Properly folded and functional PorB from *Neisseria gonorrhoeae* inhibits dendritic cell stimulation of $CD4^+$ T cell proliferation

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Neisseria gonorrhoeae* is an exclusive human pathogen that evades the host immune system through multiple mechanisms. We have shown that *N. gonorrhoeae* suppresses the capacity of antigen-presenting cells to induce $CD4^+$ T cell proliferation. In this study, we sought to determine the gonococcal factors involved in this adaptive immune suppression. We show that suppression of the capacity of antigen-pulsed dendritic cells to induce T cell proliferation is recapitulated by administration of a high-molecular-weight fraction of conditioned medium from *N. gonorrhoeae* cultures, which includes outer membrane vesicles that are shed during growth of the bacteria. *N. gonorrhoeae* PorB is the most abundant protein in *N. gonorrhoeae*–derived vesicles, and treatment of dendritic cells with purified recombinant PorB inhibited the capacity of the cells to stimulate T cell proliferation. This immunosuppressive feature of purified PorB depended on proper folding of the protein. PorB from *N. gonorrhoeae*, as well as other *Neisseria* species and other Gram-negative bacterial species, are known to activate host Toll-like receptor 2 (TLR2) signaling. Published studies have demonstrated that purified *Neisseria* PorB forms proteinacious nanoparticles, termed proteosomes, when detergent micelles are removed. Unlike folded, detergent-solubilized PorB, PorB proteosomes stimulate immune responses. We now demonstrate that the formation of PorB proteosomes from structurally intact PorB eliminates the immunosuppressive property of the protein while enhancing TLR2 stimulation. These findings suggest that gonococcal PorB present in shed outer membrane vesicles plays a role in suppression of adaptive immune responses to this immune-evasive pathogen.

Gonorrhea, the urethritis caused by *Neisseria gonorrhoeae*, has been recognized as a human infection for thousands of years and, to date, remains one of the most prevalent sexually transmitted bacterial infections, with over 78 million new cases reported worldwide in 2012 (1). Treatment of gonorrhea relies on antibiotic therapy, but there is growing concern about resistance to currently approved antimicrobial agents (2, 3). Prevention of gonococcal infections through vaccine development is an appealing strategy to address this problem but is hampered by the lack of known protective immunologic correlates (4).

Most individuals infected with *N. gonorrhoeae* do not develop protective adaptive immune responses, and thus repeated infections are common, including reinfection by the same gonococcal strain (5–7). Multiple mechanisms have been attributed to the ineffective adaptive immune response to these bacteria. *N. gonorrhoeae* major surface molecules, including pili, opacity proteins (Opa),[4] and lipooligosaccharide (LOS), undergo phase and antigenic variation at high frequency (8). Furthermore, *N. gonorrhoeae* can manipulate host immune responses through interaction with local mucosal immune cells. It has been reported that Opa_{52} binds to carcinoembryonic antigen–related cellular adhesion molecule 1 (CEACAM-1) on

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This article contains Methods and Figs. S1–S5.

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[4] The abbreviations used are: Opa, opacity protein; LOS, lipooligosaccharide; IL, interleukin; *Ng*-CM, *Neisseria gonorrhoeae* conditioned medium; CFSE, carboxyfluorescein succinimidyl ester; OVA, ovalbumin; GWM, Graver–Wade medium; OMV, outer membrane vesicle; HT, hexahistidine tag; LDAO, L-*N*,*N*-dimethylamine-*N*-oxide; LPS, lipopolysaccharide; m.o.i., multiplicity of infection; MHC, major histocompatibility complex; UNC, University of North Carolina; MW, molecular weight; BMDC, bone marrow-derived dendritic cell; ANOVA, analysis of variance.

$CD4^+$ T cells (9, 10), resulting in down-regulation of T cell proliferation in response to antigens (11). Studies of gonococcal infections in the female mouse infection model have shown that *N. gonorrhoeae* elicits T helper type 17 (Th17) responses through the induction of host transforming growth factor β. Further, the Th17 response drives induction of localized inflammation, including recruitment of host neutrophils (to which *N. gonorrhoeae* is relatively resistant) (12). Recent studies in a mouse model of gonorrhea have shown that *N. gonorrhoeae* induces production of interleukin 10 (IL-10) and regulatory T cells (Tr1) that suppress Th1- and Th2-dependent adaptive immune responses (13).

Our previous studies have shown that *N. gonorrhoeae* suppresses the capacity of dendritic cells, professional antigen-presenting cells that play a key role in promoting pathogen-specific adaptive immune responses, to stimulate antigen-specific T cell proliferation (14). *N. gonorrhoeae* causes this suppression in part by promoting secretion of inhibitory factors, such as IL-10, and expression of cell-autonomous factors, including up-regulation of programmed death ligand 1, but the key molecular component(s) of *N. gonorrhoeae* that engage in this process were not identified. In this study, we determined that gonococcus-conditioned medium carries factors that recapitulate the suppression of dendritic cell–induced T cell proliferation observed with whole bacteria. We show that properly folded, recombinant PorB, a major protein component found in conditioned medium, has similar suppressive properties. Surprisingly, prior studies of PorB from *N. gonorrhoeae* and other *Neisseria* species have demonstrated that the protein can act as an immune-stimulating adjunct. We further demonstrate that the stimulatory properties of PorB result from a loss of immunesuppressive activity as a consequence of the loss of the properly folded PorB protein structure that occurs when detergent is removed from this integral membrane protein. Taken together, our results suggest that, although the native PorB trimer from *N. gonorrhoeae* can stimulate signaling in some immune cells through activation of Toll-like receptor 2, PorB overcomes this stimulation by profoundly inhibiting dendritic cell–promoted T cell proliferation when presented to cells in its native, properly folded state.

Results

N. gonorrhoeae–conditioned medium inhibits dendritic cell–induced, antigen-specific T cell proliferation

To determine whether *N. gonorrhoeae* releases factors responsible for inhibition of antigen-pulsed dendritic cell–induced T cell proliferation, conditioned medium from *N. gonorrhoeae* cultures (*Ng*-CM) or live *N. gonorrhoeae* was added to dendritic cells during ovalbumin (OVA) exposure for 24 h, and then the dendritic cells were washed and co-cultured with carboxyfluorescein succinimidyl ester (CFSE)–labeled T cells from OT-II mice. After 7 days, T cell proliferation in each co-culture was evaluated by quantifying the dilution of CFSE fluorescence. Treatment of dendritic cells with either *N. gonorrhoeae* or *Ng*-CM resulted in a dose-dependent reduction of proliferation of co-cultured T cells compared with OVA-treated dendritic cells (Fig. 1, *A–C*). *Ng*-CM also inhibited the capacity of human monocyte-derived dendritic cells to induce proliferation of allogeneic T lymphocytes (Fig. 1, *D* and *E*), demonstrating that *Ng*-CM-mediated suppression occurs in both human and mouse dendritic cells.

To further characterize the components released from *N. gonorrhoeae* that are responsible for inhibition of T cell proliferation, *Ng*-CM was subjected to ultrafiltration through a 100-kDa cutoff filter, and the retentate was restored to its original volume by addition of Graver–Wade medium (GWM) (15). The filtrate and retentate fractions were then assessed for their capacity to inhibit dendritic cell–induced T cell proliferation. The 100-kDa retentate from *Ng*-CM demonstrated inhibition of T cell proliferation that was indistinguishable from unfiltered *Ng*-CM, whereas the 100-kDa filtrate from *Ng*-CM exhibited no detectable inhibition of T cell proliferation (Fig. 2, *A* and *B*). Although *N. gonorrhoeae* does not secrete known protein exotoxins, *N. gonorrhoeae* and other pathogenic *Neisseria* species prolifically shed outer membrane vesicles (OMVs, also called membrane blebs) into the environment during growth (16, 17). *Ng*-CM, *Ng*-CM 100-kDa filtrate, and *Ng*-CM 100-kDa retentate were analyzed by SDS-PAGE and silver staining, which revealed that the majority of proteins present in *Ng*-CM were retained by the 100-kDa filter (Fig. S1*A*). The finding that the majority of the proteins found in *Ng*-CM and the inhibitory factor are retained by a 100-kDa filter suggests that the immunosuppressive effect of *Ng*-CM is due to OMVs or proteins in the OMVs.

