gram-negative bacteria, including *Bacteroides vulgatus* or *Veillonella parvula* [50], which can induce both Th1- and Th2-type immune responses.

Porins from non-pathogenic Neisseria species share structural and functional similarities with porins from pathogenic strains [51]. They belong to the gram-negative porin superfamily and are native trimeric proteins with a predicted 16-strand β-barrel fold structure and eight surface-exposed, variable, hydrophilic loops [51]. A sequence alignment of *N. meningitidis* and *N. lactamica* PorB has determined that the intermembrane domains are mostly conserved while some extracellular loops (loop I, IV, V and VI) [51] have more variability. Their pore function in the bacteria is regulated by a similar gating mechanism, only observed in *N. meningitidis* PorB, *N. gonorrhoeae* PIA and PIB, *N.lactamica* and *N. polysaccharea*. A similar pore function, regulated by ATP and GTP, has been described in host cells upon porin insertion in the cell membrane [75].

In the light of the similarities between porin from pathogenic and non-pathogenic Neisseriae, we asked whether the porin's ability to act as immune adjuvant is also part of these functional similarities or whether it is specific for pathogenic species. Our work is focused on porin from *Neisseria lactamica* (a commensal bacterium which most frequently colonizes the nasopharynges of children [52,53]) and is aimed at characterizing its potential effect as an adjuvant of the immune system and its mechanism of action.

2. Materials and Methods

2.1. Bacteria and cell cultures

Neisseria lactamica (Nlac) strain Y92-1009 [54] (a gift from A. Gorringe, HPA, Porton Down, Salisbury, UK) was originally isolated in Northern Ireland and is part of the ST-613 clonal

complex, and *N. meningitidis (Nme)* strain H44/76 Δ1Δ4 [55] were grown on chocolate-agar plates containing 1% Isovitalex in a 37°C in a 5% CO_2 incubator. Murine B cells were isolated from C57Bl/6J mice and C3H/HeJ mice as previously described [31]. Cells were grown in RPMI (Mediatech) containing 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES and 10 mM β-ME and defined as R10.

2.2. Porin purification

N. lactamica Y92-1009 were plated as above and grown overnight. The next day, colonies were inoculated in liquid medium (50 ml total) and grown for 6h. This step was repeated once using larger volumes of growth medium (250 ml total), and the cultures were then transferred into a total of 6 liters of medium and grown overnight at 37°C [56]. PorB was purified from the bacterial cultures with the method previously described for meningococcal porin [56], which allows for removal of endotoxin and lipoprotein. At the end of the purification, proteosomes (micellar multi-molecular organized structures) free of detergent were obtained [56] and will be referred to as "*Nlac* PorB". Purity of *Nlac* PorB was examined by Coomassie Brilliant blue staining of 12% SDS-PAGE [57] and silver stain of 15% SDS-PAGE [58]. Bacterial endotoxin content was determined with Limulus Amebocyte Lysate (LAL) assay as specified by the manufacturer (Cell Sciences, Canton, MA)

2.3. Immunization of animals

For generation of anti-*Nlac* PorB antibodies, C57Bl/6 mice (n=5) were immunized with 10 μg of purified *Nlac* PorB in 100 μl of sterile PBS in the absence of other adjuvants every two weeks via the sub-cutaneous (s.c.) route for a total of three immunizations. Sera were collected prior to the first immunization (week 0) and two weeks after each subsequent immunization (week 2, 4 and 6). Another group of animals was immunized with 10 μg of *Nme* PorB. For generation of anti-OVA antibodies, C57Bl/6 mice (n=5) and C3H/HeJ mice (n=4) (a mouse strain naturally non-responsive to LOS due to a mutation in the TLR4 gene [59]) were immunized with 10 μg of ovalbumin (OVA) alone or in the presence of 10 μg of *Nlac* PorB, 10 μg of *Nme* PorB [30] or with 200 μg of alum (Sigma) [60] as adjuvants. Ovalbumin was obtained from commercial chicken egg whites by freeze-drying followed by lyophilization and resuspension of the total egg white proteins in sterile PBS. Ovalbumin obtained with this method represents ~65% of the total protein content and is LPS-free [32].

2.4. Measurement of anti-PorB specific antibodies

Specific anti-porin antibodies in the mice sera were examined by Western blot and ELISA [24], respectively. For Western blot, *Nlac* PorB was subjected to 12% SDS-PAGE [57] followed by transfer on PVDF membrane (Millipore, Bedford, MA). The membrane was blocked for 1 h with 5% non-fat dry milk in 25 mM Tris pH 8.0, 125 mM NaCl and 0.1% Tween 20 followed by overnight incubation at 4°C with a 1:1000 dilution of mouse sera raised to *Nlac* PorB as previously described. For detection of the immunoreactive bands, an alkaline phosphatatse-conjugated anti-mouse secondary antibody (Sigma) was used followed by BCIP/NBT purple substrate (Sigma). For ELISA, briefly, 96-well plates (Immulon) were coated with 2 μg/ml of purified *Nlac* PorB or *Nme* PorB in carbonate buffer pH 9.6 at 4°C for 24h, washed three times with PBS/ 0.05% Tween-20 followed by 1h blocking with 5% BSA in PBS at room temperature. Serial dilution of individual mouse sera were incubated at 4°C for 24h washed and incubated with a secondary anti-mouse IgG alkaline-phosphatase (AP) conjugated antibody (Sigma) for 2h at room temperature followed by detection with 1 step PNPP substrate (Pierce) as specified by the manufacturer. The absorbance was measured at O.D.405. To quantify the amount of anti-porin serum IgG in μg/ml, a reference standard curve was used. One plate was coated with 10 μg/ml of a goat anti-mouse IgG F(ab')2-specific antibody (Jackson Laboratories), incubated with serial dilutions of a known amount of mouse total IgG

(Sigma) followed by secondary antibody and detection as described above. The amount of anti-porin serum IgG was extrapolated from the standard curve using a linear regression function.

2.5. Measurement of anti-OVA specific antibodies

OVA-specific total IgG and IgM in the mice sera were determined by ELISA as described above. After coating plates with 5μg of OVA, serial dilutions of mice sera were added and incubated as described. Total IgG and IgM were detected using alkaline-phosphatase conjugated secondary goat anti-mouse IgG and anti-mouse IgM (Sigma). Quantification of OVA-specific IgG and IgM was performed as described above using an IgG and an IgM standard curve, respectively. Results are expressed in μg/ml of OVA-specific antibody. For determination of OVA-specific IgG subclass titers, goat anti-mouse specific anti-IgG1, IgG2a, IgG2b and IgG3 were used, followed by AP-conjugated secondary antibody as previously described. Results are expressed as sera titers at O.D. 405.