N. gonorrhoeae $PorB_{1B}$, *but not LOS, inhibits OVA-pulsed dendritic cell–induced T cell proliferation*

OMVs from *N. gonorrhoeae* contain large quantities of LOS as well as outer membrane proteins, primarily PorB (17, 18). We therefore sought to determine whether LOS or gonococcal proteins abundant in OMVs present in *Ng*-CM were responsible for inducing inhibition of dendritic cell–directed, OVA-induced T cell proliferation. The amount of LOS in *Ng*-CM was estimated following SDS-PAGE and silver staining by comparing the staining intensities to a titration series of purified LOS (Fig. S1*A*). The quantity of *Ng*-CM that conferred maximal inhibition of dendritic cell–mediated T cell proliferation (25 μl, Fig. 1*C*) was estimated to contain ~100 ng of LOS. When dendritic cells were treated with 100 ng of purified LOS in the presence of OVA prior to co-culture with $CD4^+$ T cells, no inhibition of T cell proliferation was observed (Fig. S1*B*). When 10-fold more purified LOS (1 μg) was used, a slight inhibition of T cell proliferation was observed, but this trend did not meet statistical significance (Fig. S1*C*). These data indicate that purified LOS was unable to recapitulate the inhibition of dendritic cell-induced T cell proliferation that was observed with *Ng*-CM and suggest that outer membrane proteins may be responsible for the inhibition.

N. gonorrhoeae porin (PorB) is the most abundant gonococcal protein in both outer membranes and OMVs, constituting ~50% of the total protein content (19, 20), and has been shown to have pleotropic effects on host cells through a variety of mechanisms (21–23). *N. gonorrhoeae* has two PorB serotypes, $PorB_{1A}$ and $PorB_{1B}$; the strain used in these experiments, FA1090, expresses the $PorB_{1B}$ serotype. As anticipated, immu-

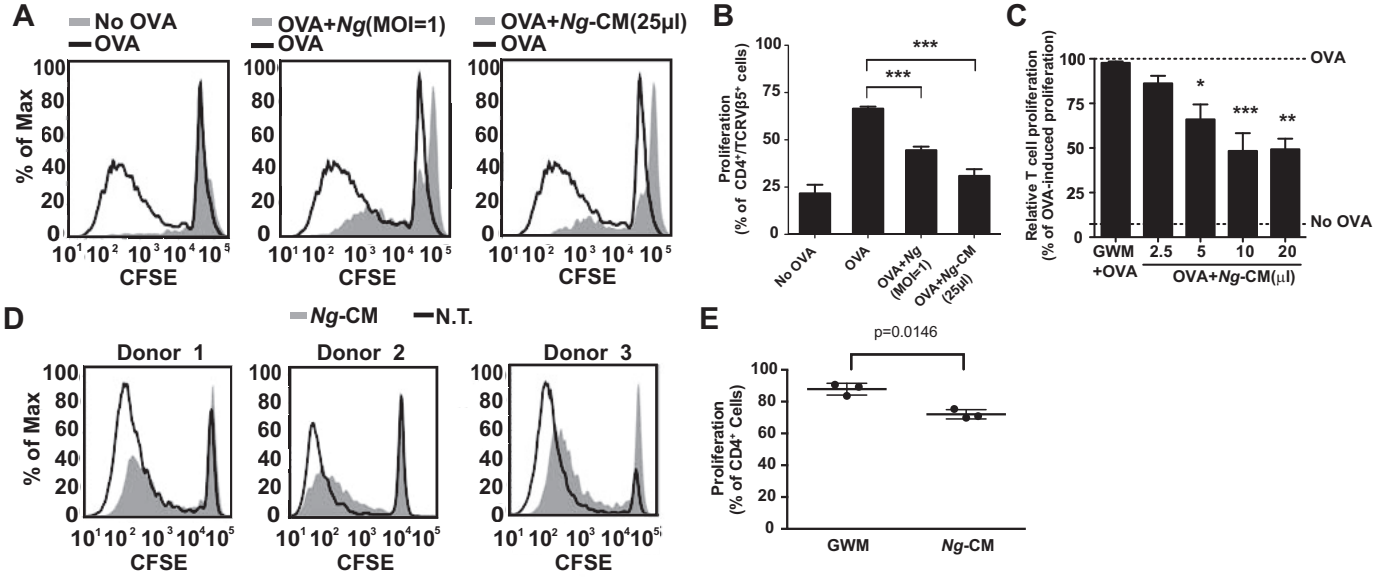

Figure 1. *N. gonorrhoeae* cells and *N. gonorrhoeae* conditioned medium inhibit OVA-primed dendritic cell–induced T cell proliferation. Dendritic cells were exposed to *N. gonorrhoeae* or *Ng*-CM in the presence of OVA for 24 h and then co-cultured with CFSE-loaded OT-II T cells for 7 days. T cell proliferation mediated by OVA stimulation was assessed by flow cytometry as described under "Experimental procedures." *A*, representative overlay histograms of OT-II T cell proliferation induced by dendritic cells, with the indicated treatment in *filled gray*. No OVA treatment is defined as GWM without OVA (*left panel*), *Ng* (m.o.i. = 1) plus OVA (*center panel*), or *Ng*-CM (25 μl) plus OVA (*right panel*). The OVA-pulsed dendritic cell control is plotted as a *black line* in each panel. *B*, the percentage of cultured OT-II T cells that underwent at least 1 generation of proliferation relative to all OT-II T cells in the culture is plotted after co-culture with dendritic cells without OVA antigen (*no OVA*) or treated with OVA, *Ng* (m.o.i. = 1) plus OVA, or *Ng*-CM (25 μl) plus OVA. Statistical significance was determined by one-way ANOVA with a *post hoc* Tukey analysis for multiple comparisons. ***, $p < 0.001$. *C*, T cell proliferation after co-culture with dendritic cells exposed to OVA and GWM or the indicated amounts of *Ng*-CM was measured as in *B*. The percentage of OT-II T cell proliferation for each condition was normalized to that observed after co-culture with OVA-pulsed dendritic cells. The *top dashed line* indicates the normalized proliferation induced by OVA, and the *bottom dashed line* marks the percentage of proliferation in the absence of antigen (*no OVA*). Data are mean ± S.E. ($n = 11$). Statistical significance was determined by one-way ANOVA with a *post hoc* Tukey analysis for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with dendritic cells treated with GWM. *D*, human dendritic cells from three separate donors were left untreated (*N.T.*) or treated with *Ng*-CM (25 μl) and co-cultured with CFSE-labeled allogeneic lymphocytes, and proliferation was assessed by flow cytometry after 7 days in culture. *E*, the percentage of cultured allogeneic T lymphocytes that underwent at least one generation of proliferation relative to all T lymphocytes in the culture after co-culture with untreated or *Ng*-CM-treated dendritic cells from the three donors shown in *D*. The *p* value was determined by ratio paired *t* test.

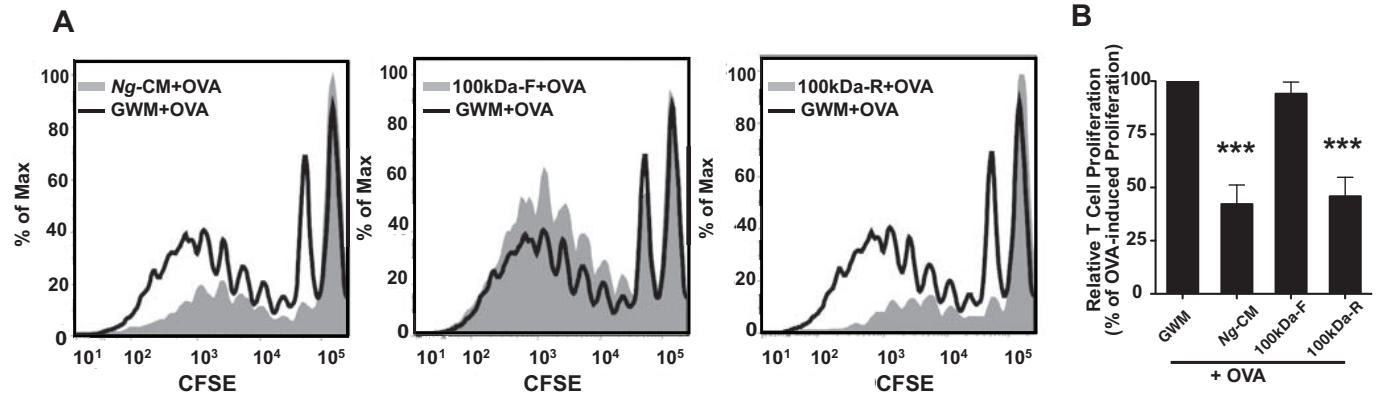

Figure 2. The >100-kDa retentate of ultrafiltered *Ng*-CM contains all of the inhibitory activity on OVA-pulsed dendritic cell–induced T cell proliferation. *Ng*-CM was separated into two fractions, retentate (>100 kDa) and filtrate (<100 kDa), by ultrafiltration through a 100-kDa cutoff filter. Dendritic cells were pulsed with OVA plus GWM, *Ng*-CM, *Ng*-CM filtrate (100kDa-F), or *Ng*-CM retentate (100kDa-R) and co-cultured with CFSE-labeled OT-II T cells, and the proliferation of T cells after 7 days in culture was assessed by flow cytometry. *A*, representative overlay histograms of OT-II T cell proliferation induced by OVA-pulsed dendritic cells treated with the indicated stimulation in *gray*: *Ng*-CM + OVA (*left panel*), *Ng*-CM 100kDa-F + OVA (*center panel*), or 100kDa-R + OVA (*right panel*). The GWM + OVA–pulsed dendritic cell control is plotted as a *black line* in each panel. *B*, the percentage of T cells proliferating relative to all of the T cells in the well induced by dendritic cells treated with OVA + GWM, *Ng*-CM, *Ng*-CM filtrate, or *Ng*-CM retentate were normalized to the OVA-pulsed dendritic cell control as described in Fig. 1*C*. Data are mean ± S.E. ($n = 6$). Statistical significance was determined by one-way ANOVA with a *post hoc* Tukey analysis for multiple comparisons. ***, $p < 0.001$ compared with dendritic cells treated with GWM.

noblot analysis demonstrated that $PorB_{1B}$ was present in *Ng*-CM and that all detectable $PorB_{1B}$ was retained by the 100-kDa cut-off filter (Fig. 3*A*). To examine the effects of $PorB_{1B}$ on dendritic cell–mediated T cell proliferation, $PorB_{1B}$ lacking its signal sequence and containing a N-terminal hexahistidine tag (HT) followed by a tobacco etch virus protease recognition site was expressed as inclusion bodies in *E. coli* and refolded into an active state in the presence of the detergent LDAO as described under "Experimental procedures." For some experiments, the hexahistidine tag was removed using tobacco etch virus prote-

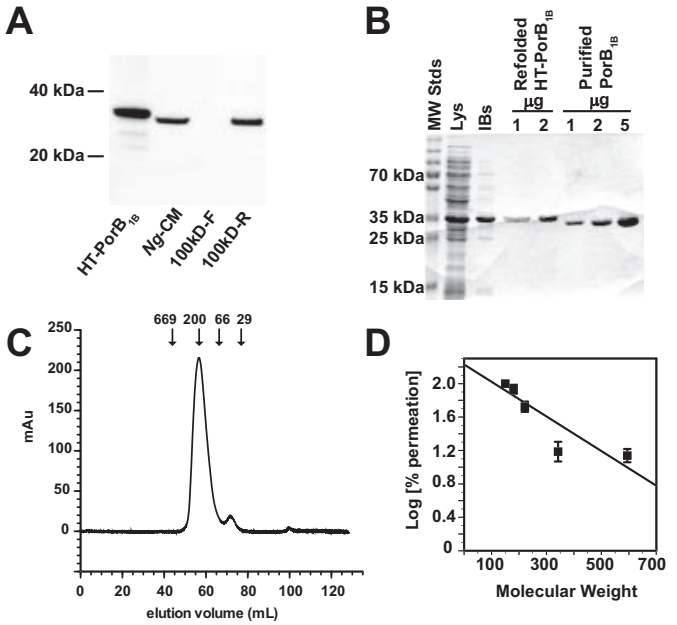

Figure 3. Refolded, purified, recombinant $PorB_{1B}$ forms functional trimeric porin complexes. *A*, *Ng*-CM, 100kDa-F, and 100kDa-R from FA1090 were prepared as described under "Experimental procedures" and analyzed by SDS-PAGE and Western blotting using an antibody against $PorB_{1B}$. A lane containing recombinant HT-$PorB_{1B}$ was run as a positive control for antibody reactivity. *B*, recombinant $PorB_{1B}$ was prepared as described under "Experimental procedures," and fractions from the indicated steps of the preparation were analyzed by SDS-PAGE after staining with Coomassie Brilliant Blue R-250. *Lys*, bacterial lysate; *IBs*, inclusion bodies. *C*, recombinant, refolded $PorB_{1B}$ was run on a Sephacryl S300 column, and the A_{260} was plotted *versus* the volume of eluent. The elution position for protein standards of known molecular mass (in kilodaltons) are indicated by *arrows*. *D*, the log of the ratio of the rate of swelling of liposomes containing purified recombinant $PorB_{1B}$ promoted by saccharides to the rate induced by arabinose (defined as 100%) is plotted against the molecular weight of the saccharide.

ase (Fig. 3*B*). Refolded HT-$PorB_{1B}$ and $PorB_{1B}$ in LDAO micelles were highly pure and eluted as homogenous single peaks in size exclusion chromatography. The estimated molecular mass of purified, refolded $PorB_{1B}$ was ~250 kDa (Fig. 3*C*), consistent with two PorB trimers (~114 kDa), the known tertiary structure for native *N. gonorrhoeae* $PorB_{1B}$, and an LDAO detergent micelle (~25 kDa). Functional activity of refolded and purified $PorB_{1B}$ was demonstrated by its capacity to promote size-dependent permeation of a series of saccharides following reconstitution into liposomes (Fig. 3*D*) (24).

The addition of purified $PorB_{1B}$ (50 μg/ml) to dendritic cells during OVA exposure led to profound inhibition of T cell proliferation in our co-culture system, recapitulating the effects observed with live *N. gonorrhoeae* treatment (Figs. 4, *A* and *B*). The addition of an equivalent volume of LDAO-containing buffer, the vehicle for $PorB_{1B}$, caused a small, reproducible decrease in dendritic cell–induced T cell proliferation, but this decrease was not statistically significant (Fig. S2). The $PorB_{1B}$ preparations were found to have low levels of endotoxin contamination (between 1.5 and 6 endotoxin units/mg of protein), which, at the highest amount of $PorB_{1B}$ added, corresponds to a maximum concentration of ~ 2.5 ng of *E. coli* LPS/ml in the dendritic cell culture. To ensure that *E. coli* LPS contamination of the recombinant $PorB_{1B}$ was not responsible for the $PorB_{1B}$-mediated inhibition of T cell proliferation observed, OVA-pulsed dendritic cells were exposed to *E. coli* LPS at concentrations between 1 and 100 ng/ml and assayed for the ability to stimulate OT-II T cell proliferation. *E. coli* LPS at these concentrations demonstrated no detectable effect on OVA-pulsed dendritic cell–induced T cell proliferation (Fig. S3).

Although these experiments demonstrated an inhibitory effect on dendritic cell–mediated T cell proliferation when recombinant $PorB_{1B}$ was added to the culture, it did so at concentrations much higher than the levels of $PorB_{1B}$ present in either the *Ng*-CM or live gonococci that produced an equivalent inhibitory effect (data not shown). However, because $PorB_{1B}$ is an integral membrane protein, a substantial percentage of the $PorB_{1B}$ is likely to aggregate or bind to the tissue culture plate when the protein is diluted from its detergent-containing buffer into cell culture medium. Therefore, we sought to determine whether the amounts of dendritic cell-associated $PorB_{1B}$ were similar between cells treated with recombinant purified $PorB_{1B}$ and those treated with live *N. gonorrhoeae* by performing immunoblot analysis of the dendritic cells prior to their use in the co-culture T cell proliferation assay. The amount of $PorB_{1B}$ associated with dendritic cells after treatment with 50 μg/ml of purified $PorB_{1B}$ was similar to that observed in cells treated with live *N. gonorrhoeae* at an m.o.i. of 10 (Fig. 4*C*). We observed more cell-associated $PorB_{1B}$ in cells treated with 5 μg/ml of recombinant porin than with live *N. gonorrhoeae* at an m.o.i. of 1, even though the former caused little inhibition, whereas the latter decreases T cell proliferation by over 50%. These data indicate that *N. gonorrhoeae* likely produces additional inhibitory factors that complicate the comparison between live bacteria and a single purified protein. One such factor is the inhibitory interleukin IL-10, which has been shown to be secreted in response to live *N. gonorrhoeae* (10).