2.6. Measurement of cytokines

INF-γ and IL-12p70 (Th1 cytokines), IL-4 and IL10 (Th2 cytokines), IL-6 and TNF-α were examined by ELISA using Opt-EIA kit (BD Biosciences) specific for each cytokine according to manufacture's protocol.

2.7. Cell incubations and flow cytometric analysis

Purified mouse B lymphocytes (5×10^6/ml) [26] were stimulated in R10 for 24h with *Nlac* PorB or *Nme* PorB (10 μg/ml), the TLR2/TLR1 synthetic ligand Pam3CSK4, the TLR2/TLR6 synthetic ligand Pam2CSK4 (100 and 10 ng/ml) and the TLR4 ligand *N. meningitidis* LOS (100 ng/ml). The culture supernatants were collected and analyzed for cytokine production as described above. For expression of cell surface antigens, the following anti-murine FITC-labeled MAbs were used: anti-rat IgG, anti-CD86, anti MHC-class II and anti-CD40 (PharMingen, San Diego, CA). Cells were analyzed by flow cytometry on a FACScan(TM) flow cytometer using CellQuest acquisition and analysis software (Becton Dickinson, Mountain View, CA). Gating was used to exclude cellular debris. All the histograms shown are representative of three separate experiments.

2.8. Lymphocytes proliferation assay

Whole splenocytes were obtained from immunized mice and cell proliferation was measured by 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyl tetrazolium bromide (MTT, Sigma) incorporation assay [60]. Briefly, after erythrocytes lysis with NH_4Cl, the splenocytes were seeded in R10 medium in 96-well flat-bottom plates in quadruplicate at a concentration of 5×10^6 cell/ml in 100 μl of medium. OVA (10 μg/ml), Neisserial LOS (10 μg/ml) and concanavalin A (ConA) (10 μg/ml) were added and incubated for 72h followed by addition of 10 μl of MTT to each well for further 3–4h. MTT solvent (100 μl) was added and the absorbance was measured in an ELISA reader at 570 nm with a 690 nm reference. Alternatively, splenic B cells from naive C57Bl/6 mice and TLR2 knockout mice [26] were incubated with *Nlac* PorB and *Nme* PorB at 10 μg/ml, LOS (100 ng/ml) and ConA (5 μg/ml) for 44h and cell proliferation was determined as described above. Cell proliferation was expressed in arbitrary units as Stimulation Index, calculated by dividing the average cell proliferation in the wells incubated with each stimulus by the average cell proliferation in the wells incubated with medium alone.

2.9. Statistic analysis

ELISA were performed in duplicate and repeated three times to ensure reproducibility. Statistical analysis of the data were performed with Graphpad InStat software.

3. Results

3.1. Purification of PorB from N. lactamica

Neisserial porins are native trimeric proteins composed by monomers of approximate molecular weight of ~ 34–42 kDa [51]. Most of their properties *in vitro* are dependent on an intact trimeric structure, including their ability to activate immune cells, modulate apoptosis and form functional pores, as shown for *Nme* PorB by our group [56]. PorB was purified from *N. lactamica (Nlac)* strain Y92-1009 [54] (a gift from Dr. A. Gorringe, HPA, Porton Down, Salisbury, UK) with a method that we have previously optimized for purification of PorB from *N. meningitidis (Nme)* and *N. gonorrhoeae (Ngo)* [56]. Purified *Nlac* PorB was first examined by SDS-PAGE followed by Coomassie staining (Figure 1A) and it appears to have a m.w. of approximately 35–37 kDa (indicated by the arrow) in reducing conditions (monomeric form) [61,62]. Higher molecular weight bands corresponding to trimers and oligomers were detected by non-denaturing gel electrophoresis performed in the absence of SDS (data not shown), suggesting a presumed native trimeric structure similar to *Nme* PorB [56]. Traces of a protein contaminant of approx. m.w. of 25 kDa were consistent with Reduction Modifiable Protein (Rmp) [63] (Figure 1A), recognized by an anti-meningococcal RmpM MAb by Western blot (data not shown). Silver stain SDS-PAGE was consistent with negligible presence of LOS contamination (Figure 1B), confirmed by LOS quantification with Limulus Amebocyte Lysate (LAL) assay [56] (data not shown).

3.2. Immunogenicity of Nlac PorB

C57Bl/6 mice and C3H/HeJ mice were immunized with *Nlac* PorB in the absence of other adjuvants and aliquots of sera were collected prior to the first immunization (Preimmune) and two weeks after each subsequent immunization for Western blot and ELISA analysis. *Nlac* PorB is detected by a representative anti-*Nlac* PorB mouse serum (week 6, 1:1000 dilution) as a band of approx. 35 kDa in Western blot (Figure 2A, lane 2). The pre-immune serum failed to recognize the porin (Figure 2A, Lane 1), indicating that specific antibodies are generated to *Nlac* PorB. We also examined the mouse sera by ELISA. Plates were coated with *Nlac* PorB and specific mouse anti-porin total IgG levels were measured. Immunization of mice with *Nlac* PorB induced production of specific antibodies to *Nlac* PorB (Figure 2B, open square), while the preimmune sera did not recognize the porin (Figure 2B, closed square). Similar results were obtained when the sera of C3H/HeJ mice were analyzed (data not shown).

3.3. Adjuvant effect of Nlac PorB

Porins from several bacterial species have been demonstrated to act as immune adjuvants, including *Nme* PorB [19,20,25]. To examine whether *Nlac* PorB has a similar property, its ability to increase antibody production to a prototype antigen, ovalbumin (OVA), was determined. To insure absence of LPS contamination in the OVA preparation, OVA was obtained from chicken egg whites as described [32], and was found to be free of LPS by LAL assay (data not shown). C57Bl/6 mice were immunized with OVA alone or mixed with adjuvants i.e. *Nlac* PorB, *Nme* PorB and alum. Sera were collected two weeks after each immunization and specific anti-OVA antibodies production was examined by ELISA. In Figure 3A, the concentration in micrograms per ml of anti-OVA total IgG in the sera of mice immunized with OVA alone, *Nlac* PorB/OVA and alum/OVA is shown and demonstrates that *Nlac* PorB significantly enhanced IgG production after two immunizations, similar to alum. Furthermore, *Nlac* PorB proved to be as efficient as *Nme* PorB as an adjuvant, since mice immunized with *Nme* PorB/OVA produced similar amounts of specific anti-OVA IgG (data not shown).