To further assess the loss of the suppressive effect of $PorB_{1B}$ at concentrations lower than 50 μg/ml, we tested the capacity of $PorB_{1B}$ to inhibit dendritic cell–induced T cell proliferation at three different subcytolytic concentrations of LDAO detergent. We observed minor but consistent concentration-dependent decreases in proliferation with detergent alone (~30% at the highest concentration of detergent). Although complete suppression of T cell proliferation was observed with 50 μg/ml $PorB_{1B}$ regardless of the detergent concentration, we also observed that, as the concentration of LDAO was increased, the inhibitory potency of $PorB_{1B}$ increased (Fig. 4*D*). These data suggest that, when $PorB_{1B}$ loses association with detergent micelles and presumably loses its native, membrane-associated trimeric structure, it also loses the capacity to inhibit dendritic cell–induced T cell proliferation.

To ensure that $PorB_{1B}$ was not simply competing with OVA for proteolytic digestion or loading into antigen-presenting MHC molecules, we tested whether other proteins, either BSA or an isolated recombinant gonococcal protein (the transcriptional regulator GdhR) expressed in *Escherichia coli*, could inhibit dendritic cell–induced CD4 T cell proliferation. In contrast to recombinant $PorB_{1B}$, neither protein impacted the capacity of OVA-pulsed dendritic cells to induce OT-II T cell proliferation (Fig. 4*E*) (25).

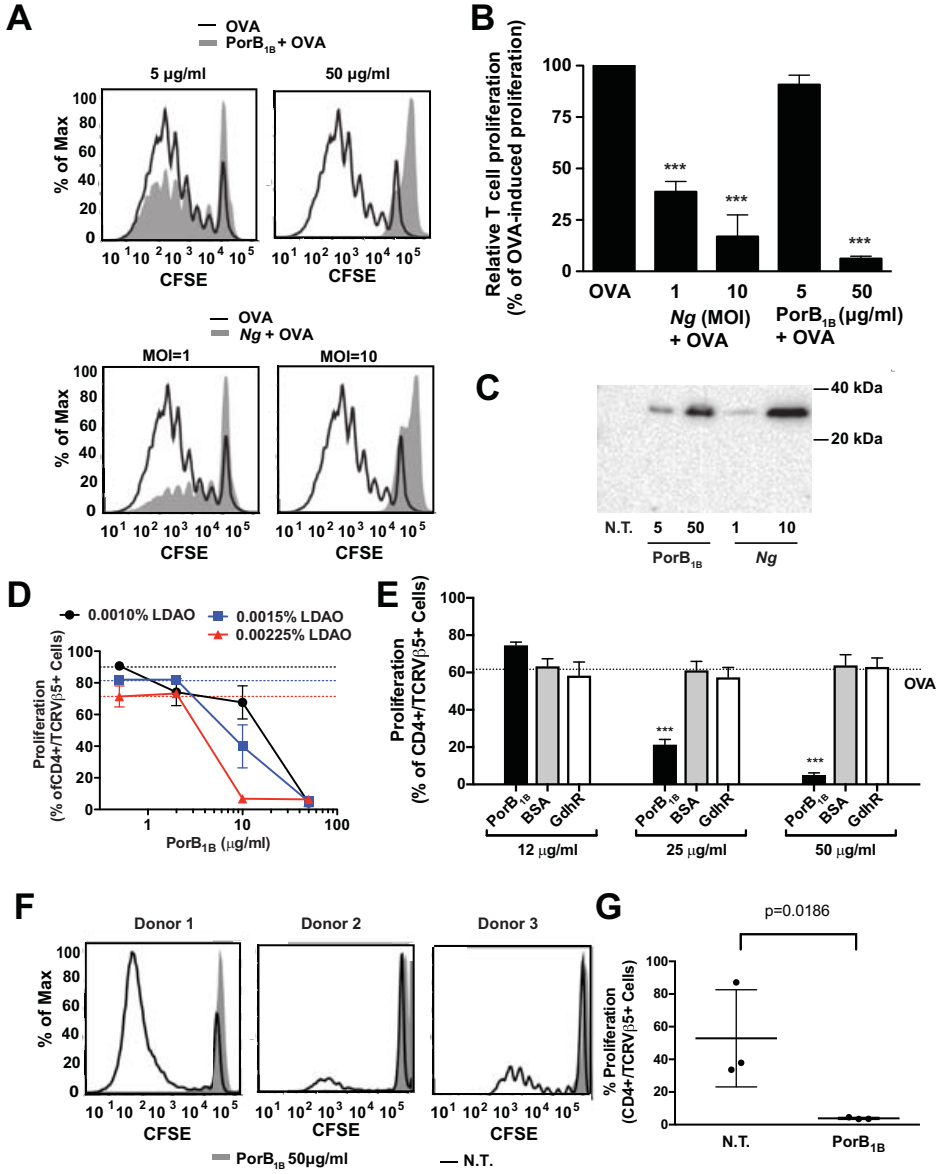

Figure 4. Refolded, purified, recombinant $PorB_{1B}$ inhibits OVA-pulsed dendritic cell–induced T cell proliferation. *A* and *B*, dendritic cells were pulsed with OVA alone or OVA plus either purified recombinant $PorB_{1B}$ (at 5 and 50 μg/ml) or *N. gonorrhoeae* (m.o.i. = 1 and 10), and the proliferation of CFSE-labeled OT-II T cells was assessed after 7 days by flow cytometry. *A*, the dilution of CSFE fluorescence in T cells following incubation with dendritic cells pulsed with the indicated treatments. *B*, the percentage of T cells undergoing proliferation relative to all of the T cells in the culture is plotted for each of the indicated treatments of the dendritic cells. Data are expressed as mean ± S.E. (n = 4–11 replicates). Statistical significance was determined by one-way ANOVA with a *post hoc* Tukey analysis for multiple comparisons. ***, $p < 0.001$ compared with dendritic cells treated with OVA alone. *C*, dendritic cells were not treated (*N.T.*) or exposed to either purified recombinant $PorB_{1B}$ (5 and 50 μg/ml) or *N. gonorrhoeae* (m.o.i. = 1 and 10) for 24 h. The cells were washed, whole-cell lysates were prepared as described under "Experimental Procedures," and equal amounts of protein were subjected to Western blotting using an antibody against $PorB_{1B}$. *D*, dendritic cells were pulsed with OVA in the presence of the indicated final concentration of LDAO (*black circles*, 0.0010%; *blue squares*, 0.0015%; *red triangles*, 0.00225%) and recombinant $PorB_{1B}$, the pulsed dendritic cells were co-cultured with CFSE-labeled OT-II T cells, and the proliferation of OT-II T cells was determined using flow cytometry. *Points* represent the mean ± S.E. (n = 4–7 replicates). *Dashed lines* indicate the proliferation induced by OVA-pulsed dendritic cells treated with the indicated LDAO concentration in the absence of $PorB_{1B}$. *E*, dendritic cells were pulsed with OVA in the presence of LDAO (final concentration, 0.0015%) and the indicated proteins at 50 μg/ml: recombinant $PorB_{1B}$, BSA, or recombinant refolded GdhR, a transcriptional regulator. The pulsed dendritic cells were co-cultured with CFSE-labeled OT-II T cells, and the proliferation of OT-II T cells was determined using flow cytometry. The percentage of T cells undergoing proliferation relative to all of the T cells in the culture is plotted for each of the indicated treatments of the dendritic cells. *F* and *G*, human dendritic cells from three separate donors were left untreated or incubated with purified $PorB_{1B}$ (50 μg/ml) for 24 h, and then they were washed and co-cultured with CFSE-labeled allogeneic lymphocytes for 7 days. Lymphocyte proliferation was assessed from the dilution of CSFE fluorescence by flow cytometry. Representative overlay histogram plots of CFSE-labeled allogeneic T cells after co-culture with the indicated dendritic cells are shown for each donor (*F*), and the percentage of cultured allogeneic T lymphocytes that underwent at least one generation of proliferation relative to all T lymphocytes in the culture is plotted from each of the three donors (*G*). Data are expressed as mean ± S.E. (n = 3 replicates). The p value was determined by ratio paired t test.

Because *N. gonorrhoeae* is an exclusive human pathogen, we also assessed whether addition of $PorB_{1B}$ would inhibit T cell proliferation in a model of human dendritic cell–induced T cell proliferation. As shown previously for treatment with live *N. gonorrhoeae* and with *Ng*-CM (Fig. 1, *D* and *E*), T cell proliferation induced by co-culture with allogeneic dendritic cells

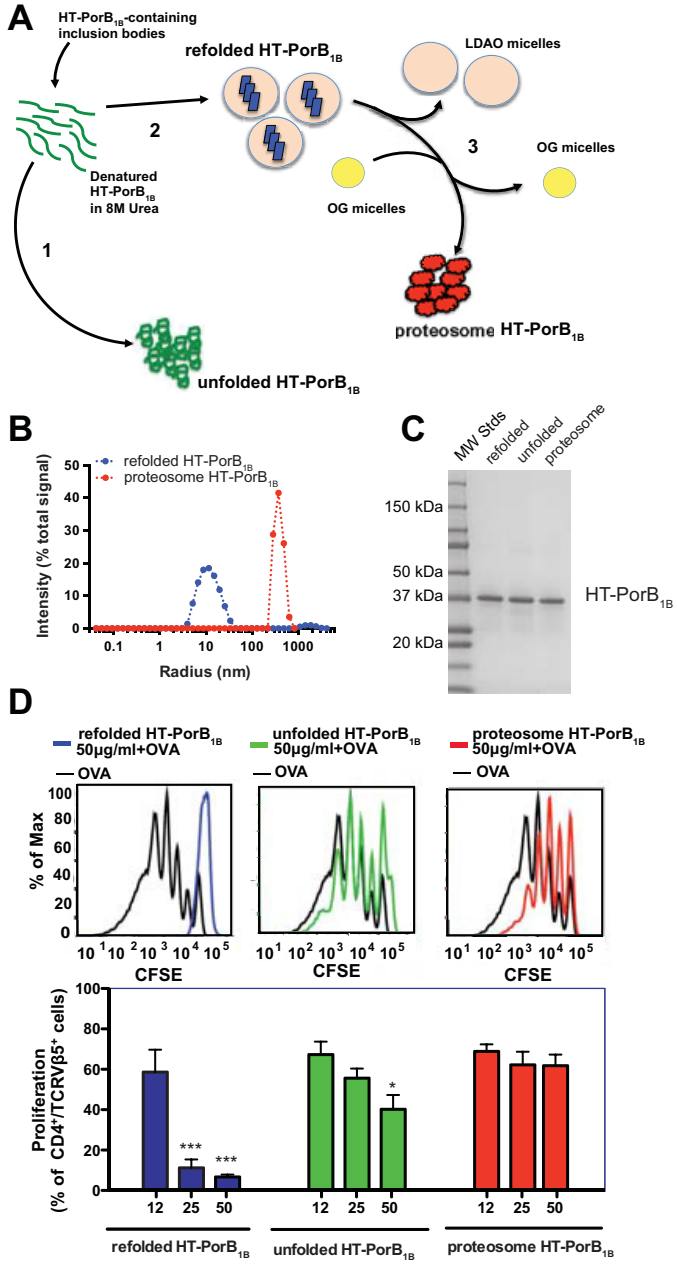

Figure 5. Recombinant $PorB_{1B}$ must be properly folded to inhibit dendritic cell–induced T cell proliferation. *A*, schematic of the preparation of unfolded, refolded, and proteosome recombinant HT-$PorB_{1B}$ from urea-solubilized recombinant HT-$PorB_{1B}$–containing inclusion bodies. *1*, urea removed from unfolded HT-$PorB_{1B}$ by dialysis. *2*, refolded HT-$PorB_{1B}$ generated by dilution of urea-solubilized protein into buffer containing LDAO detergent micelles, followed by size exclusion chromatography. *3*, HT-$PorB_{1B}$ proteosomes were prepared by ethanol precipitation of refolded HT-$PorB_{1B}$, resuspension in octyl glucoside (*OG*), followed by removal of octyl glucoside by dialysis. *B*, the Stokes radii determined from dynamic light scattering of refolded and proteosome HT-$PorB_{1B}$. *C*, equal quantities (2 μg) of unfolded, refolded, and proteosomal HT-$PorB_{1B}$ were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250. *D*, dendritic cells were pulsed with OVA in the presence of LDAO (final concentration, 0.0015%) and the indicated concentrations of recombinant unfolded, refolded, or proteosomal HT-$PorB_{1B}$. The pulsed dendritic cells were co-cultured with CFSE-labeled OT-II T cells, and the proliferation of OT-II T cells was determined using flow cytometry. The *top panels* show representative flow cytometry histograms from a single experiment in which the dendritic cells were treated with 50 μg/ml of the indicated protein preparation. The *bottom panels* show the percentage of T cells undergoing proliferation relative to all of the T cells in the culture plotted for each of the indicated treatments of the dendritic cells. Data are expressed as mean ± S.E. ($n = 3$ replicates). Statistical significance was determined by one-way ANOVA with a *post hoc* Tukey analysis for multiple comparisons. *, $p < 0.05$; ***, $p < 0.001$ compared with dendritic cells treated with OVA alone.

was inhibited when the dendritic cells were first treated with recombinant $PorB_{1B}$ (Fig. 4, *F* and *G*). Moreover, neither BSA nor GdhR were capable of inhibiting T cell proliferation when added at the same concentration of purified $PorB_{1b}$.

$PorB_{1B}$ suppression of dendritic cell–mediated T cell proliferation requires properly folded $PorB_{1B}$ and is not mediated through TLR2 signaling

Purified *Neisseria* PorB proteins have been shown previously to have immunostimulatory activity in both cultured immune cells and when injected into whole animals (23, 26–28). In these reports, the protein, which was either purified from *N. gonorrhoeae* or refolded from *E. coli* inclusion bodies, had the detergent used to solubilized this integral membrane protein removed through a process of ethanol precipitation and dialysis during the final steps of preparation. The resulting detergent-free protein aggregates have been termed PorB "proteosomes" (29). We sought to test whether the suppressive effect on dendritic cell-induced T cell proliferation we observed with $PorB_{1B}$ treatment of dendritic cells was independent of the folded structure of $PorB_{1B}$. Unfolded $PorB_{1B}$ was prepared by dialysis into PBS of urea-solubilized recombinant $PorB_{1B}$ inclusion bodies, whereas $PorB_{1B}$ proteosomes were prepared from our refolded, detergent-containing $PorB_{1B}$ preparations by ethanol precipitation and dialysis using the methods of Massari *et al.* (29) (Fig. 5*A*). Dynamic light scattering performed on refolded and proteosome preparations of recombinant $PorB_{1B}$ clearly demonstrated that refolded $PorB_{1B}$ in LDAO micelles exists as a single homogenous species with an estimated Stokes radius of 10 nm, whereas proteosome $PorB_{1B}$ preparations without detergent formed much larger particles with a Stokes radius of ~700 nm (Fig. 5*B*). Based on the crystal structure of *Neisseria meningitidis* PorB, the size estimate generated by dynamic light scattering for the refolded protein is consistent with two $PorB_{1B}$ trimers sitting within an LDAO micelle, which is also consistent with estimates generated by retention time in size exclusion chromatography (Fig. 5*C*). Each of these protein preparations resulted in highly purified $PorB_{1B}$, as determined by SDS-PAGE (Fig. 5*C*). These preparations were then tested for their effects on OVA-pulsed dendritic cell stimulation of OT-II T cell proliferation. As we had observed previously, refolded $PorB_{1B}$ demonstrated suppression of T cell proliferation, but neither the unfolded nor the proteosome preparation of $PorB_{1B}$ suppressed T cell proliferation in this system (Fig. 5*D*). These data suggest that the suppressive properties of PorB are disrupted when the structure of the protein is not maintained.

Neisseria PorB proteins have been shown previously to activate host TLR2 (28, 30). Structural and functional studies indicate that the seventh extracellular loop of $PorB_{1B}$ appears to be the $PorB_{1B}$-derived ligand for TLR2 (31). Unfolded, refolded, and proteosomal HT-$PorB_{1B}$ was applied to a reporter cell line expressing human TLR2. Each $PorB_{1B}$ protein preparation activated TLR2 under these conditions. Refolded $PorB_{1B}$ generated