In Figure 3B, specific anti-OVA IgM levels induced by *Nlac* PorB and alum are shown, demonstrating that *Nlac* PorB also elicited a significantly enhanced IgM production to OVA.

Collectively these data support the effect of *Nlac* PorB as a protein with immune adjuvant ability, similar to *Nme* PorB, with the difference of being derived from a non-pathogenic bacterial specie. We also examined the response of C3H/HeJ mice (LPS-hyporesponsive mice) [59] immunized with OVA alone and *Nlac* PorB/OVA, and determined a very similar response to C57Bl/6 mice (data not shown).

3.4. Analysis of IgG subclasses and Th1/Th2 response

To determine whether *Nlac* PorB induced a Th-biased response, we measured IgG subclasses, IgE and Th1 and Th2 cytokines in the sera of immunized mice. The different IgG subclasses were examined by ELISA and are shown in Figure 4. Significantly higher titers ($p = < 0.05$) of OVA-specific IgG1 were induced by alum (Figure 4, white bars) and by *Nlac* PorB (Figure 4, black bars) as compared to OVA alone (Figure 4, gray bars). *Nme* PorB also induced similar IgG1 levels (data not shown). In addition, *Nlac* PorB elicited significantly higher titers ($p = < 0.05$) of IgG2b and, to a lesser extent, of IgG3 and IgG2a (Figure 4), which were not induced by alum.

As induction of high IgG1 titers and of IgE is considered indicative of a Th2-type immune response while high IgG2a, IgG2b and IgG3 and low IgE titers are typical of a Th1-type response [64], we examined whether Th1- and Th2-type associated cytokines were induced in the mice sera after receiving two immunizations (week 4). As shown in Table 1, mice immunized with *Nlac* PorB/OVA exhibited high levels of IL-4 and IL-10 (Th2 cytokines), similar to mice immunized with alum/OVA (and with *Nme* PorB/OVA, data not shown). Furthermore, the Th1 cytokines INF-γ and IL-12p70 were also augmented in response to *Nlac* PorB (and *Nme* PorB, not shown), but not by alum (Table 1), supporting the hypothesis that *Nlac* PorB can induce both a Th1- and a Th2-type response when is used as adjuvant. Mice immunized with *Nlac* PorB alone produced cytokine levels equal or slightly higher than mice immunized with OVA alone (data not shown), and we also determined a similar cytokines pattern in the immune sera of C3H/HeJ mice (data not shown).

3.5. Splenocyte proliferation in OVA-immunized mice

In vitro proliferation of splenocytes from mice immunized with OVA alone, *Nlac* PorB/OVA, *Nme* PorB/OVA and alum/OVA in response to stimulation with OVA was measured. Mice were immunized every two weeks as previously described and the spleens were collected two weeks after the second immunization (week 4). Splenocytes were incubated with OVA (10μg/ml) for induction of proliferation. Neisserial LOS (10 μg/ml) and the mitogen Concanavalin A (10 μg/ml) were used as non-specific inducers of cell proliferation while medium alone was used as a negative control. The cells were incubated for 72h as described in the Methods and proliferation was measured by addition of a chromogenic substrate (MTT), which forms intracellular crystals detectable spectrophotometrically at 570nm with 690nm correction. Cell proliferation was calculated by dividing the average cell proliferation in the wells incubated with each stimulus by the average cell proliferation in the wells incubated with medium alone and expressed in arbitrary units as Stimulation Index, shown in Figure 5. Specific proliferation in response to OVA (Figure 5, black bars) was determined in splenocytes from mice immunized with *Nlac* PorB/OVA and *Nme* PorB/OVA, while it was not induced in cells from the OVA alone and alum/OVA mice groups. Non-specific proliferation was induced in all the splenocyte groups by of ConA (Figure 5, white bars) and by LOS (Figure 5, gray bars), appearing to be further increased in cells from mice immunized with *Nlac* PorB/OVA and *Nme* PorB/OVA. These data suggest that immunization with a mixture of *Nlac* PorB and OVA sensitizes mouse splenocytes for induction of *Nlac* PorB-mediated proliferation in response to OVA stimulation.

3.6. Activation of immune cells in vitro and role of TLR2

To assess whether the mechanism of adjuvanticity of *Nlac* PorB was consistent with APC activation, similar to what has been described for *Nme* PorB [31], we examined its effect on B cells *in vitro*. Although dendritic cells are mostly responsible for initiation of a primary immune response, activation of both DCs and B cells has been extensively characterized in response to *Nme* PorB [26,31,32] and is a crucial comparison for the effect of *Nlac* PorB. Purified mouse splenic B cells were incubated with different concentrations of *Nlac* PorB (20, 10, 1, 0.1 μg/ml) for 24h and a dose-dependent increase of surface expression of the co-stimulatory molecule CD86, of CD40 and of MHC II was determined by flow cytometry (data not shown). In all the histograms in Figure 6, the isotype control is shown by the gray area and medium-incubated cells are shown by the thin line. In Figure 6A, we compared the effect of *Nlac* PorB (10 μg/ml, thick line) and *Nme* PorB (10 μg/ml, dotted line) [26] on B cell activation and a similar up-regulation of CD86, MHC II and CD40 was determined.

As a TLR-mediated cell activation has been described for adjuvants of bacterial origin (i.e. TLR9 for CpG DNA [13] and, most importantly, TLR2 for *Nme* PorB [65]), we examined whether B cell activation mediated by *Nlac* PorB was also TLR2-dependent using B cells from TLR2 knockout mice. Figure 6B shows that the expression of CD86, MHC II and CD40 was abrogated in B cells from TLR2 knockout mice in response to *Nlac* PorB (Figure 6B, thick line) similar to *Nme* PorB (Figure 6B, dotted line). B cells from wild type mice and TLR2 knockout mice were also incubated with the TLR2/TLR6 ligand, Pam2CSK4 (100 ng/ml), the TLR2/TLR1 ligand Pam3CSK4 (100 ng/ml) as positive controls and with the TLR4 ligand LOS (100 ng/ml) as a negative control. Figure 6C shows up-regulation of CD86, MHC II and CD40 in response to Pam2CSK4 (Figure 6C, dotted line), Pam3CSK4 (Figure 6C, dashed line) and LOS (Figure 6C, thick line) in B cells from wild type mice. However, expression of these markers was abrogated in B cells from TLR2 knockout mice in response to Pam2CSK4 and Pam3CSK4 (Figure 6D, dotted line and dashed line, respectively) while the ability of the TLR4 ligand, LOS, to induce surface markers upregulation was unaffected (Figure 6D, thick line). These data suggest that *Nlac* PorB activates immune cells *in vitro* in a TLR2-dependent manner.

Next, we determined whether *Nlac* PorB induced cytokine secretion *in vitro* in B cells and whether this was also mediated by TLR2. Purified B cells from wild type mice and TLR2 knockout mice were incubated with *Nlac* PorB, Pam3CSK4 and LOS as described above and IL-6 and TNF-α were measured in the cell supernatant by ELISA. Table 2 shows that *Nlac* PorB induced increased levels of both IL-6 and TNF-α, similar to Pam3CSK4 and LOS. This response was dependent on TLR2 expression, as B cells from TLR2 knockout mice failed to produce them in response to PorB and Pam3CSK4, although they were responsive to the TLR2-independent positive control, LOS.

As an additional method for correlating the ability of *Nlac* PorB to activate immune cells *in vitro* with its immune adjuvant activity, we measured induction of B cell proliferation. LOS and ConA were used as positive controls and medium alone was used as a negative control. As shown in Figure 7, incubation with *Nlac* PorB for 48h induced proliferation of B cells from wild type mice (Figure 7, black bars) in a similar manner to *Nme* PorB, LOS and the mitogen ConA. However, B cells from TLR2 knockout mice failed to proliferate in response to *Nlac* PorB and *Nme* PorB, while TLR4-dependent proliferation was induced by LOS and non-specific proliferation was induced by ConA (Figure 7, white bars). Collectively, these data strongly indicate that the immune stimulatory effects of *Nlac* PorB on mouse B cells are dependent on expression of TLR2.

4. Discussion

Although bacterial porins have been characterized as adjuvants, purified *N. lactamica* porin PorB has not been examined before. In this work we describe the purification of PorB from *N. lactamica* Y92-1009 (a particularly relevant strain, as outer membrane vesicles derived from it are being explored as an alternative approach for a meningococcal vaccine [54]). Purified Neisserial porins mediate cell activation and signaling pathways *in vitro* and *in vivo*; however, if their native trimeric structure is disrupted into monomers, these activities are lost [56]. We have supporting evidence that the presumed native trimeric structure of *Nlac* PorB is maintained upon purification, suggested by the presence of high molecular weight bands in non-denaturing SDS PAGE, in a similar manner to PorB from *N. meningitidis* [56]. In fact, a high degree of structural and functional similarities between neisserial porins has been described [51]; they share a high content of β-pleated sheet, a predicted 16-strand β-barrel fold and multiple surface-exposed, variable, hydrophilic loops [51,67]. However, an aminoacid sequence alignment between PorB from *N. meningitidis* and *N. lactamica* has determined that the regions of homology do not include some surface exposed loops (loop I, IV, V and VI) [51]. Mice immunized with purified *Nlac* PorB in the absence of other adjuvants mount a robust specific immune response to the porin.

The immune adjuvant effect of bacterial porins, including Neisserial porins from pathogenic strains, has been previously characterized [19,20,25]. Both native and recombinant pathogenic neisserial porins have demonstrated to induce similar immune responses, in terms of generation of anti-porin antibodies and bactericidal activity and are considered for a potential anti-meningococcal vaccine [28,76,77]. However, potential difficulties in the recombinant porin's expression and structure re-folding or the use of large amounts of pathogenic bacterial cultures (although it could be argued that the use of non-capsulated strains would lower the risks of growing hundred liters of culture), might limit their development as adjuvants. We asked whether porins from non-pathogenic *N. lactamica* have equivalent immune adjuvant properties and propose that they can be safely used as an alternative to pathogenic strains. Our data demonstrate that *Nlac* PorB induces increased antibodies production to a prototype antigen (ovalbumin) in mice, similar to other adjuvants such as alum and *Nme* PorB. Alum typically induces high IgG1 and IgE titers and Th2-type cytokine IL-4 and IL-10 in mice. *Nlac* PorB not only induces high IgG1 titers, similar to alum, but also higher titers of IgG2b and IgG3, which are events associated with induction of a Th1 type response in mice [64]. We also demonstrate that secretion of both Th1-type cytokine IL-12p70 and INF-γ and Th2-type cytokines IL-4 and IL-10 was increased by *Nlac* PorB. Induction of both Th1 and Th2 type immune responses is a very desirable characteristic for an immune adjuvant, making it potentially suitable for formulation of vaccines directed not only towards bacterial pathogens but also viral pathogens or helmints.

Achievement of a long-lasting immunity against vaccine antigens via antigen-specific lymphocytes proliferation and induction of memory cells is also induced by immune adjuvants. In our experimental system, *Nlac* PorB (and *Nme* PorB) induced antigen-specific splenocytes proliferation, while OVA alone and alum [60] failed to induce such proliferation, suggesting a specific effect for both *Nlac* PorB and *Nme* PorB. However, splenocytes from mice immunized with *Nlac* PorB/OVA (and *Nme* PorB/OVA) also appeared more susceptible to non-specific proliferation induced by both ConA and LOS, maybe due to a synergistic effect.

There is a growing body of evidence suggesting that bacterial components with immune adjuvant activity, including CpG DNA [13], *Nme* PorB [31] or Shigella porins[20], act via Toll-like receptors (TLRs) and can induce both a Th1- and a Th2- immune response [45,68–70]. In addition, it has been recently shown that TLR2 mediates Th1 responses via direct stimulation of Th1 cells and via IL-12 production by APCs, and that TLR2-mediated INF-γ

production is crucial for protective immunity against several infectious pathogens [71]. As *Nme* PorB has been shown to activate immune cells via a TLR2-mediated mechanism [31], we have examined whether the immune adjuvant effect of *Nlac* PorB might have a similar mechanism of action. *Nlac* PorB appears to induce immune cell activation (i.e. up-regulation of CD86, CD40 and MHC II on murine B cells, *in vitro* production of IL-6 and TNF-α, *in vitro* B cell proliferation) similar to other known TLR2 ligands, such as *Nme* PorB, Pam2CSK4 and Pam3CSK4. However, these effects are abrogated in B cells from TLR2 knockout mice, suggesting a role for TLR2 in *Nlac* PorB-mediated immune cell activation although it is derived from a non-pathogenic bacterium which does not elicit much inflamation.