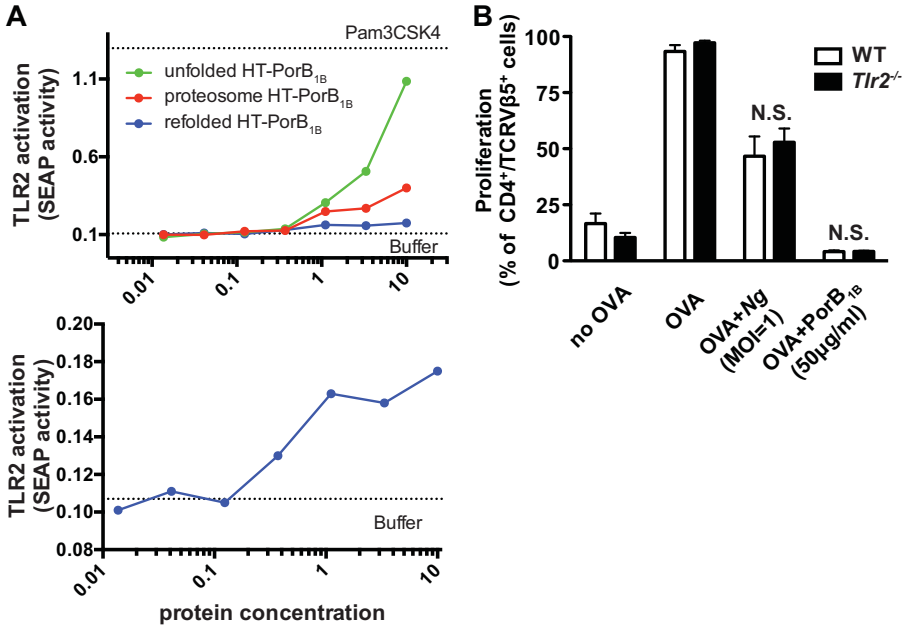

Figure 6. $PorB_{1B}$ does not inhibit dendritic cell–induced T cell proliferation through TLR2. *A*, HEK-Blue hTLR2 reporter cells were treated with LDAO-containing buffer (0.0075%) alone or with the indicated concentrations of recombinant unfolded, refolded, and proteosome HT-$PorB_{1B}$. After 24 h, cell culture supernatants from the treated cells were assayed for the activity of secreted alkaline phosphatase as described under "Experimental procedures." The *top panel* shows the dose response for all three $PorB_{1B}$ preparations, whereas the *bottom panel* shows only refolded $PorB_{1B}$ with an expanded *y* axis. *B*, WT or *Tlr2*$^{-/-}$ dendritic cells were treated with GWM (*no OVA*), OVA, OVA + *Ng* (m.o.i. = 1), or OVA + $PorB_{1B}$ (50 μg/ml) and then co-cultured with CSFE-labeled OT-II T cells. T cell proliferation was assessed by flow cytometry. The mean ± S.E. ($n = 4$) of the percentage of OT-II T cells that underwent proliferation over 7 days is plotted for cells co-cultured with WT dendritic cells (*black columns*) or *Tlr2*$^{-/-}$ dendritic cells (*white columns*) for the indicated treatments. Statistical significance was assessed by two-way ANOVA with a *post hoc* Bonferroni analysis for multiple comparisons. No statistically significant difference was found between WT and *Tlr2*$^{-/-}$ cells.

a maximal TLR2 activation of a roughly 1.5-fold increase over baseline reporter activity. In contrast, proteosome $PorB_{1B}$ preparations induced a nearly 7-fold activation, and unfolded $PorB_{1B}$ induced a nearly 15-fold activation (Fig. 6*A*). These data suggest that high levels of TLR2 activation previously reported for proteosome $PorB_{1B}$ preparations from a number of *Neisseria* species could be the result of high-binding valency associated with an aggregated protein ligand, whereas actual native $PorB_{1B}$ stimulation of TLR2 is much more modest.

Because TLR2 activation by capsular polysaccharide is known to mediate the immunosuppressive effects of outer membrane vesicles from the gut commensal *Bacteroides fragilis*, we sought to determine whether TLR2 engagement by $PorB_{1B}$ was activating tolerogenic signaling pathways in dendritic cells by utilizing dendritic cells generated from *Tlr2*$^{-/-}$ mice (32). T cell proliferation following co-culture with *Tlr2*$^{-/-}$ dendritic cells was indistinguishable from the proliferation observed when co-cultured with WT dendritic cells (Fig. 6*B*). Furthermore, *N. gonorrhoeae* or refolded $PorB_{1B}$ treatment of dendritic cells prior to co-culture with T cells resulted in equivalent inhibition of T cell proliferation regardless of the genotype of the dendritic cell. Taken together, these data suggest that, although TLR2-activating capacity is present in isolated *N. gonorrhoeae* PorB regardless of the folded structure of the protein, the immunosuppressive effects of the protein, which requires functionally folded PorB, are dominant and overcome any TLR2-activating activity.

Inhibition of dendritic cell–induced T cell proliferation by *N. gonorrhoeae* PorB was observed even 3 days into the dendritic cell/T cell co-culture, suggesting that PorB acts directly on the dendritic cells rather than by inducing inhibitory T cell signaling, such as T regulatory cell differentiation, within the co-culture (Fig. S4). To test whether PorB treatment impacted the maturation of dendritic cells exposed to OVA prior to co-culture with T cells, we examined the fraction of cells with high CD11c and MHC class II expression and found no significant differences between cells treated with refolded, unfolded, or proteosome PorB (Fig. 7, *A* and *B*). In contrast, we observed that treatment of dendritic cells with refolded PorB resulted in a significant decrease of the co-stimulatory dendritic cell surface protein CD86 compared with treatment with unfolded or proteosome PorB (Fig. 7*C*). Treatment of dendritic cells at the highest doses of refolded PorB (50 μg/ml) also caused a significant decrease in surface expression of the costimulatory molecule CD40 compared with treatment with proteosome PorB (Fig. 7*D*). Taken together, these studies support the finding that properly folded *N. gonorrhoeae* PorB acts directly on dendritic cells to reduce their capacity to stimulate antigen-driven T cell proliferation.

Discussion

N. gonorrhoeae is a highly adapted human pathogen that is closely related to commensal *Neisseria* species. Like commensal *Neisseria* that live on human mucosal surfaces without eliciting protective immunologic responses, *N. gonorrhoeae* has the ability to escape the human immune response. Mechanisms involved in successful evasion of *N. gonorrhoeae* from human immune responses are multifactorial and complex. Recent findings have suggested that *N. gonorrhoeae* can actively suppress host immune responses through interaction with differ-