TLR ligands are not only expressed by pathogenic organisms; for example the TLR4 ligand, LPS or the TLR5 ligand, flagellin are present on all Gram-negative bacteria and on most gut commensals, respectively, and can activate immune cells. One could speculate that purified bacterial components from both pathogenic and non-pathogenic species could equally signal via TLRs *in vitro* but they might require different adaptor molecules (i.e. MyD88 or TRIF–TRAM [72]) or even initiate distinct intracellular cascades of events when they are integral part of live bacteria [73]. Furthermore, pathogenic signals might require different co-receptors for TLR signaling: for example, porins from pathogenic *N. meningitidis* signal via TLR2/TLR1 [65]. Whether TLR2 engagement by *Nlac* PorB expressed on intact live bacteria is followed by NF-κB nuclear translocation and cytokine secretion [39–41] still remains undetermined, and further analysis of *Nlac* PorB interaction with TLRs will be required to elucidate its ability to activate cells, to act as an adjuvant and ultimately even potentially relate to its non-pathogenic nature. Finally, commensals also might lack additional virulence factors to initiate pro-inflammatory responses.

In conclusion, development of efficient adjuvants is of great importance for successful vaccinations to improve the control of diseases. Adding adjuvants to vaccines potentiates their immune stimulating effect, as demonstrated by *N. meningitidis* serogroup C conjugate vaccines, which have almost eliminated serogroup C meningitis in the UK in 1999 [74]. Moreover, adjuvants allow to induce an effective protection using fewer doses of vaccine, which is particularly advantageous for vaccines targeted to infants and elderly. We have characterized PorB from commensal *Neisseria lactamica* as an immune adjuvant with equivalent properties to PorB from pathogenic *N. meningitidis* and to alum. An additional advantage of using neisserial porins as adjuvants is that these proteins can be stored at room temperature for prolonged periods of time without losing activity, decreasing cold chain requirements for shipment and storage in sub-optimal conditions (i.e. small spaces with poor refrigeration in Third World countries). There is no doubt that improvement of vaccine formulations is essential to fight numerous diseases and the development of new adjuvants is key to advance the field of vaccination, our understanding of biological systems to improve the control of diseases and to enhance health.

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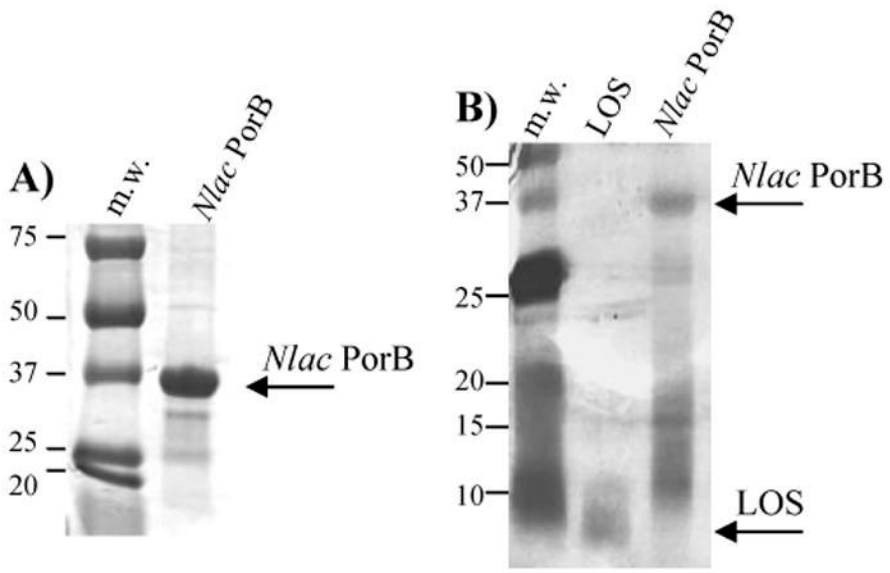

Figure 1.
Purification of PorB from *N. lactamica*. PorB was purified from liquid cultures of *N. lactamica* as previously described [56]. **A)** SDS-PAGE and Coomassie staining of purified *Nlac* PorB. The reduced monomeric form is indicated by the arrow. **B)** SDS-PAGE and silver staining of *Nlac* PorB. Neisserial LOS was used as positive control for detection of potential contaminant lipopolysaccharide. Reduced PorB and LOS are indicated by the arrows.

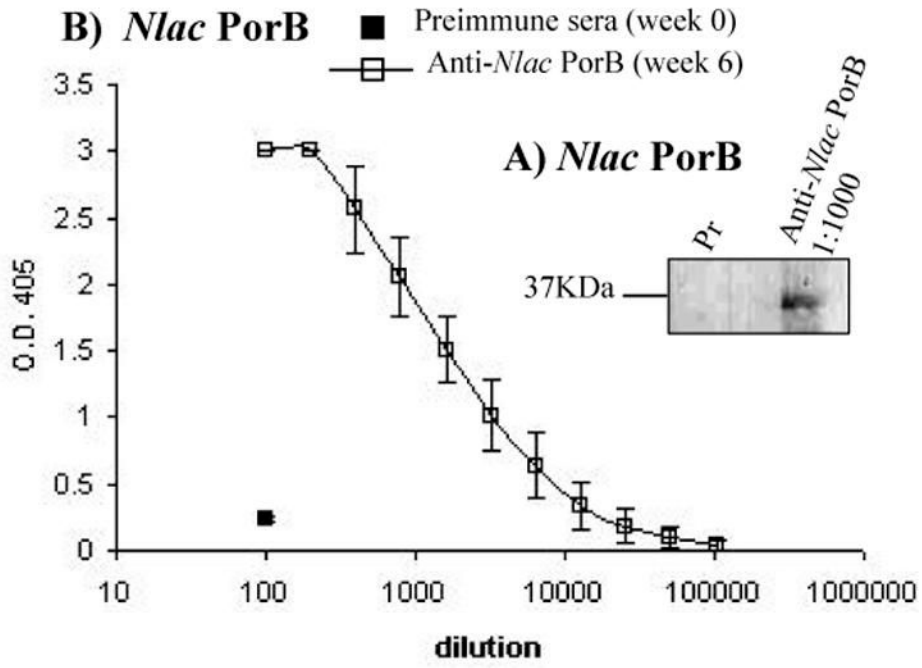

Figure 2.