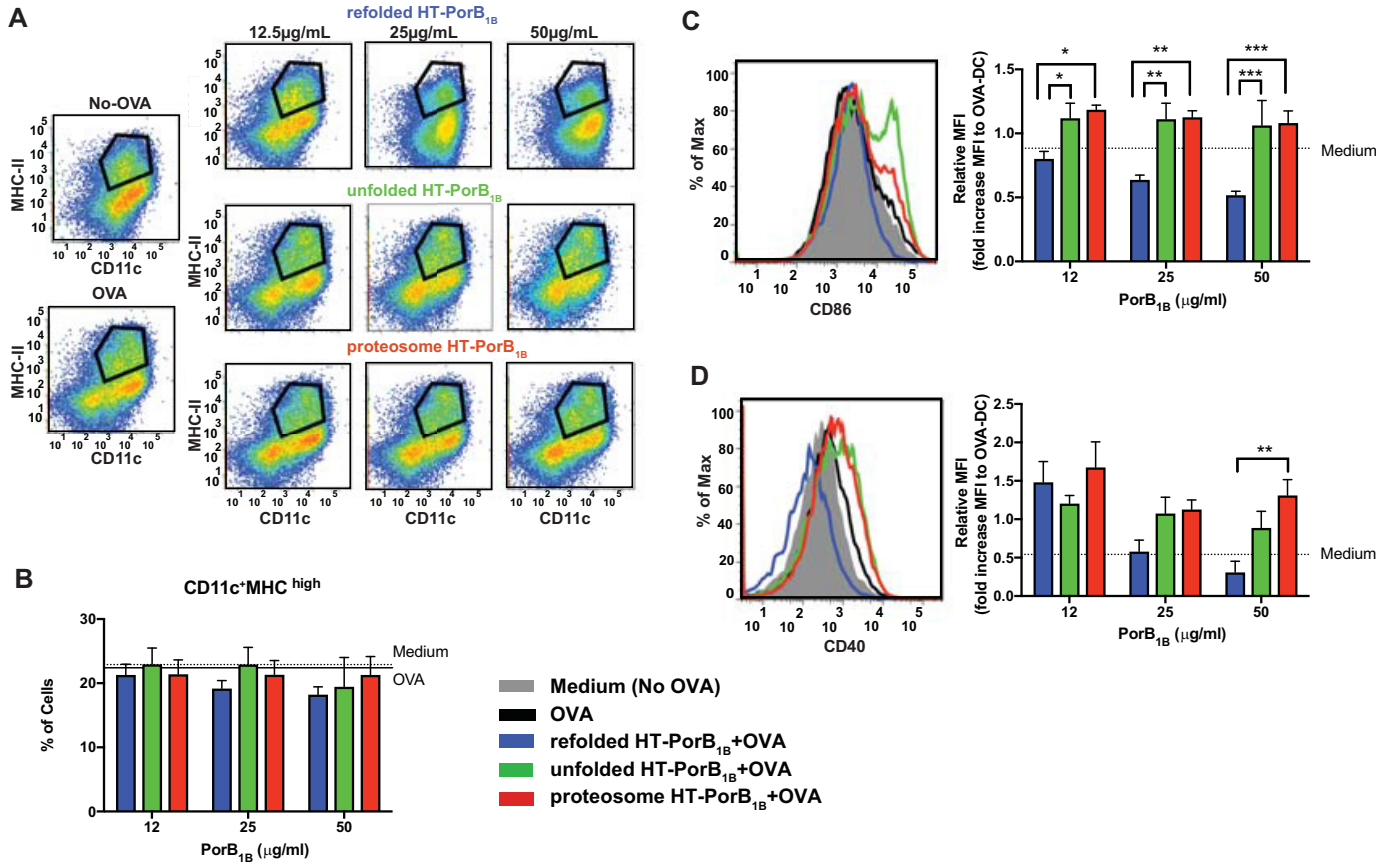

Figure 7. Properly folded $PorB_{1B}$ causes a decrease in surface expression of costimulatory molecules on dendritic cells. Dendritic cells were pulsed with OVA for 24 h in the presence of LDAO (final concentration, 0.0015%) and the indicated concentrations of recombinant unfolded, refolded, or proteosome HT-$PorB_{1B}$, as was done prior to co-culture of dendritic cells with T cells in Fig. 5. Cells not pulsed with OVA were provided culture medium with LDAO (final concentration, 0.0015%) as a control. After 24 h, the cells were stained for the indicated dendritic cell surface markers and analyzed by flow cytometry. *A*, representative scatterplots for MHCII *versus* CD11c from each treatment group, with the gating used to assess the fraction of cells with high MHC and CD11c indicated by the *black lines*. *B*, the *columns* represent the percentage of dendritic cells that stained positively for high expression of both MHCII and CD11c after the indicated treatments from seven separate dendritic cell cultures. *C*, surface expression of the co-stimulatory molecule CD86 in the $MHCII^{high}$,$CD11c^{high}$ dendritic cell populations was determined. A representative histogram of CD86 expression is shown for cells treated with 25 μg/ml of the indicated PorB preparation (*left panel*). Also shown is a bar graph representing the mean fluorescence intensity (*MFI*) of CD86 staining relative to cells pulsed with OVA without addition of PorB for all treatment groups (*right panel*). *D*, surface expression of the co-stimulatory molecule CD40 in the $MHCII^{high}$,$CD11c^{high}$ dendritic cell populations was determined. A representative histogram of CD40 expression is shown for cells treated with 25 μg/ml of the indicated PorB preparation (*left panel*). Also shown is a bar graph representing the mean fluorescent intensity of CD86 staining relative to cells pulsed with OVA without addition of PorB for all treatment groups (*right panel*). Statistical significance was assessed by a two-way ANOVA with a *post hoc* Bonferroni analysis for multiple comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for the noted comparisons.

ent types of immune cells. Antigen-presenting dendritic cells direct host immune responses toward either immunity or tolerance (33, 34). Studies have shown that the human vaginal mucosa contains four major subsets of myeloid-derived dendritic cells, each displaying unique functions that direct different types of immune responses (35). Therefore, understanding how *N. gonorrhoeae* interacts with dendritic cells to produce an end response is an important step in devising strategies to overcome the immune evasion of this pathogen.

Prolific blebbing of OMVs is a characteristic of *Neisseria* species; however, the consequences of this process are not fully understood (16, 17). A recent study demonstrated that the polysaccharide capsule associated with OMVs from the commensal bacillus *B. fragilis* is capable of inducing a tolerogenic phenotype in dendritic cells (32). Our studies demonstrate that an OMV-containing fraction of conditioned medium from *N. gonorrhoeae* is also capable of inducing immune suppression in dendritic cells. Unlike *B. fragilis*, *N. gonorrhoeae* does not produce capsular polysaccharide, which led us to further investigate the key components of *Ng*-CM that harbor these immunosuppressive properties. Yu *et al.* (36) recently demonstrated that dendritic cells exposed to *N. gonorrhoeae* suppress $CD4^+$ T cell responses to HIV-derived antigens presented by those dendritic cells. This suppression appears to be mediated largely by activation of the immune-inhibitory CEACAM1 receptor on dendritic cells by *N. gonorrhoeae* Opa proteins, which have also been implicated in promoting production of the immune-suppressive cytokines IL-10 and transforming growth factor β, thereby inhibiting T cell proliferation (13). However, because *N. gonorrhoeae* Opa proteins bind only to human CEACAM-1 but not mouse CEACAM-1 (37), mechanisms exclusive of Opa-CEACAM-1 interactions must exist that are responsible for suppression of antigen-dependent T cell proliferation by mouse dendritic cells exposed to *N. gonorrhoeae* or *Ng*-CM. Although OMV-based vaccines have demonstrated that OMVs are capable of inducing immune responses to proteins and polysaccharides associated with OMVs, the relative antigenicity of OMV-associated proteins compared with those of the

isolated proteins or proteins in association with bacterial pathogen-associated molecular patterns (PAMPs) that are also found in OMVs is not known. It is possible that physiologic levels of OMV shed by pathogenic and commensal bacteria actually suppress immune responses, as suggested by this study and studies published previously (32).

In addition to PorB, LOS is another major component of OMVs shed by *Neisseria* species. LOS is a potent inducer of the inflammatory cytokine response, and *N. meningitidis* LOS has adjuvant properties that presumably are mediated by TLR4 activation by the lipid A moiety of LOS (38). The oligosaccharide component of gonococcal LOS can vary structurally because of phase-variable expression of enzymes in the synthetic pathway (39), and these LOS variants have been reported to alter the cytokine secretion profiles of dendritic cells because of their stimulation of different surface lectin receptors, with changes in the cytokine profile influencing $CD4^+$ T cell subtype differentiation (40). We found that, unlike *N. gonorrhoeae* cells or OMV-containing *Ng*-CM, purified gonococcal LOS was unable to elicit inhibition of T cell proliferation by OVA-primed dendritic cells.

PorB is the most abundant outer membrane protein in *N. gonorrhoeae*. Importantly, we found that purified recombinant $PorB_{1B}$ has the capacity to inhibit dendritic cell-mediated $CD4^+$ T cell proliferation. *Neisseria* PorB proteins have been shown to activate host TLR2 responses (28, 30, 41), and purified PorBs from several commensal *Neisseria* species have been shown to stimulate immune responses when injected in mice and to induce dendritic cell maturation when added to cells in culture (26, 28, 42). The major difference between those published studies and this study is the preparation of PorB. All published studies demonstrating immune activation by PorB have been carried out with a PorB preparation in a detergent-free particle termed a proteosome (28, 43). Because PorB is an integral membrane protein, it is unlikely that it maintains its native functional structure under these conditions. The purified recombinant PorB in the nontoxic detergent (LDAO) micelles we use in this study mimics native PorB both structurally and physiologically, as shown by its performance on gel filtration chromatography and its capacity to facilitate sugar permeation when reconstituted into liposomes (Fig. 4*C*). The potency of detergent-solubilized $PorB_{1B}$ for inhibiting dendritic cell–mediated T cell proliferation increases as the amount of LDAO in the culture medium is increased, which, we suspect, suppresses the tendency of PorB to aggregate when LDAO micelles disperse upon dilution. Importantly, recombinant $PorB_{1B}$ in the absence of refolding or refolded $PorB_{1B}$ taken through the precipitation and detergent removal procedure used to generate PorB proteosomes in other published reports eliminates its immunosuppressive activity. These data strongly suggest that the native micelle/membrane-associated structure of PorB is required for its inhibitory function.

Unfolded, refolded, and proteosome $PorB_{1B}$ all have the capacity to stimulate TLR2, as has been reported previously, but at markedly different levels. Our data suggest that proteosome $PorB_{1B}$ retains its TLR2-stimulating activity while simultaneously eliminating the immunosuppressive activity of $PorB_{1B}$, which would explain the reported immunologic adjuvant properties of *Neisseria* species PorB that populate the current biomedical literature. Interestingly, PorB proteosomes have been shown to induce much more robust anti-PorB antibody responses than an equivalent quantity of native gonococcal PorB administered in naturally derived outer membrane vesicles (43). Given that poor immune responses to *N. gonorrhoeae* in infected humans are well-documented, there is certainly indirect evidence that naturally occurring gonococcal PorB associated with the bacteria or OMVs does not support a robust immune response in the natural host.

Unlike *B. fragilis* OMV–associated capsular polysaccharide, which induces a tolerogenic dendritic cell phenotype through host TLR2 activation (32), *N. gonorrhoeae* cells and purified recombinant $PorB_{1B}$ inhibited the capacity of dendritic cells derived from $Tlr2^{-/-}$ or WT mice to induce T cell proliferation. These data demonstrate that properly folded PorB from *N. gonorrhoeae* present in whole bacteria or in OMVs shed from the bacteria are capable of exerting immunosuppressive effects on dendritic cells. The mechanism underlying this suppression has yet to be determined. Gonococcal PorB has been shown to traffic to host cell mitochondria. In some studies, this interaction has been reported to promote host cell apoptosis, whereas, in others, it has been reported to inhibit host cell apoptosis (21, 44, 45). Some gonococcal PorB proteins have been shown to interact with host cell surface receptors, including a scavenger receptor expressed in endothelial cells and Gp96, both of which have been implicated in immune cell signaling and immune tolerance (46–48). These and potentially novel signaling pathways clearly need to be investigated further for their potential role in mediating immunosuppressive signaling downstream of gonococcal PorB.

Experimental procedures

Ethics statement

All experiments involving the use of mice were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at UNC. Human dendritic cells were obtained from subjects enrolled in a UNC Institutional Review Board–approved study (Study 05-2860) who were deidentified prior to use in this study. The UNC Office of Human Research Ethics reviewed the proposed use of deidentified human subject-derived dendritic cells and determined that the use described (Study 12-0024) does not constitute human subject research as defined under federal regulations (45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(l)) and does not require further IRB approval.

Preparation of N. gonorrhoeae and N. gonorrhoeae conditioned medium

Inocula of a predominantly Opa^+ frozen stock of FA1090 of *N. gonorrhoeae* were prepared as described previously (14). To minimize phase or antigenic variation, passage of FA1090 and its derivatives was kept to a minimum. The bacterial density of each inoculum was estimated by measuring A_{600} and confirmed by plating of serial dilutions.

To generate *Ng*-CM, *N. gonorrhoeae* strain FA1090 was grown overnight from frozen stock streaked on GC medium broth (GCB) plates. The cells were swabbed from the plates, introduced into 10 ml of GWM at an A_{600} of 0.2, and grown for 4.5 h in a shaking incubator at 37 °C with 5% CO_2. Bacteria were removed by centrifugation at 1200 × *g* for 10 min, followed by filtration through a sterile 0.22-μm filter. The *Ng*-CM was further fractionated for some experiments by ultrafiltration using a Centriprep device with a 100-kDa cut-off filter (EMD Millipore, Billerica, MA) according to the manufacturer's instructions.

Purification and functional activity of recombinant N. gonorrhoeae PorB$_{1B}$

Recombinant $PorB_{1B}$ was produced in *E. coli* as inclusion bodies and refolded using a modification of the method described by Olesky *et al.* (24). Full details are provided in supporting Materials and Methods, but a brief description is as follows. $PorB_{1B}$ expression was induced in BL21*(DE3) harboring pT7-HTb-por1090, which replaces the PorB signal sequence with a 28-amino acid extension (HT) containing a hexahistidine tag and a tobacco etch virus protease cleavage site at the N terminus of the protein (HT-$PorB_{1B}$). HT-$PorB_{1B}$–containing inclusion bodies were isolated and solubilized in 8 M urea. Unfolded HT-PorB was prepared from urea-solubilized inclusion bodies by dialysis into PBS. Refolded HT-$PorB_{1B}$ was prepared by slowly diluting urea-solubilized inclusion bodies into refolding buffer (200 mM 3-(cyclohexylamino)propanesulfonic acid, 400 mM NaCl, 50 mM Tris-HCl, 0.3% LDAO (pH 11)), and after stirring overnight, the solution's pH was lowered to 8.0 with concentrated HCl, and any precipitated protein was removed by centrifugation and filtration. The protein was purified by immobilized Ni^{2+} chromatography. In most experiments, the protein used had the HT tag removed by digestion with tobacco etch virus protease, and the untagged, properly folded $PorB_{1B}$ was isolated in the flow-through of a nickel-nitrilotriacetic acid column run in the presence of 15 mM imidazole, followed by size exclusion chromatography on a Sephacryl S-300 column in 1× PBS, 0.1% LDAO (PBSL). Preliminary experiments showed no difference between $PorB_{1B}$ and HT-$PorB_{1B}$ for inhibition of dendritic cell–mediated T cell proliferation (Fig. S5); therefore, in later experiments, the HT tag was left attached to the protein. Proteosome HT-$PorB_{1B}$ was prepared from refolded HT-$PorB_{1B}$ by precipitation with ethanol, resuspending the precipitated protein in 8% β-octyl glucoside, and then dialyzing in PBS to remove the detergent. Purified PorB preparations were tested for the presence of LPS using a chromogenic *Limulus* amebocyte lysate endotoxin assay (Toxinsensor™, Genscript) and human TLR4-expressing reporter cells (HEK-Blue™ hTLR4, Invivogen) according to the manufacturer's protocol.

Functional activity of refolded and purified $PorB_{1B}$ trimers was demonstrated by the swelling assay originally described by Nikaido and Rosenberg (49). Permeation rates of arabinose (MW 150), glucose (MW 180), galactose (MW 180), GlcNAc (MW 221), sucrose (MW 342), and raffinose (MW 595) were quantified as a ratio of the rate of permeation of the indicated sugar to the permeation rate of arabinose. Stachyose (MW 660) was used to find the isoosmotic concentration of the liposome preparation as described previously (24, 49).

Generation, infection, and stimulation of bone marrow–derived dendritic cells

Bone marrow–derived dendritic cells (BMDCs) were prepared from 9- to 12-week-old C57BL/6 or C57BL/6 *Tlr2*$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME) as described previously (14). After 7 days of growth and differentiation, BMDCs were washed, resuspended, and incubated with 100 μg/ml OVA (Sigma-Aldrich, St. Louis, MO) and either *N. gonorrhoeae* FA1090 at the indicated m.o.i., GWM, *Ng*-CM, purified gonococcal LOS purified from FA1090, recombinant $PorB_{1B}$, or PBSL (vehicle). After incubation for 24 h, the cells were collected and washed for co-culture with T cells or downstream assays.

BMDC–T cell co-culture/proliferation assay

T cells were isolated from spleens and lymph nodes of OT-II mice (C57BL/6-Tg(TcraTcrb)425Cbn/J, The Jackson Laboratory) and labeled with CFSE (Life Technologies) as described previously (14). Labeled, enriched T cells (5×10^5 cells/ml) were co-cultured with BMDCs at a density of 5×10^4 cells/ml in 48-well plates or plates containing transwell inserts (Costar, Corning, NY). After 7 days in co-culture, T cell proliferation was assessed by measuring the dilution of CFSE fluorescence in $CD4^+$,$TCRV\beta5^+$ lymphocytes using flow cytometry on a BD FACSCanto or a BD LSRII-SOS (BD Biosciences, Palo Alto, CA) as described previously (14). Data from 10,000–100,000 events were acquired for each sample and saved as an FCS 3.0 file that was subsequently analyzed with FlowJo software (Tree Star, Ashland, OR).

Assessment of TLR2 activation

HEK-Blue™ hTLR2 reporter cells were acquired from Invivogen (San Diego, California). The cells were cultured in growth medium (Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), glucose (4.5 g/liter), penicillin/streptomycin (50 units/ml and 50 μg/ml), and Normocin (100 μg/ml)) and then maintained in selection medium (growth medium supplemented with 100 μg/ml neocin). Cells were seeded in 96-well plates at a density of 5×10^4 cells in 200 μl per well, and after 24 h, they were exposed to vehicle, the indicated concentrations of $PorB_{1B}$ preparations, or 30 ng/ml Pam3CSK4 (Invivogen). Secreted alkaline phosphatase activity was assessed after 24 h of incubation by adding 20 μl of cell culture supernatant to 180 μl of Quanti-blue™ reagent (Invivogen) in a new 96-well plate and measuring optical density at 625 nm after a 2 h incubation at 37 °C.

Statistical analysis

Statistical analyses were performed using Prism 5 or 6 software (GraphPad, La Jolla, CA). Significance of the differences between multiple groups was assessed using one-way or two-way ANOVA with Tukey or Bonferroni as a *post hoc* test for multiple comparisons. In all cases, $p < 0.05$ was considered statistically significant.

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