Immunogenicity of *Nlac* PorB. **A)** Sera from C57Bl/6 mice immunized s.c. with *Nlac* PorB were used in Western blot (1:1000 dilution) for detection of *Nlac* PorB (lane 2). Preimmune serum (Pr) is shown in lane 1. **B)** Serial dilution (1:1000 initial dilution) of anti-*Nlac* PorB (open square) were used in ELISA for detection of *Nlac* PorB. Preimmune serum is shown by the closed square. The results represent the mean of two experiments performed in triplicate wells ± standard deviations.

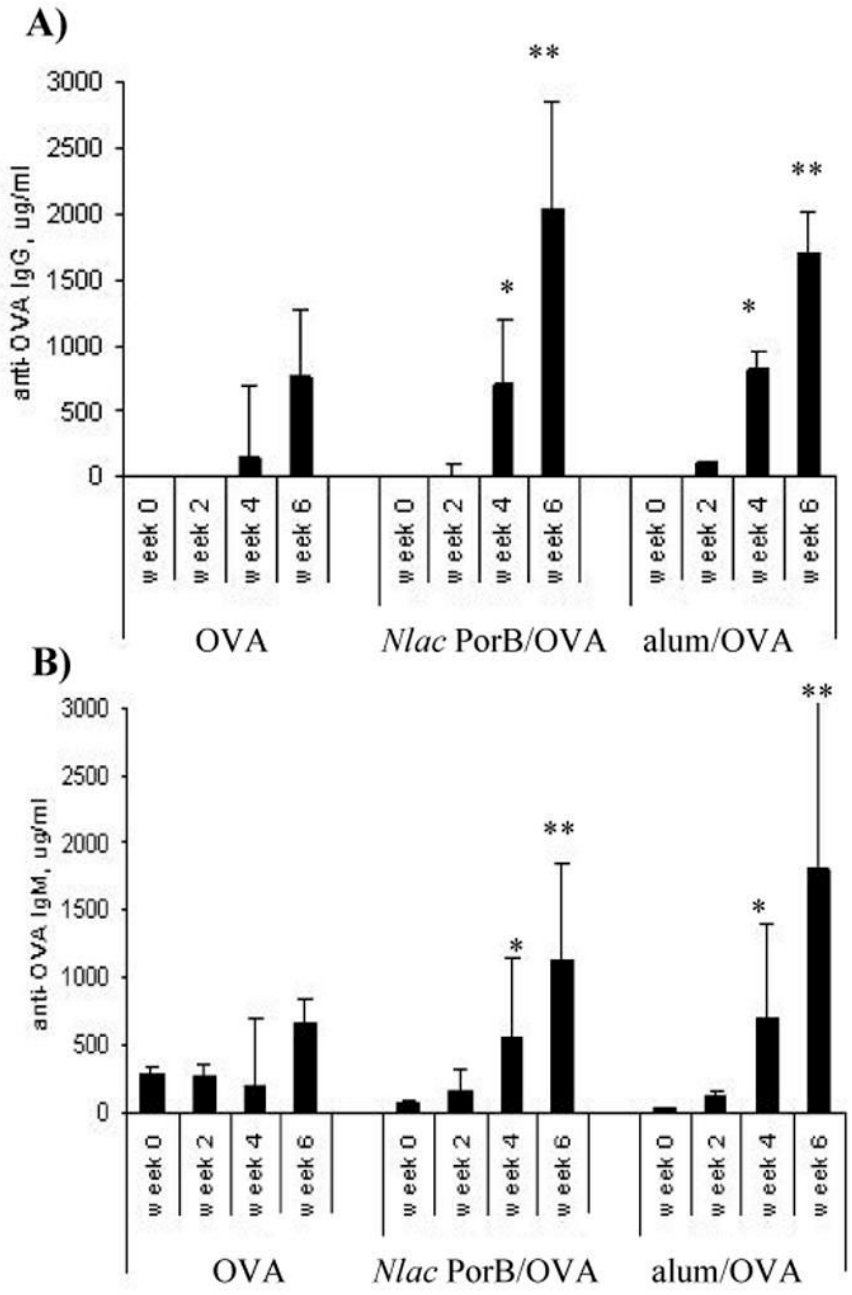

Figure 3.

Effect of *Nlac* PorB on induction of OVA-specific total IgG and IgM in OVA-immunized mice. Groups of five mice were immunized s.c. with 10 μg of OVA alone or dissolved in saline containing 200μg of alum or 10 μg of *Nlac* PorB every two weeks for a total of three immunizations. Sera were examined prior to the first immunization (week 0) and two weeks after each immunization (week 2, 4 and 6). **A)** and **B)** OVA-specific total IgG and IgM antibodies in the sera were measured by ELISA, quantified by using standard IgG and IgM reference curves and expressed in μg/ml. The values represent the mean of triplicate wells for each individual mouse serum ± standard deviation. Significant differences ($p = < 0.05$) with the OVA group were calculated by Mann Withney test and are indicated by the asterisks.

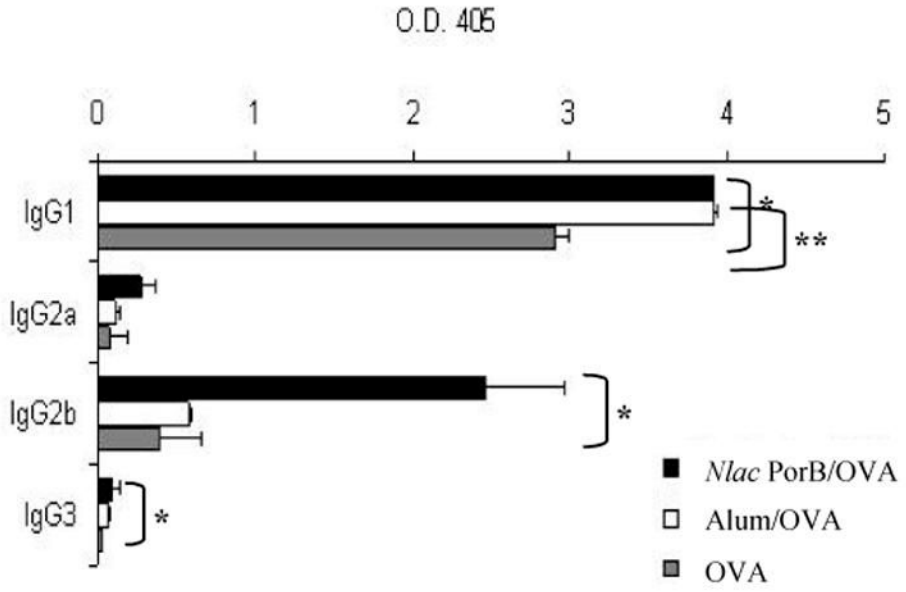

Figure 4.

Effect of *Nlac* PorB on induction of OVA-specific IgG subclasses. OVA-specific IgG1, IgG2a, IgG2b and IgG3 titers were measured by ELISA in the sera of immunized mice. The results represent the mean of triplicate wells ± standard deviation and are expressed as O.D.405. Significant differences ($p=<0.05$) with the OVA group were calculated by Mann Withney test and are indicated by (*) for *Nlac* PorB and (**) for alum.

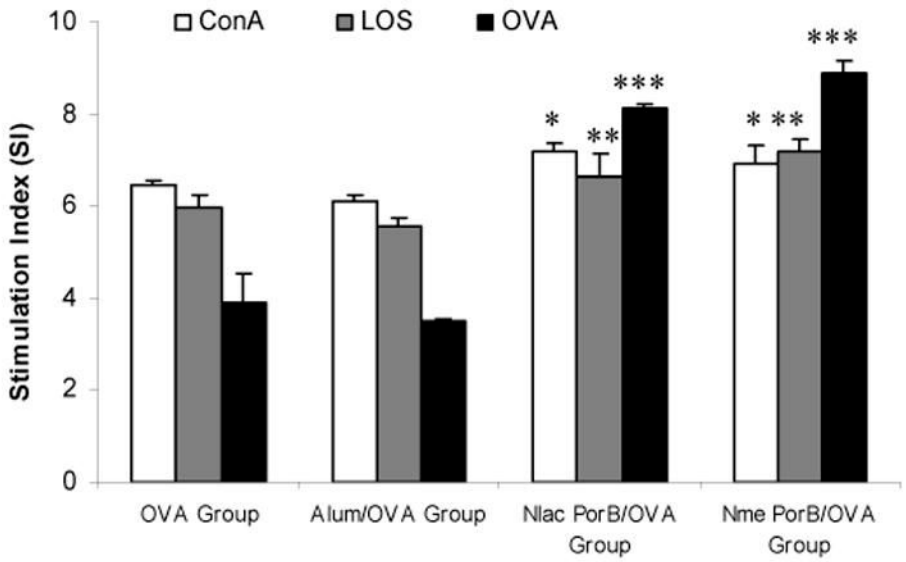

Figure 5.

Effect of *Nlac* PorB on specific and non-specific splenocyte proliferation. Splenocytes from mice immunized with OVA alone, alum/OVA, *Nlac* PorB/OVA or *Nme* PorB/OVA were incubated for 72h with OVA (10 μg/ml, black bars) for induction of specific proliferation or with LOS (10 μg/ml, gray bars) and Concanavalin A (ConA, 10 μg/ml, white bars) for induction of non-specific proliferation. Medium alone was used as a negative control. Splenocytes proliferation was determined by the MTT assay. The values represent the mean of quadruplicate wells ± standard deviation and expressed as Stimulation Index (SI) as described in the text. Significant differences ($p=<0.05$) with the OVA-immunized group were calculated by Mann Withney test and are indicated by (*) for ConA stimulation, (**) for LOS stimulation and (***) for OVA stimulation. No significant differences were detected between the OVA group and the alum/OVA group.

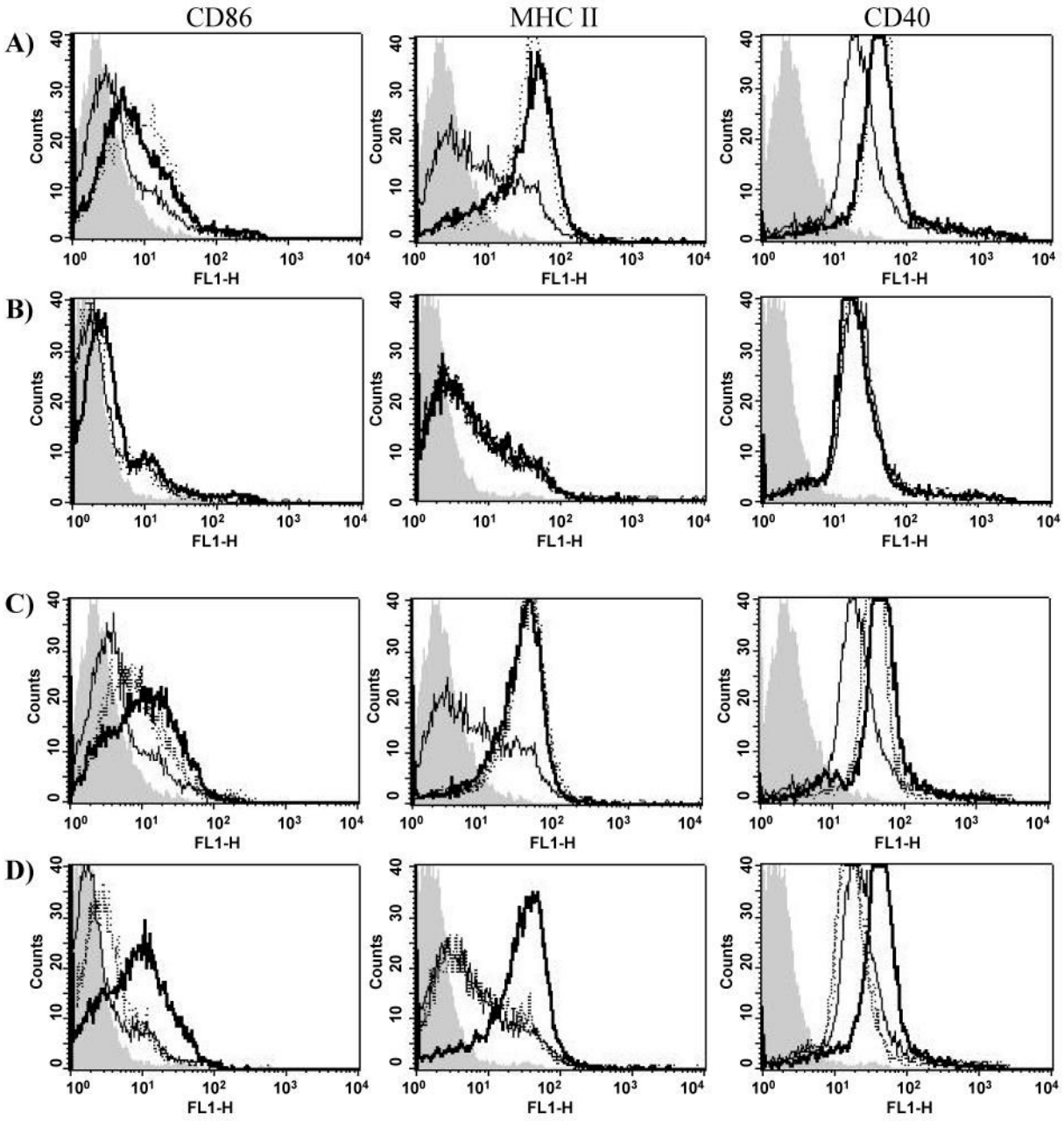

Figure 6.

Nlac PorB stimulates B cells *in vitro*. Surface upregulation of CD86, MHC II and CD40 was measured by flow cytometry. In all the histograms the isotype control is indicated by the gray area and the medium is indicated by the thin line. B cells from **A)** C57Bl/6 wild type mice and **B)** TLR2 knockout mice incubated for 24h with *Nlac* PorB (10 μg/ml, thick line) and *Nme* PorB (10 μg/ml, dotted line). B cells from **C)** wild type mice and **D)** TLR2 knockout mice incubated for 24h with Pam2CSK4 (100 ng/ml, dotted line), Pam3CSK4 (100 ng/ml, dashed line) and LOS (100 ng/ml, thick line).

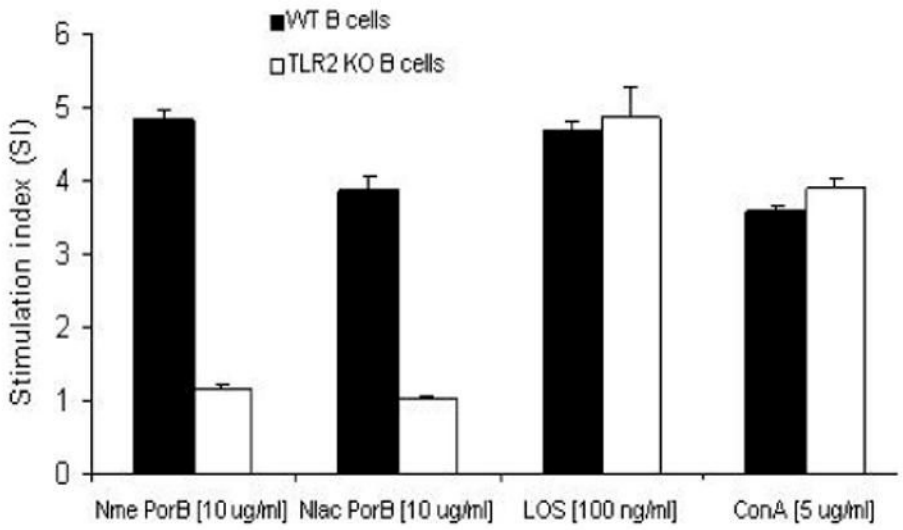

Figure 7.

Effect of *Nlac* PorB on B cell proliferation. B cells from C57Bl/6 mice (black bars) and TLR2 knockout mice (white bars) were incubated for 44h with *Nlac* PorB or *Nme* PorB (10 μg/ml), LOS (100 ng/ml) or the mitogen Concanavalin A (ConA, 5 μg/ml) as previously described. Medium alone was used as a negative control. Proliferation was determined by the MTT assay as previously described. The values represent the mean of quadruplicate wells ± standard deviation and expressed as Stimulation Index (SI) as described in the text.

Table 1

Serum levels of Th1 and Th2 cytokine in immunized mice

The sera of mice after receiving two immunizations (week 4) were examined by ELISA for production of cytokines. The values are presented as mean ± standard deviation of triplicate wells for each individual mouse serum. Significant differences with the OVA group were calculated by Mann Withney test and are indicated by (*)

Groups	INF-γ (ng/ml)	IL-12p70 (ng/ml)	IL-10 (pg/ml)	IL-4 (pg/ml)
OVA	17.09 ± 5.8	47.2 ± 15.2	223.3 ± 171	219.1 ± 0.2
Nlac PorB/OVA	31 ± 9.7*	89.7 ± 26*	530 ± 178	451.2 ± 3.4*
Alum/OVA	6.36 ± 6.9	49.3 ± 40	421 ± 148.4	441.5 ± 2.5*

Table 2

Levels of Th1 and Th2 cytokine produced by B cell *in vitro*

B cells from C57Bl/6 wild type mice and TLR2 knockout mice were incubated for 24h with *Nlac* PorB, Pam3CSK4, Pam2CSK4 and Neisserial LOS as indicated. The levels of IL-6 and TNF-α were measured by ELISA, quantified by using standard reference curves and expressed in pg/ml. The results represent the mean of triplicate wells ± standard deviations.

	WT B cells		TLR2 KO B cells	
(pg/ml)	**TNF-α**	**IL-6**	**TNF-α**	**IL-6**
Medium	n.d.	0.6 ± 0.3	n.d	6.3 ± 1.5
Nlac PorB 10μg/ml	122.4 ± 16	13.6 ± 1.8	n.d.	4.8 ± 2.1
Pam3CSK4 0.1 μg/ml	216.6 ± 39	19.2 ± 7.3	n.d.	3.4 ± 0.9
Pam2CSK4 0.1 μg/ml	180 ± 59	22.6 ± 12.8	n.d.	4.7 ± 5.4
LOS 0.1 μg/ml	95 ± 31	11.8 ± 5.6	93.9 ± 47.5	12.9 ± 4.9